Journal of Research in Pharmacy

Review Article

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Three-dimensional cell culture systems in drug development studies

Fersu Gül Asya ÇALIŞIR[1,2], Bilge DEBELEÇ BÜTÜNER[1*]

1 Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Ege University, Izmir, Turkiye
2 Department of Pharmaceutical Biotechnology, Institute of Health Sciences, Ege University, Izmir, Turkiye
* Corresponding Author. E-mail: bilge.debelec@ege.edu.tr (B.D.B.) Tel. +90-232-311 34 26.

Received: 22 April 2024/ Revised: 27 May 2024/ Accepted: 5 June 2024

ABSTRACT: Three-dimensional (3D) cell culture methods have been widely used in many research areas including the investigation of disease pathology such as carcinogenesis mechanisms, tissue engineering, and drug discovery. It has been known that 3D cell culture models have many advantages in comparison to both the monolayer two-dimensional (2D) cell culture methods in terms of better representing the *in vivo* cellular conditions and animal models due to their lower cost and higher applicability. 3D cell culture systems consist of validated models such as spheroids and organoids generated w/w.o. extracellular matrix components. Applications of these models can be seen in cancer research, high-throughput drug screening, and prediction of drug response on patient-derived 3D cellular models. Establishing novel 3D systems also has great potential to support the development of biotechnological therapeutics such as cellular therapies or therapeutic efficacy tests of various candidates on 3D models. This review article aims to briefly describe the recent literature on types, advantages, limitations, and applications of 3D cell culture models in various stages of drug development studies such as disease models, and drug response tests.

KEYWORDS: Three-dimensional cell culture; 3D disease models; 3D drug testing; spheroid; organoid

1. INTRODUCTION

Cell culture methods have played an important role in basic molecular research and drug research improving our understanding of cellular mechanisms for many years. *In vitro* cell culture methods have many advantages in terms of both ethical and laboratory applicability compared to animal experiments. In addition, the 3R principles published by Russell and Burch [1]. They introduced "replacement, reduction, and refinement", which implies replacing animal models with alternative methods, decreasing the numbers, and ruling the inhumane procedures out to alleviate the potential downsides of animal use in biomedical research [2,3]. Thus, the use of cell culture techniques instead of animal experiments by researchers has gradually increased, as *in vitro* cell culture systems can be changed flexibly and are open to the use of molecular analysis by new methods. Experimental designs such as gene silencing/overexpression and drug treatment can be performed where their molecular and cellular effects such as alterations in protein expressions and signaling pathways, cell proliferation, cell communications, and cell death mechanisms can be analyzed easily and cost-effectively in comparison to animal studies. Therefore, we see that cell culture practices have become standard nowadays in drug development, gene therapy, cancer biology/therapeutics, and regenerative medicine [4–6].

Cell culture practices have advanced significantly since the establishment of the first cell line in the 20th century and have been an important source of models and data for basic and health sciences over the past century. With the use of cell lines, a research material with a stable and homogeneous cell distribution has started to be used. This has allowed experiments and studies to be standardized and optimized so that scientists can obtain more precise and reproducible results [7,8].

In two-dimensional (2D) cell culture, cells form a monolayer structure by attaching to the neighboring cells and the surface of a flat bottom cell culture equipment. Although this is a useful method for culturing cells and performing experiments, increasing concerns about the ability of the method to mimic a living organism have been raised in recent years as the cells are not on a flat surface in a living organism and they are a collection of cells clinging to each other in a three-dimensional (3D) order. Therefore, it is known that in many drug development studies, *in vitro* test results differ considerably from *in vivo* test results [9].

How to cite this article: Çalışır FGA, Debeleç Bütüner B. Three-dimensional cell culture systems in drug development studies. J Res Pharm. 2024; 28(6): 2236-2242.

