RESEARCH ARTICLE

Editorial Process: Submission:10/26/2021 Acceptance:02/13/2022

Recapitulating Tumor Microenvironment Using AXTEX-4D™ for Accelerating Cancer Research and Drug Screening

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Abstract

Objective: The formation of three-dimensional spheroid tumor model using the scaffold-based platforms has been demonstrated over many years now. 3D tumor models are generated mainly in non-scalable culture systems, using synthetic and biological scaffolds. Many of these models fail to reflect the complex tumor microenvironment and do not allow long-term monitoring of tumor progression. This has resulted in inconsistent data in drug testing assays during preclinical and clinical studies. **Methods:** To overcome these limitations, we have developed 3D tissueoids model by using novel AXTEX-4D platform. **Results:** Cancer 3D tissueoids demonstrated the basic features of 3D cell culture with rapid attachment, proliferation, and longevity with contiguous cytoskeleton and hypoxic core. This study also demonstrated greater drug resistance in 3D-MCF-7 tissueoids in comparison to 2D monolayer cell culture. **Conclusion:** In conclusion, 3D-tissueoids are more responsive than 2D-cultured cells in simulating important tumor characteristics, anti-apoptotic features, and their resulting drug resistance.

Keywords: Tissueoid- 3D/4D culture- cancer- drug discovery

Asian Pac J Cancer Prev, **23 (2)**, 561-571

Introduction

Globocan 2018 estimated incidence of 18.1 million new cancer cases and 9.6 million cancer deaths. Cancer is a global health issue that continues to be a challenge to treat and demand action (Zugazagoitia et al., 2016). The development of effective anticancer drugs significantly requires and depends on reliable in vitro high-tech screening systems (Kitaeva et al., 2020). The absence of reliable and effective in vitro screening models that could mimic key aspects of the tumor microenvironment, such as drug resistance and phenotypic changes to cells, impedes the reliable translation of in vitro findings into in vivo clinical models. The poor correlation between preclinical in vitro and in vivo data with clinical trials has an adverse impact on drug development costs, with the trial cost vary from $3 million to $347 million (Moore et al., 2018) (Martin et al., 2017). Only ~7% of anticancer drugs gain clinical approval which is much lower than drugs (~18%) for other diseases (Hay et al., 2014). Therefore, the action is required to develop effective and reliable in vitro models that reflect the in vivo tumor microenvironment and in vivo efficacy more accurately.

Two-dimensional (2D) monolayer cell culture has contributed immensely to understanding basic cancer research, disease modelling, drug discovery, and toxicity studies. However, 2D cell culture-based assays are mostly non-indicatory, non-predictive, and non-representative of the real tissues (Jensen and Teng 2020; Kapalczynska et al., 2018). To this end, 3-D tumor spheroids are an attractive alternative to 2-D cell culture as they can recapitulate many aspects of the tumor microenvironment, including paracrine effects, cell-cell interactions, and extracellular matrix deposition (Breslin and O'Driscoll, 2013; Nyga and Loizidou, 2011). Furthermore, many environmental factors inducing metabolic and oxidative stress in tumor cells such as hypoxia and the formation of a necrotic core can also be recapitulated by 3D systems (Sutherland, 1988). Additionally, 3-D spheroids also reduce the time and costs associated with translating laboratory findings into animal models and can accelerate the process of anticancer drug development (Kitaeva et al., 2020).

The establishment of 3D cell culture methods has been based on either scaffold-based, scaffold-free gels, bioreactors/or microchips (Benien and Swami, 2014; Jensen and Teng, 2020). Various scaffold-based platforms have been described in the literature, which can be biological (hydrogels such as collagen, gelatin, alginate, or chitosan), synthetically engineered (Polyethylene glycol (PEG), polylactic acid (PA), polyglycolic acid (PGA)) to

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emulate key properties of ECM. However, these systems have been investigated mainly for tissue engineering purposes, where they can be used as delivery vehicles for cells and drugs (Abdalla, 2019). Moreover, a synthetic scaffold-based 3D system pose challenges related to oxygen availability, heterogeneities present in the synthetic cellular microenvironment, and complication in imaging and cell analysis (Fang and Eglen, 2017). Imaging is difficult in 3D cell culture due to plate incompatibility with microscopes and uncentered spheroids in well plates. Similarly, fluorescence microscopy is also a challenge in 3D cell cultures because unlike 2D cell culture where only a single xy image is taken, 3D cell cultures must obtain a z stack by taking a series of xy images at fixed intervals in the vertical direction by automated microscopes. Having to take a series of xy images to obtain a z stack at high magnification objectives often increases the time, storage space and are not suitable for high throughput setting. Therefore, continued improvement in the imaging of 3D culture system is needed (Jensen and Teng, 2020).

Similarly, scaffold-free systems, often referred to as spheroids, can be produced in various ways, such as the hanging drop technique. However, the routine use of such models for drug development has been hampered due to the destruction of spheroids during their analysis, manipulation, and by a lack of standardized procedures to perform downstream assays (Courau et al., 2019; Langhans, 2018).Therefore, the lack of a robust 3D cell culture model led to the continued improvement in the 3D culture system and technology.

The study addresses the existing challenges in the prior art to achieve the functional outcome by utilizing a three-dimensional model that closely recapitulate the tumor microenvironment. The model has been developed on AXTEX-4D™ platform. The fabric of AXTEX-4D™ includes non-woven fabric, porous, an inert polymer that allows the cells to attach, grow into its spaces and, help in acquiring 3D like features (Baru et al., 2021a). Patterns like intercellular bridges were observed in 3D spheroids which mimic the real tissue morphology(Baru et al., 2021a). Therefore, we called the system a 3D tissueoids cell culture system. 3D-tissueoids attain the three dimensions with time, which is provided by the system's ability to generate the 3D tissueoids in much less time and for an extended period (365 days). We have previously provided an explanation comparing AXTEX-4D™ with its peer platform (Baru et al., 2021b).