ISSN: 2630-6344

http://dx.doi.org/10.29228/jrp.897

2. WHY DO WE USE 3D CELL CULTURE TECHNOLOGY?

Although 2D cell culture has been of great benefit in understanding the biology of cancer, identifying carcinogenic markers, and developing new drugs, the 3D structure and complexity of the tumor and its environment cannot be adequately mimicked in the 2D cell culture. In the 2D system, the cells are in a single layer and passaged regularly paving the way for genetic mutations. In addition, they have access to unlimited oxygen and nutrient sources leading to unnatural cellular events and interactions. So, 2D cell culture cannot adequately reflect the *in vivo* tumor tissue [10].Therefore, 3D cell culture systems are suggested to be a useful tool as cells can be cultured much more similarly to the *in vivo* tumor in terms of metabolic and physiological properties leading to better analysis of the anticancer drug responses more closely *in vivo* conditions [11,12].

Another disadvantage of 2D culture is the way the cells are attached to themselves. In 2D culture, cells attach to the neighboring cells in a single dimension and adhere to the surface of the culture equipment depending on adherence properties. In 3D culture, cells adhere to themselves in all dimensions and are in communication with each other. So, the extracellular matrix (ECM) elements found in 2D culture are quite different from the ECM elements formed by the cell in 3D culture [13]. The differences between cell-to-cell and ECM interactions in 2D and 3D models result in major differences in terms of morphological characteristics of cells, protein expression patterns, proliferation as well as cell death mechanisms. It has been observed that there are notable differences in cell-cell and cell-matrix interactions between 2D and 3D culture models [14] Specifically, when cells are grown as a monolayer in a flat and rigid cell culture plate, they tend to create an artificial polarity that is not present in their *in vivo* environment [4]. It can be observed that 2D cell culture models may present dissimilarities in cell communication, morphology, extracellular matrix (ECM), and protein expression when compared to *in vivo* conditions. It is advisable to take these limitations into account when interpreting results obtained from 2D models and therefore, 3D cell culture is necessary to better mimic the *in vivo* cellular interactions than 2D cell culture [15].

In 2D culture models, nutrients, growth factors, and drug candidates act uniformly on all cells due to their monolayer arrangement. However, it is worth noting that substances administered to tissue through the vascular system may not reach all regions of the tissue equally for *in vivo* systems. This variability is particularly evident in tumor tissues, where there is a proliferative zone at the outermost, followed by a quiescent zone and a necrotic (hypoxic) zone inside. This is also the case in 3D spheroids. As a result, oxygen, nutrients, growth factors, and vascularisation decrease toward the inner parts of the tumor tissue [16], so the distribution of nutrients or drug candidates in the 3D complex structure is not uniform, unlike in the two-dimensional culture model. Consequently, 2D culture models are more susceptible to drug candidates or other substances applied than 3D culture models. This suggests that the dose rates of substances administered in 2D models differ from those *in vivo* and therefore may present certain limitations in accurately reflecting *in vivo* doses, which could potentially pose challenges in subsequent clinical research stages [15].

Moreover, 3D culture models are also attractive because of their advantages over animal models. Firstly, animal studies need to be supported by further steps in human concept for the translation of the results into human medicine. Use of human cells in 3D culture systems allow the evaluation of the pathological mechanisms and specific diseases considering the cellular interactions of human tissue. In addition, high throughput screening by 3D models is simpler and more cost-effective than animals producing comparable experimental results and without any ethical limitations in drug discovery research. 3D cell culture tumor models also allowed researchers to investigate tumor biology in a time-saving way by giving similar results in gene expression, cell cycle/proliferation/motility/cytoskeleton/adhesion/death, and tumor vascularization [5,6,17].

In particular, there is a huge advantage in the evaluation of the data related to specific diseases or pathological mechanisms that could be misunderstood if analyzed just with an animal model.

3. TYPES OF 3D CELL CULTURE SYSTEMS FOR DRUG DEVELOPMENT STUDIES

Currently, there are several ongoing studies aimed at improving *in vitro* cell culture models. To achieve a more accurate simulation of *in vivo* events *in vitro*, researchers are developing various artificial extracellular matrix (ECM) components that replicate the physical and biochemical properties of ECM components from both biological and non-biological sources. Cells have varying access to oxygen, nutrients, and other substances, similar to the conditions in living organisms. Further, methods such as co-culturing multiple cell lines, which interact *in vivo* are also utilized to better replicate the tumor microenvironment. By incorporating additional ECM-like structures and co-culture techniques, researchers achieve reliable results that are increasingly more representative of *in vivo* conditions [18].