In this study, cancer tissueoids demonstrated the basic features of 3D cell culture, such as universality, rapid attachment, longevity, viability, hypoxia, and cytoskeleton arrangement, which are required for the 3D growth of cell lines and biopsies. Additionally, the platform can be utilized for multiple downstream assays such as monitoring and quantification of cell growth by microscopy and absorbance-based methods. Tissueoids generated on AXTEX-4D™ platform can be easily transferred from the platform to any desired format of ELISA plates. We have further compared the efficacy of drug sensitivity with 2D-monolayer culture. Overall, the current study characterizes and highlights the utility of the preclinical 3D tissueoids model, which may be useful for analyzing features of growth and drug sensitivity of cancer cells.

Materials and Methods

A methodological flowchart describing methods to measure characteristics of three- dimensional (3D) tissueoids model is shown in Figure 8.

Cell Lines

The various human cancer cell lines (MCF-7 (Breast Cancer), PC3 (Prostate cancer), A375 (Skin Melanoma), HepG2 (Liver Cancer), NIH-3T3 (Mouse fibroblast cells), CHOK1 (Chinese hamster ovary), HEK-293 (Human embryonic kidney) were obtained from ATCC. All the cancer cell lines were grown in EMEM (MCF-7 and HepG2), F12K (PC3 and CHOK-1), and DMEM (A-375, NIH-3T3,and HEK-293) supplemented with 2mM glutamine (Sigma- Aldrich, St; Louis, MO, USA) and 10% FBS (Gibco) at 37°C humidified condition with 8% CO_2 under static condition.

2D cell culture

For 2D culture, cells were seeded at around 60-70% confluency, i.e., approximately 0.8 x 10^6 in a 60 mm dish. At 80-90% confluency of cells, media was removed, and cells were washed with PBS, trypsinized, and centrifuged at 1,000 rpm for 5 minutes. Finally, cells were resuspended in an appropriate volume of respective media and plated in 96 well plate (5,000 cells/well), depending upon the experiment.

3D cell culture

Tissueoids were formed on AXTEX-4D™ platform as described previously (Ambica Baru 2020). Briefly, cells were seeded at around 60-70% confluency, i.e., approximately 0.8 x 10^6 in a 60 mm dish. The cells were washed with PBS at 80-90% confluency, trypsinized, and centrifuged at 1,000 rpm for 5 minutes. The cell suspension was made in such a way that 1 ml of respective media contain 0.25 X 10^6 cells so that 20µl of the media contained a cell number of 5,000 cells per drop. The drop was pipetted onto the inverted lid of 60mm dish and dish was filled with PBS. Following that the lid was inverted, and the drops were poured on AXTEX-4D™ platform for tissueoids formation in the desired format and analyzed for attachment and growth by using phase contrast inverted microscope (Nikon Eclipse TS100) and SEM at various magnifications. The analysis of tissueoids assembly was done by measuring diameter, area, and perimeter of tissueoids using Biowizard software (Dewinter optical Inc). Shape P2A = (Perimeter)2/4π × (Area) represents the object roundness and is equal to 1 for a perfect circle; for less spherical objects, P2A becomes larger than 1 (Mittler et al., 2017).

Scanning Electron Microscopy (SEM)

Tissueoids grown on AXTEX-4D™ platform was fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in PBS, pH 7.4. After that, samples were vacuum dried for 10 mins with 0.1 mbar pressure followed by mixing of argon

gas. Finally, the samples were coated with gold particles using Agar Sputter Coater (AIIMS, New Delhi, India). The coated samples were analyzed using SEM (EVO-18 Research, Zeiss) at 500, 1,000, and 1,500X magnification.

Confocal Microscopy

Briefly, samples were fixed in 3.7% formaldehyde solution permeabilized with 0.1% TRITON X-100and washed three times with 1X PBS. To observe cytoskeleton of tissueoids, cells were stained with phalloidin–tetramethylrhodamine B isothiocyanate conjugate solution (50µg/ml) for 45 minutes and counterstained with NucSpot Live 488 nuclear stain (1:1,000) for 10-15 minutes at RT.

For viability, samples were washed with PBS and stained with Calcein AM (100ng/ml) for 30 minutes. Similarly, for hypoxia, samples were stained with HIF-1α specific antibody (Cat#ab2185). Briefly, cells were fixed with 4% PFA for 15 minutes and washed with 1X PBS. After permeabilization with 0.1% Triton-X for 4 minutes, samples were blocked with 1% BSA for 1hour at RT and next incubated with 1ug/ml HIF-1α antibody for overnight at 4˚C. Finally, tissueoids were incubated with goat anti-rabbit FITC conjugated secondary antibody (Abcam#150077) at 37˚C for 2 hours and washing was done with PBS. All the samples were mounted with prolong gold antifade reagent (Thermo) and analyze by confocal microscopy. Images were acquired using Nikon's confocal microscope (A1 R HD 25) and analyzed with the NIS Elements software (Nikon Corporation, Tokyo, Japan). To avoid inter-channel mixing (405 nm, 488 nm, and 561 nm), pictures were captured separately with individual laser and were merged later.

Treatment with clinical Drug

Both 2D and 3D tissueoids (5,000/well, n=3) were treated with doxorubicin and 5FU for 48 hours at the various doses. Finally, Prestoblue assay has been done as described previously (Baru et al., 2021a). Briefly, prestoblue reagent (1/10th of the total volume of media) was directly added to the control and treated cells and incubated for 2 hours at 37 °C. The metabolic rates were measured by the amount of relative fluorescence unit (RFU) at Excitation 560 nm and Emission 590nm using a spectrofluorometer plate reader (SPECTRA MAX GEMINI EM, Molecular Devices).

Results

Universality of AXTEX-4D™: Formation of 3D tissueoids from various mammalian cell lines and primary culture

The universality of 3D culture systems is particularly attractive as cells of different origins could be grown to enhance cancer research and drug development significantly. Therefore, we decided to define the universality of AXTEX-4D™ platform by growing cancer and transformed cells/tissues of different origins. This study used four mammalian cell lines (cancer: MCF-7, PC3, A-375; transformed: HEK-293,) and four primary tumor tissues (Gastric, Thyroid, and colon). Approximately one to two days after seeding on AXTEX-4D™ platform, tissueoids were formed by all cell lines and tissues of different origins.

Previous findings have shown that tumor cell lines of diverse origin, when cultured in 3D spheroid conditions, form two distinct groups according to the architecture of spheroid shapes: i) tight spheroids and ii) loose spheroids (Vinci, Box and Eccles 2015; Vinci et al., 2012). However, the criteria used in the literature

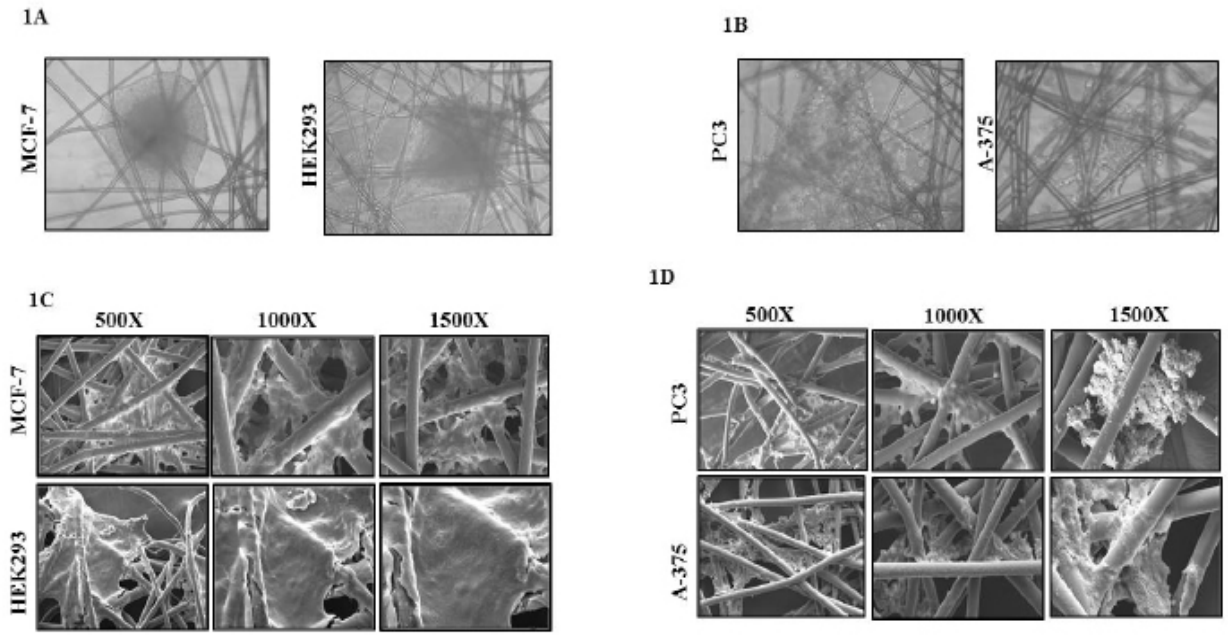

Figure 1. Formation of Compact and Loose 3D Tissueoids on AXTEX-4D™ platform. Bright field microscopy images of A) MCF-7, HEK293 and B) PC3, A-375 tissueoids. Scanning electron microscope (SEM) images of A) compact and B) loose 3D tissueoids of indicated cell lines on the AXTEX-4D™ platform at 500X, 1000X and 1500X magnification. Each experiment was repeated four times.

Table 1. P2A of Various Cell Lines

SN	Cell line	Area	Perimeter	P2A (Perimeter)^2/4π × (Area)
1	MCF-7	1079193.282 Micron Square	3847 microns	~1.0
2	HEK293	1064552.407 Micron Square	3835 microns	~1.0
3	PC3	1569082 Micron Square	5123	1.3
4	A-375	182543.110 Micron Square	1882	1.5

are rather visual and arbitrary (Mittler et al., 2017). We therefore decided to define a measurable parameter of spheroid assembly. Spheroid formation was analyzed using Biowizard software (Dewinter Optical Inc, India) (Figure 1). Different parameters such as perimeter and area were analyzed to calculate P2A. We found Shape P2A is the best direct morphometric parameters to characterize spheroid roundness; when it attains the minimum, the aggregates are considered round and spheroid(Mittler et al., 2017). The value of P2A was found to be close to one in case of HEK-293 and MCF-7 in comparison to PC3 and A-375 cells (Table 1). Therefore, the aggregates of HEK-293 and MCF-7 are considered round and spheroid (Figures 1A,B). 3D tissueoids assembly on AXTEX-4D™ platform was further confirmed using scanning electron microscopy (SEM) at higher magnification. Visual examination revealed that HEK-293, and MCF-7, cells started to form compact tissueoids (CTs) (Figure 1C). While PC3 and A-375, cells grow in the AXTEM-4D™ platform, and although tissueoids formed, they were loose (Figure 1D). Interestingly, patterns like grooves and folds, which mimic the real tissue morphology were also observed in 3D tissueoids.

In addition, we tried tissueoid culture with a small biopsy tissue of different origins to determine whether our platform is applicable to samples obtained from the biopsies. As shown in Figure 2 the cells from a biopsy successfully cultured to form tissueoids on AXTEM-4D™ platform. These data collectively indicate that cell growth in the 3D tissueoids model allows the cells to retain cellular organization, resembling the in vivo condition more closely.

Tissueoids formation with minimal number of cells with rapid attachment

The ability to generate spheroids from small cell numbers is particularly relevant when dealing with rare patient-derived cells or cells with high mortality rates (Raghavan et al., 2015). SEM analysis showed that the tissueoids formed from 25 cells are suitable for image