Spheroid is a cellular aggregate, that includes one or more cell types preventing attachment of cells to a flat surface. The formation of spheroids is performed by various techniques either scaffold-based or scaffold-free.

In scaffold-based methods, cells are first grown in a matrix. This material can be synthetic, metallic, or natural. Scaffolds create an environment similar to ECM materials for cells and they grow using this usually reticulated matrix as a support material and form a 3D structure. Hydrogel is one of the most preferred scaffolds with its hydrophilic structure. It is also advantageous that it can be made from both natural and synthetic sources [18,19] and can be made of many different structures such as gelatin, matrigel, collagen, and fibrinogen [20]. Hydrogels are reticulated structures composed of non-crosslinked polymers. Because they are hydrophilic, they absorb large amounts of liquid and form structures similar to the soft tissues of a living organism [18]. Hydrogels mimic the ECM while allowing the penetration of substances by diffusion. Thus, growth factors, biomolecules, or cytokines secreted by cells can be delivered from cell to cell within the hydrogel. In addition, the structure of the hydrogels can be modified concentration-dependently providing the use of different types of cells in a wide range of experimental designs for multiple research needs [21,22].

In scaffold-free methods, cells are grown in a 3D tissue-like structure without using an external scaffold material to support the ECM environment. There are various techniques, which commonly lead cells to form acellular aggregates without attaching to the surface of the cell culture equipment. The hanging drop technique is a method in which the liquid placed on a flat surface remains suspended due to the surface tension and the cells inside collapse to the bottom of the liquid with gravity [18]. Cells gather together at the bottom of the suspended liquid and form a spherical structure due to the lack of a ground to attach. Although this method is cheap and easy to create 3D cell culture, it also has disadvantages such as the small size of the spheroid, the difficulty of drug testing as well as high-throughput screening [5,23].

The ultra-low attachment (ULA) plate method consists of coatings or polymers applied to the bottom of the plate to prevent cells from adhering to the plate surface [24]. These plates can be formed with polymers such as poly-HEMA or agarose. ULA plates are usually U-bottom to facilitate the cells to aggregate together and form a spherical structure. Spheroids created by this method generally have consistent sizes and maintain their stability during further experimental processes [25]. Conducting a larger volume of cell culture than the hanging drop method provides an advantage for high throughput drug screening. In a recent study conducted by Imamura et al. in 2015 using the ULA method, it was shown that 3D breast cancer cells were much more resistant to paclitaxel and doxorubicin treatment than 2D culture [26]. Recently, commercial companies have also developed ULA plates, contributing to the increasing demand for this method [21,27].

The pellet culture method is used in 3D culture to ensure that the cells stick together by centrifugation. In this method, cells are collected at the bottom of the tube with a relatively low centrifugal force (500 g, 5 min.). After the supernatant is discarded, it is resuspended in the medium formulated for the spheroids. The disadvantage of this method is exposing the cells to centrifugation, which may cause physical damage to the cells due to the shear stress [18]. In a drug screening study by [28], it was shown that spheroids of different sizes could be obtained by using the pellet culture method and the results demonstrated the reproducibility of pellet culture and the stability of spheroids is a reliable tool for drug development studies.

The spinner method includes the cells suspended in a bottle being mixed with circular movements via a mixer. In this way, the cells start to gather and form spheroids by the continuous work of a mixer to form spheroids stable and in similar sizes. Excessive speed of the mixer may damage the spheroids whereas low speed may result in exiguous formation and aggregation of spheroids. As it is a closed system, it is difficult to observe the formation stage of spheroids and further waste accumulation in a continuous culture is a notable disadvantage. However, the low risk of contamination and high yield of spheroids still make this method a useful tool for 3D spheroid culture [4].

An organoid is a 3D cellular structure that is derived from stem cells and resembles the relevant organ structurally or functionally. Although many scientists define organoids differently, most generally, an organoid can be defined as 3-dimensional tissue-like structures that can be created with more than one cell line, or created using stem cells, and thus can turn into different cells and tissue-like structures [29]. Organoids can be formed from different tissues of many different organs. These features make it a promising practice, as it mimics the *in vivo* environment very well and can involve several different tissues at the same. [30]. Organoids are more complex structures than spheroids as they resemble the multicellularity of the original organ. It was first created as a primary culture from organ or tissue pieces in a scaffold [4].