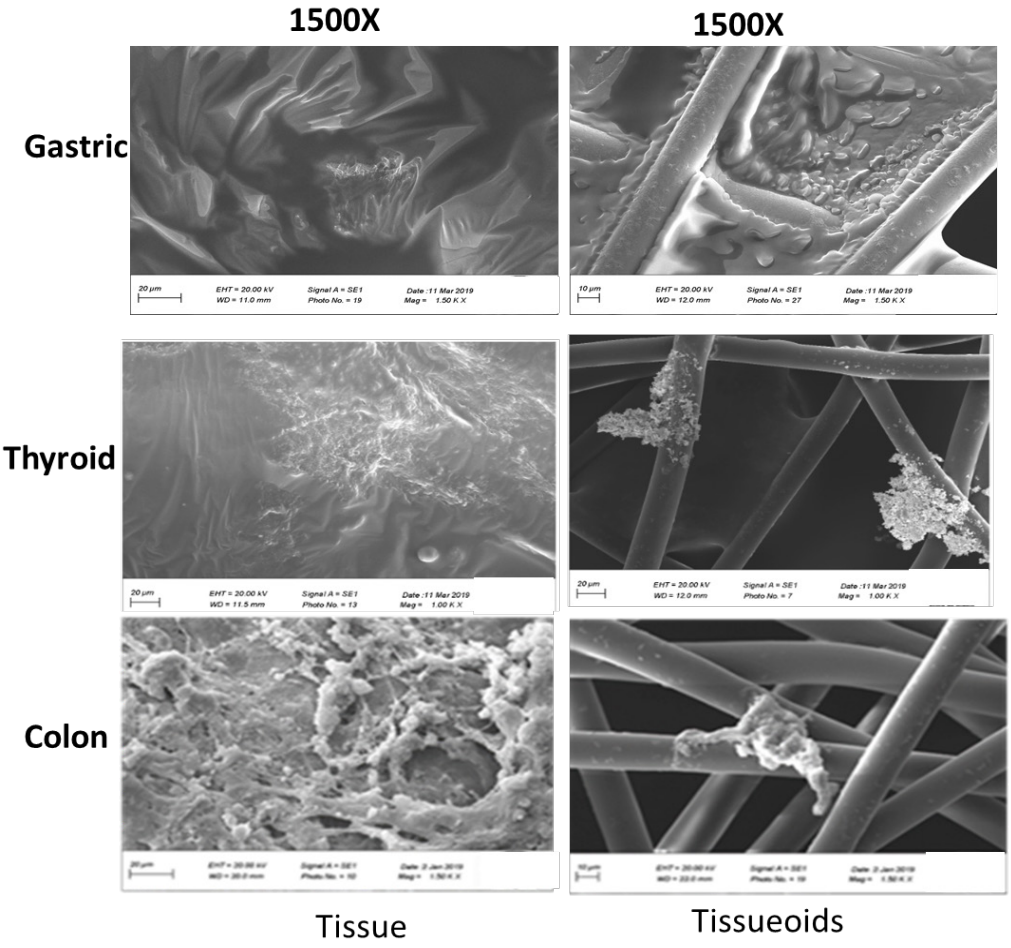

Figure 2. SEM Images of Primary Tumors Grown on AXTEX-4D™ Membrane at 1500X Magnification.

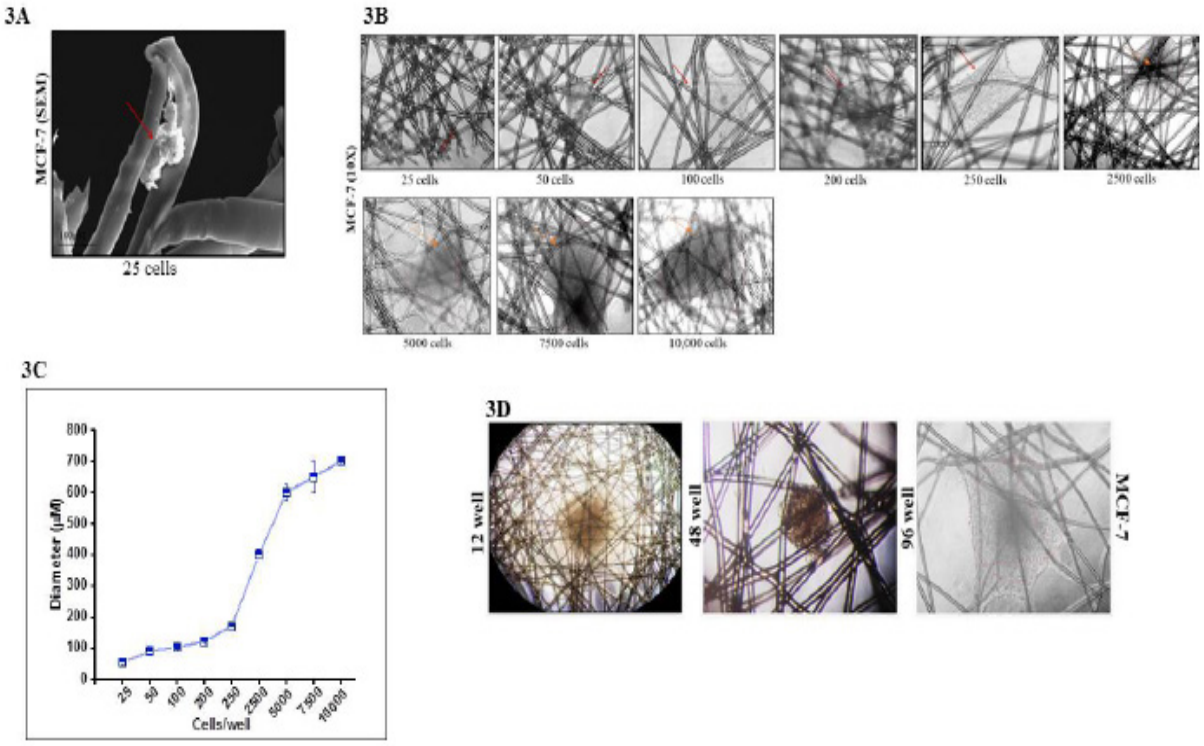

Figure 3. Diameters of 3D Tissueoids with Various Cell Densities. SEM image of MCF-7 3D tissueoids formed with 25 cells/well. B) Phase-contrast microscope images showing the diameter of MCF-7 3D tissueoids formed with indicated cell densities C) Graphical representation of diameters formed with various cell densities. Values are means ± S.E.M. from n=3 D) Phase-contrast microscope images showing the formation of MCF-7 3D tissueoids in indicated cell formats. Each experiment was repeated four times.

acquisition and analysis (Figure 3A). At this density, the average diameter of tissueoids was consistent with a value of 55 ± 5µm, yielding a coefficient of variation of 9.0%. These results suggest the adaptability of the AXTEX-4D™ platform in forming 3D tissueoids when the sample availability is minimal.