4. APPLICATIONS OF 3D CELL CULTURE MODELS

Although the use of laboratory animals for drug research has been a very common tool for drug discovery research, alternative methods have been developed recently to reduce the use of animals in testing drug candidates by high-throughput screening. For this reason, 3D methods attract much more attention as they are quite advantageous in drug research in terms of enabling appropriate disease modeling close to *in vivo* conditions and thus reducing the use of animals [31]. The use of animals is not only costly but also has ethical problems. However, tests performed in 2D cell culture do not provide enough information to continue clinical research. Therefore, 3D cell culture methods can be a tool that reduces costs, does not cause ethical problems, but still allows us to obtain enough data to continue clinical research [6,32].

3D cell culture is widely used in cancer studies. The reason for this is that when creating a tumor model, tumor spheroids are found in layers like a real tumor; In other words, there is a proliferative zone with a high oxygen content in the outer part, which is immediately exposed to stimuli from the environment, a quiescent zone in the middle, which is exposed to relatively less oxygen and external stimuli, and a hypoxic and necrotic zone in the inner part, where external stimuli almost do not affect. Thus, the structure of 3D cell culture enables this method to mimic *in vivo* conditions of tumor, where it is not possible in 2D cell culture [33].

The design of a 3D culture model should be performed according to the purpose of the study and the analysis methods to be used. Utilization of biological materials such as collagen, alginate, gelatin, matrigel, and hyaluronic acid is a well-known method to mimic ECM in scaffold-based 3D method. Although natural materials are biologically compatible with living organisms, it is more difficult to keep these materials stable. On the other side, synthetic materials, which are structurally stable are not as adaptable to cells as biological materials. To solve this problem, researchers created hybrid scaffolds. In this way, a hybrid structure can take 3D cell culture technology much further allowing tissue compatibility and stability [34]. It can potentially be used in genetic studies, cancer research, and drug development studies [35].

In addition, structural changes in ECM depending on the interactions of cells with each other in 3D culture lead to both the altered protein expression pattern and drug responses in comparison to 2D cell culture. Further, as the interaction of different cell types resident in the same tissue also results in altered protein expression and drug response, it is valuable to design culture models that better mimic *in vivo* cellular conditions by not only utilizing a 3D culture model w/w.o ECM components but also including different cell types interacting in tissue such as cancer epithelial cells and either fibroblast or T-cells [36]. As results obtained from monocultures using a single cell line might lead to major changes in drug research or mechanistic studies, combining the co-culture approach with 3D cell culture enables researchers to better model a wide range of diseases *in vitro* [37].

Many studies in the literature have proved the results of 3D culture modeling achieve better *in vivo* mimicking outcomes of drug screening tests for many cancer types such as glioblastoma [38] and bladder cancer [39]. Further, in the study by Imamura et al. [26], it was shown that 3D breast cancer cells were much more resistant to drug treatment compared to 2D culture. In addition, development studies of exosome-based therapeutics for breast cancer also showed that exosomes derived from 3D culture improve therapeutic effects in breast cancer treatment [40] . In a recent study, 3D cell culture-derived exosomes exhibit greater effects than 2D exosomes in cartilage repair in a rabbit cartilage defect model [41] . The 3D culture of MSC exosomes emerges as a potential therapeutic strategy for pro-angiogenesis through the activation of the HMGB1/AKT signaling pathway [42].

Similarly, patient-derived 3D organoids have also been shown to be a valuable tool for *ex vivo* drug testing and prediction of drug treatment response for many cancer types [43,44].

5. CONCLUSIONS AND FUTURE PERSPECTIVES

3D culture models are valuable tools and quite promising for applications in *in vitro* disease models, cell-based therapy strategies, and drug discovery by bridging the gap between 2D cell culture methods to animal models. Moreover, it is worth noting that cell culture provides additional benefits compared to animal models, particularly in terms of reducing the use of animals. Variable factors can be more easily controlled during *in vitro* testing, while the effects of complex systems and factors of *in vivo* models are uncertain.