To observe the effect of cell density on tissueoids formation, tissueoids of different cell numbers of

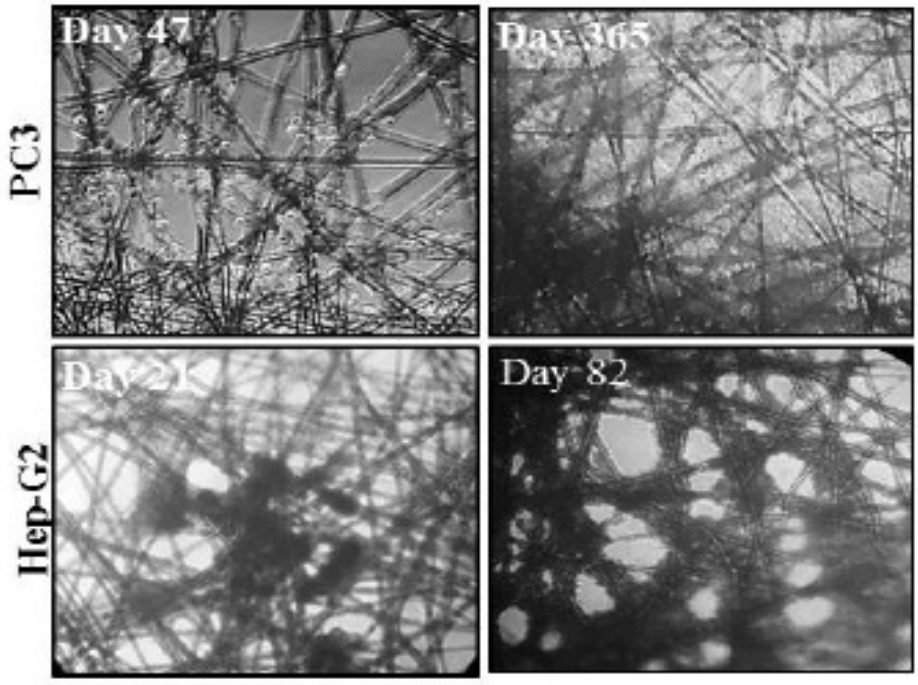

Figure 4. Longevity of 3D Tissueoids. 3D tissueoids of indicated cell lines were grown on AXTEX-4D™ platform and phase contrast microscope images at 10X magnification showing longevity of PC3 and Hep-G2 3D tissueoids at indicated time points

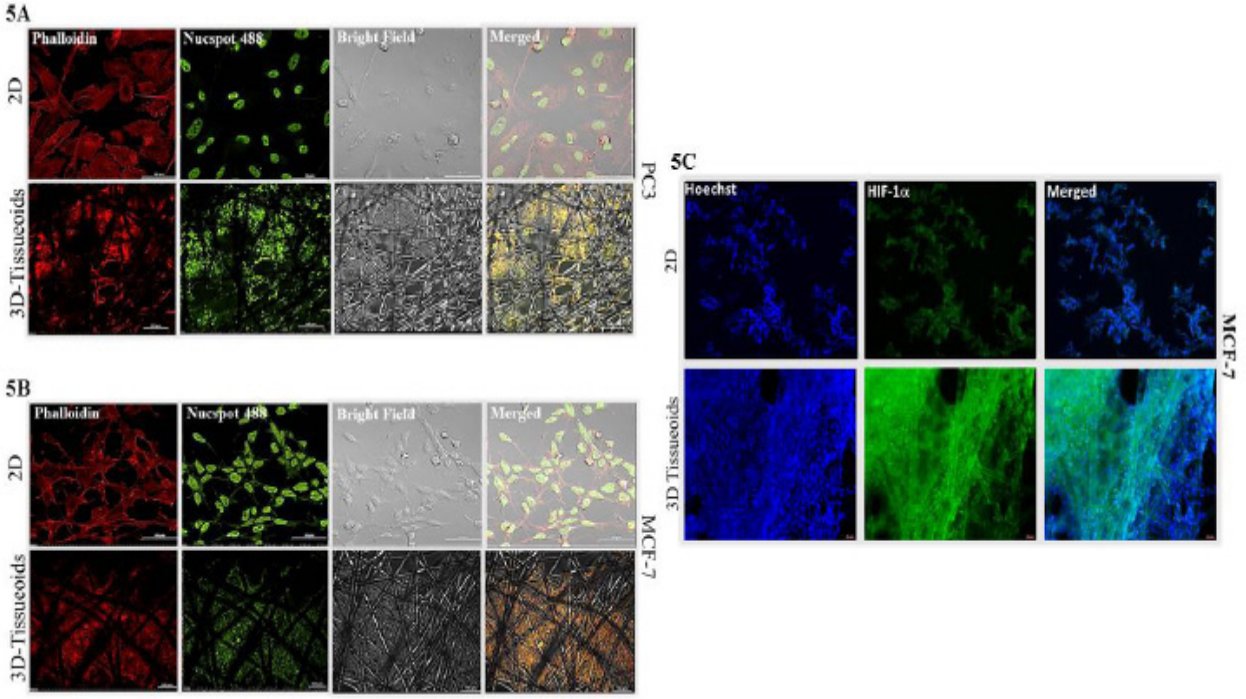

Figure 5. Cytoskeleton Arrangement of 2D and 3D Tissueoids.Confocal microscope images depicting cytoskeletal arrangement in 2D monolayer and 3D tissueoids using (A) PC3 and (B) MCF-7. Staining was performed with anti-Phalloidin antibody (Red) and Nuncspot Live 488 (green- nucleus stain) C) Immunofluorescence of MCF-7 cells depicting HIF-1α expression (green) in 2D and 3D tissueoids. Each experiment was repeated three times.

MCF-7 were formed on AXTEX-4D™ platform using phase-contrast microscopy. Obviously, the larger the original cell sheet, the bigger the 3D tissueoids obtained from the MCF-7 cell line. Our results support the obvious previous findings where it has been shown that increasing the density of cell seeding resulted in a linear increase in spheroid diameter (Dubois et al., 2019) (Figures 3B-3C). The optimal conditions required to form tissueoids of approximately 500-600 µm in diameter (Ivanov et al., 2014; Mittler et al., 2017) were determined to be 5,000 cells/well for 24-48 hrs. The diameter of >500 µm has been shown to have more pronounced necrotic core, deviate from linearity(Ivanov et al. 2014), and optimum for microarray assays (Hardelauf et al., 2011).

We have also tested the scaffold utility in forming the tissueoids with different plate formats from 12, 48, and 96 well plate and found consistency with all the plate format suggesting the versatility of the AXTEX-4D™ platform to form tissueoids for desirable experimental needs (Figure 3D).

Longevity and viability of 3D tissueoids

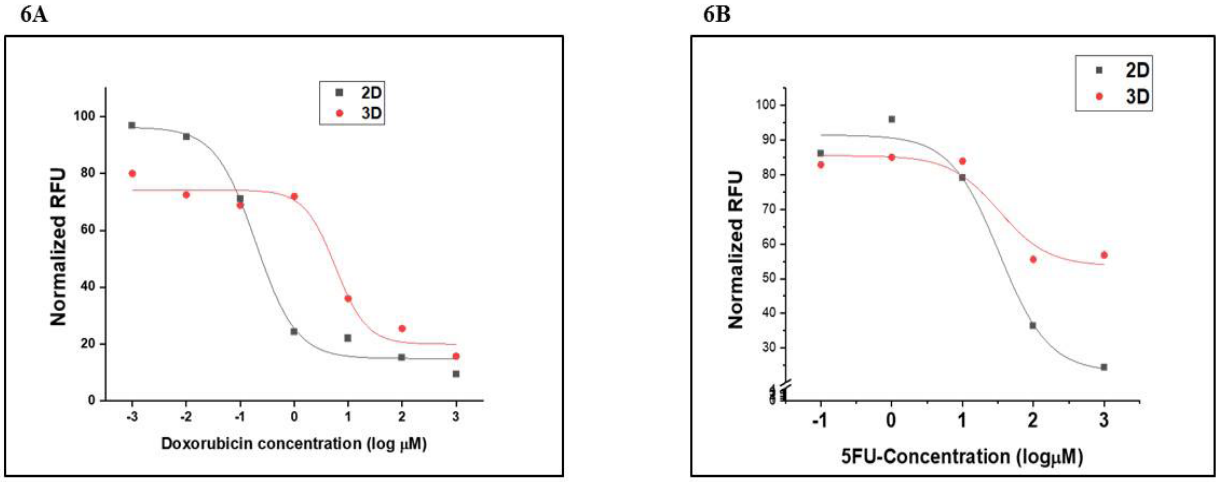

Figure 6. Effect of Doxorubicin and 5FU Drug Sensitivity in Conventional 2D and Indicated 3D Cell Culture Models. MCF-7 3D tissueoids were treated with indicated concentrations of doxorubicin and 5FU for 48hrs and cell viability using PrestoBlue. Each experiment was repeated three times. Values are means±S.E.M.

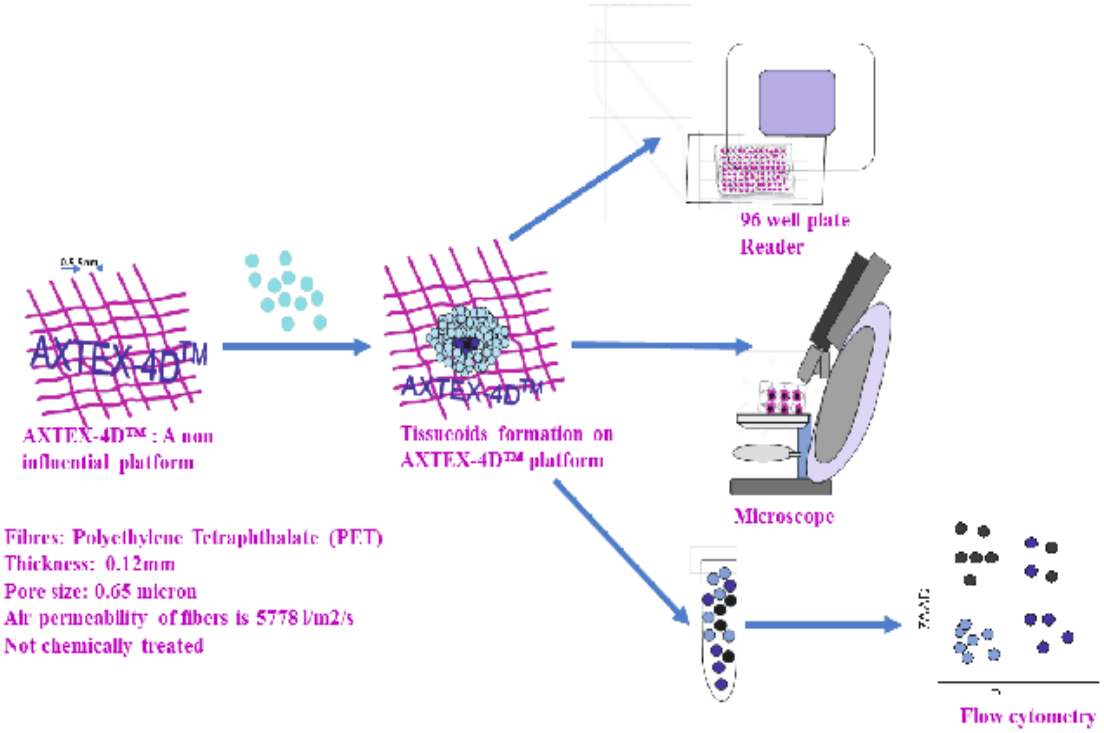

Figure 7. Applications of AXTEX-4D™ Platform

Longevity of the spheroid, a crucial attribute, is required for increasing flexibility and robustness of the 3D system to design the experiments (Christopher R. Cox). To this end, the longevity and viability of the tissueoids were observed in two cancer cell lines: Hep-G2 and PC3. To show the length in time during which tissueoids remained in culture, the longevity of tissueoids derived from HepG2 and PC3 cells was observed. Tissueoids were generated in 12 well formats with media change after every third day. Different fields on different days were captured. This study demonstrates that tissueoids of HepG2 and PC3 were able to survive till 82 and 364 days with an increased number of cells and density, hence allowing the three dimensions to the tissueoids with time. The fourth dimension is provided by the AXTEX-4D™ platform to generate the 3D tissueoids for an extended period of time(Kundu P US 20200326330 A1 2020). We speculate that the smaller life span of Hep-G2 cells