The specificity and controllability of all conditions in cell culture also increase the reproducibility of experiments. While *in vivo* experiments may provide a more accurate reflection of real-life physiology compared to cell culture, it is important to acknowledge that physiological differences between humans and animals can be overlooked when testing treatments developed for humans on animals. Therefore, it is crucial

to consider these factors when evaluating the efficacy of treatments. Conversely, using human cells directly in cell culture eliminates such issues [15]. However, standardization of 3D cell culture techniques by using validated 3D cell culture models is crucial to assure the reproducibility of the experiments and applicability of the high throughput analysis especially for drug discovery research. As gene expression quantification is an essential part of testing drug response, new protocols adjusted to use for 3D cultured cells are needed such as direct lysis of 3D cell cultures for RT-qPCR gene expression quantification. Further, gene editing using CRISPR/Cas9 technology has been used to generate genetically engineered organoids for related disease models and drug response studies [45]. In conclusion, when various parameters, which are significant in choosing the optimal research tool, are taken into consideration, 3D cell culture can be evaluated as the optimal tool by being advantageous in many ways without downsides in comparison to 2D cell culture and animal studies. A brief comparison on indicated parameters mentioned in this review were summarized in Table 1.

Table 1. Comparison between 2D cell culture, 3D cell culture, and animal studies (darker blue indicates that the method is advantageous in this parameter)

2D Cell Culture	3D Cell Culture	Animal models
Monolayer	Multiple layer	Multiple layer
Cells grow up 2-dimentionally	Cells grow up 3-dimentionally similar to *in vivo*	Cells grow up 3-dimentionally in tissue organization
Cells have to attach plastic base	Cells attach to each others	Cells attach to each other in tissue organization
Morfologically flat and elongated cells	Cell shape is closer to *in vivo*	Cell shape in tissue organisation
All cells exposed same nutrients and oxygen	Different nutrient and oxygen content (outer layer of the cells have higher nutrient and oxygen content)	Different nutrient and oxygen content in tumor tissue (outer layer of the cells have higher nutrient and oxygen content)
Less mimicking *in vivo* condition	Better mimicking *in vivo* condition	Represent *in vivo* condition
Less expression of cell-cell extracellular matrix components	More cell-cell extracellular matrix components	Cell-cell extracellular matrix components in tissue organisation
Different drug response compared to *in vivo*	Similar drug response to *in vivo*	Represent *in vivo* drug response
Gene expression is different from *in vivo*	Similar gene expression to *in vivo*	Represent *in vivo* gene expression
Signaling pathways represent human cells	Signaling pathways represent human cells with better cell-cell communication	Signaling pathways do not represent human cells
Experimental results directly represent human cells	Experimental results directly represent human cells with better cell-cell communication	Experimental results need to be translated into human concepts
Easy experimental protocols such as transfection for gene silencing/overexpression	Experimental protocols are available but need to be improved	Difficult experimental protocols for genetic manipulation
Easy to handle in laboratory and low cost	More expensive than 2D, cheaper than animal studies	Hard to handle in laboratory and high cost

Overall, 3D cell culture models are unsurprisingly a great opportunity for personalized medicine making it more achievable by 3D culturing techniques for a wide range of patient-derived cell types to first test any drug candidate on cells *in vitro*, before exposing it to the human body. Thus, this approach can be a standard practice to observe adequate treatment outcomes of personalized medicine. Upon these advantages, it is suggested that research for developing 3D cell culture models is necessary to appropriately represent *in vivo* cell microenvironment and disease pathology.

Acknowledgements: This study was supported by the Scientific and Technological Research Council of Turkey (222S863).

Author contributions: Concept – F.G.A.Ç., B.D.B..; Design – F.G.A.Ç., B.D.B.; Supervision – B.D.B.; Resources – B.D.B.; Materials – B.D.B.; Data Collection and/or Processing – F.G.A.Ç.; Analysis and/or Interpretation – F.G.A.Ç., B.D.B.; Literature Search – F.G.A.Ç., B.D.B.; Writing – F.G.A.Ç., B.D.B.; Critical Reviews – F.G.A.Ç., B.D.B.

Conflict of interest statement: The authors declared no conflict of interest.

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