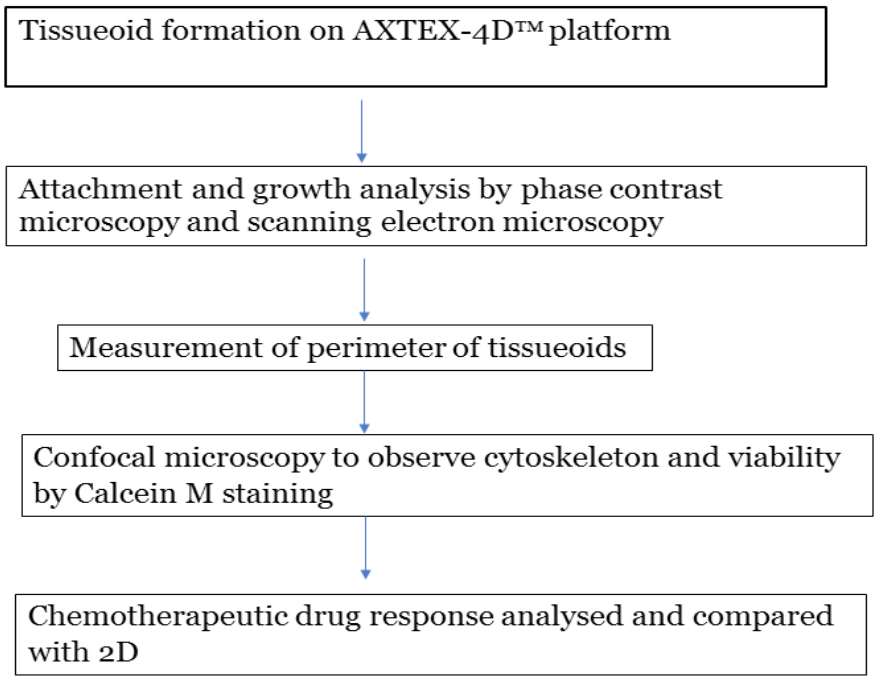

Figure 8. Flowchart Describing Methods to Measure Characteristics of Three- Dimensional (3D) Tissueoid Model

may be due to their high metabolically active nature and due to which these cells grow rapidly and, after a certain period, come out of the matrix (Chen et al., 2018) (Figure 4). On the other hand, prostatic cell metabolism does not conform to the "classical" Warburg metabolic phenotype exhibited by other solid tumors (Abu El Maaty et al., 2017). These results suggest the applicability of the platform for time-dependent single and multi-drug studies. In our previous study (Baru et al., 2021a) we have shown the viability of tissueoids at different time points (5, 25, 108, and 365 days) by Calcein AM staining, which is used to determine cells' viability. The increase in fluorescence over the period suggesting the long-term viability of tissueoids.

Cytoskeleton arrangement and hypoxic core in 3D Tissueoids

The dimensionality of a cell's environment greatly influenced the structure and distribution of the cytoskeleton (Walker et al., 2020). To determine how a 3-D arrangement of cells influences cytoskeletal organization, we investigated the arrangement of actin in PC3 and MCF-7 cells in comparison with 2D cells. 3D cultures of prostate cancer cells (PC-3) and breast cancer cells (MCF-7) were used to determine whether the AXTEX-4D™ platform was suitable for two cell types forming loose or compact tissueoids respectively. PC-3 and MCF-7 tissueoids were fixed and stained with Phalloidin to visualize actin filaments (F-actin) and Nucstop-488 to stain the nuclei. A regular F-actin arrangement around the nucleus was observed (Figures 7a and b). Confocal analysis also showed significant changes in the architecture of 3D MCF-7 and PC3 tissueoids, where contiguity of the cells was visible. In contrast, the cells in 2D were elongated and scattered with defined edges and margins (Figures 5A and 5B). These results suggest that AXTEX-4D™ platform can be used on cell types forming compact and loose spheroids.

Furthermore, multiple hypoxia-driven cell behaviors and phenotypes reflect key characteristics of in vivo growing tumors (Liverani et al., 2019). Hypoxia-inducible factor (HIF) functions as a master transcriptional regulator for adaptation to hypoxia by inducing adaptive changes in gene expression for regulation of proliferation, angiogenesis, apoptosis and energy metabolism. Cancers with high expression of the alpha subunit of HIF (HIFα) are often malignant and treatment resistant. Therefore, the development of a molecular probe that can detect HIF activity has great potential value for monitoring tumor hypoxia. We observed that HIF-1 α fluorescence was significantly higher in the core than the periphery of the 3D tissueoids for MCF7 cells when compared with 2D monolayer culture. As hypoxia is a pro-metastatic factor enhancing the malignancy of various tumors, our platform can be used to investigate the role of hypoxia in tumor cell types.

Effect of chemotherapeutic agents on viability

To explore the mechanism of differential sensitivity to two different chemotherapeutic agents in the 2D- and 3D-cultures, the viability of the MCF-7 cell line was compared in 3D tissueoids model. The relative growth rate of the MCF-7 breast cancer cells in the presence or absence of the 2 chemotherapeutic agents (Doxorubicin and 5FU) in 2D- or 3D-cell culture is shown in Figure 6. The range of drug concentrations used was set based on clinical pharmacokinetic data in order to explore the clinical relevance of the results. Specifically, 48-hour treatment with seven doses (0.001, 0.01, 0.1, 0, 1, 10, 100 μM) of doxorubicin and five doses of 5-FU (0.1, 0, 1, 10, and 100μM) was carried out in 96 well plate. Results of prestoblue staining for doxorubicin indicated that the MCF-7 3D tissueoids that developed on AXTEX-4D™ platform tended to show high IC50 of 5.82μM than 2D culture (IC_{50}=0.19μM) (Figure 6A). For 5FU, there was no clear difference in IC-50 between the 2D- and 3D-culture of MCF-7 (Figure 6B). These results support the previous findings (Imamura et al., 2015; Koch et al., 2021) and suggest that the 2D and 3D cultures responded differently to chemotherapy; the 3D cultures were more resistant to doxorubicin, but not to 5FU than the 2D cultures. Taken together, MCF-7 cells cultured as 3D tissueoids displayed markedly higher resistance to selected chemotherapeutic drugs than 2D cultures. These results further suggest that 3D tissueoids could be an optimal drug-testing model, that mimic human disease.

Discussion

In the last decade, multiple attempts have been made towards the improvement of preclinical models to accelerate cancer research. Despite these efforts, most of the available models fail to recapitulate several important attributes of the tumor microenvironment and do not reflect the complexity of human tumors (Estrada et al., 2016; Hirt et al., 2015; Weigelt et al., 2014; Kitaeva et al. 2020). However, against the optimism of these exciting possibilities, critical challenges for 3D cell cultures include assay validation, destruction of spheroids, and a lack of standardized procedures to perform downstream assays (Courau et al., 2019; Fang and Eglen, 2017; Langhans 2018). Besides, most in vitro 3D experiments can only be carried out for few weeks (~4 weeks), which impedes examination of long-term effects of the microenvironment or drug treatments on disease progression (Bialkowska et al., 2020; Edmondson et al., 2014).

In this work, we aimed to describe the characteristics of a 3D tissueoids model that was developed using AXTEX-4D™ platform (Baru et al., 2021a). This study showed that 3D tissueoids possessed cellular morphology with evident actin cytoskeleton arrangement along with hypoxic core and showed longer lifespans compared to their counterparts cultured on the standard 2D monolayer. The longer life span of tissueoids (~365 days) makes them a suitable model for long-term cell cultures and allows for time-dependent studies, drug combinations, and multi-dosing studies with the potential for developing novel therapeutics. In contrast to this, other time-intensive 3D models have been reported to have a short life span due to the unavailability of the restricted flow of nutrients and gases (Edmondson et al., 2014). Furthermore, cell seeding density and geometry also play an important

role in experiments that require long-term spheroid monitoring. These two parameters determine the size of spheroid and total imaging time. Though the optimal spheroid size depends on the assay requirements, such as the presence or absence of hypoxic core, it usually falls within a range of 200–800 μm diameter (Ivanov et al., 2014; Mittler et al., 2017). Furthermore, the hypoxic core rapidly created by the fast-growing spheroids can interfere with phenotypic measurements. As shown previously, fast-growing spheroids produced an apoptotic signal comparable to or even greater than that induced by a drug, thus interfering with cytotoxicity measurement (Mittler et al., 2017). In such cases, the analysis of smaller spheroids may be preferable. Thus, we want to observe if the platform can be used for fast growing cells such as MCF-7 to form 3D tissueoids using minimal number of cells. We have demonstrated that cells even as low as 25/well, could form tissueoids efficiently in less than 24hours with high reproducibility. This feature suggests the flexibility and adaptability of the platform to generate 3D tissueoids when the sample availability is minimal.

Several previous studies have shown that 2D-cultured cells tend to overestimate the efficacies of chemotherapeutic drugs compared with 3D-cultured cells (Bleul et al., 1996; Herter et al., 2017)and hence numerous anticancer drugs are eliminated during clinical development. In contrast, 3D-culture systems have been shown to better simulate the in vivo tumor microenvironment than a 2D-culture (3–7). Consistent with these studies, our present study showed that the cell lines producing round 3D tissueoids are more resistant to doxorubicin in a 3D- than in 2D-culture (Figure 2A). These findings suggest that a 3D-culture potentially avoids the overestimation of antitumor efficacy observed in a 2D-culture. It is noteworthy that the phenomena were observed with the clinically achievable drug concentrations calculated based on the AUC in cancer patients, which were less than the maximum drug concentrations (Cmax, Table I). However, we did not find any difference in IC_{50} values when the same cells were treated with 5FU. Our results are in line with the already reported literature where 5FU tested on different 3D models and found to be sensitive like 2D models(Imamura et al., 2015; Koch et al., 2021). This in vitro difference must be considered in future approaches for determining the ideal in vitro systems that mimic human disease. Therefore, 3D-tissueoids model could be applied as a drug testing platform to screen large compound libraries.

However, several limitations of our study warrant mention. Firstly, this study was derived from the nature of an in vitro model. Despite several 3D-culture systems, none of them are considered a standard method, and it is unclear which system is the most clinically relevant (Breslin and O'Driscoll, 2013; Lovitt et al., 2014; Rimann and Graf-Hausne, 2012; Weigelt et al., 2014). One observation is that 3D-culture of cell lines will never accurately, fully represent the tumor microenvironment in vivo, because the latter have interactions with stromal tissues or blood perfusions (Imamura et al., 2015). Co-culture with stromal cells or vascular cells may partially solve these issues. Secondly, patients derived tumors (PDXs) are considered a potential drug screening platform for the next generation (Rizzo et al., 2021; Williams, 2018). Therefore, we are further investigating the application of our platform with PDXs and co-culture with stromal cells, and the process is under investigation in our laboratory.

Overall, the current study highlights the characteristics and utility of the newly developed 3D tissueoids model, which possess basic features for the 3D growth of cell lines and could be beneficial in drug screening assays (Figure 7). Further studies are required extensively to elucidate the mechanistic aspects of the tumor microenvironment, sensitivity towards various clinically relevant treatments with special reference to biopsy tissue from patients.

Author Contribution Statement

AB: Designed and planned the experiments, conducted experiments, analyzed the results, and writing. SM: Concept design, interpretation of data, and critically reviewed the manuscript. PK: Study concept, design, interpretation of data, providing scientific inputs SS: Execution and data analysis. BPD: Planning and execution of experiments. RG: Data analysis and drafted the manuscript. NM: Study concept, design, interpretation of data, providing scientific inputs and drafting of the manuscript.

Acknowledgements

We thankfully acknowledge SAIF-Facility, AIIMS New Delhi for Scanning Microscopy facility, Confocal facility, IGIB, New Delhi for assistance. We also thank Avijit Das, Chairman for his critical discussion on experimental study. The work was funded by Premas Biotech Private Limited, India. There is no conflict of interest among authors of the manuscript. The study is approved by the Institutional Ethics committee at Premas Biotech Pvt. Ltd. AB: Designed and planned the experiments, conducted experiments, analyzed the results, and writing. SM: Concept design, interpretation of data, and critically reviewed the manuscript. PK: Study concept, design, interpretation of data, providing scientific inputs SS: Execution and data analysis. BPD: Planning and execution of experiments. RG: Data analysis and drafted the manuscript. NM: Study concept, design, interpretation of data, providing scientific inputs and drafting of the manuscript.

Funding

The work was funded by Premas Biotech Private Limited, India.

Competing Interests

All authors are employees of Premas Biotech Private Limited.

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