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Overcoming obstacles in three-dimensional cell culture model establishment: Approaches for growing A549 non-small cell lung cancer spheroids using a clinostat system

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ABSTRACT

Introduction: Non-small cell lung cancer (NSCLC) accounts for 80–85 % of lung cancer cases globally. And the A549 cell line is widely used in pharmacological and toxicity screening. Due to its popularity as a NSCLC model, it was inevitable that three-dimensional (3D) cultures of A549 cells would be established. 3D models increase physiological relevance, and their advanced structure allows researchers to obtain more translatable and reliable results. However, establishing this cell line as a 3D model may come with challenges, like clumping.
Methods: In this study, A549 spheroids were established using a clinostat-based rotating bioreactor system and were characterised in terms of morphology, planimetry, and viability.
Results: The main challenge faced included continuous aggregation of the spheroids, which constrained growth and development. This challenge was successfully overcome by supplementation with ascorbic acid, foetal bovine serum coating, and minimising handling, and a NSCLC mini-tumour model was established and semi-characterised. The spheroids survived for 25 days and had a significant increase in growth.
Conclusion: The A549 spheroid model cultured in a clinostat-based microgravity system was shown to be stable, viable, and suitable to be used in pharmacological and toxicological investigations.

1. Introduction

Lung cancer affects millions of people worldwide, and it is predicted that the number of new cases will increase over the next few years (Luo et al., 2023). Non-small cell lung cancer (NSCLC) accounts for about 80–85 % of lung carcinoma cases, with a relatively low survival rate (Yang et al., 2018). Despite many years of research efforts, lung cancer remains a global burden and causes more deaths than any other cancer (Bray et al., 2018). The A549 human lung adenocarcinoma spheroids are used as a preclinical screening cancer model for pharmacological effects and toxicity assessments (Du et al., 2021; Eguchi et al., 2022; Ito et al., 2021; Naso et al., 2021). Additionally, A549 spheroids are utilised to simulate the alveolar type II pulmonary epithelium, facilitating the study of the metabolic processing of lung tissue to identify mechanisms of drug delivery (Katsumiti et al., 2020) and to serve as a model to study respiratory infections, including influenza and severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) (Saleh et al., 2020; Sasaki et al., 2021). The A549 cell line has been established as a three-dimensional (3D) model before, utilising either static or dynamic systems. The static 3D culture systems grow spheroids on a scaffold or matrix without continuous culture media flow. Dynamic 3D culture techniques generate constant motion, encouraging cells to aggregate together to form spheroids (Wu et al., 2021). Notable static methods include ultra-low adhesion plates and collagen-coated plates, and these methods can typically be used to grow A549 spheroids for 9–12 days (Meenach et al., 2016; Pant et al., 2021). The static hanging drop method is also used to grow monoculture A549 spheroids. However, this approach resulted in the formation of rugged spheroids with rough edges and low viability (Amann et al., 2014). Dynamic culture systems such as microfluidics and spinning or rotating bioreactors have been shown to offer better nutrient and oxygen supply to the cells through irrigation and allow the cells to interact with the culture environment and each other (Zanoni et al., 2016a; Fuentes et al., 2022). Dynamic spheroid culture technology such as clinostat-based systems can generate large quantities of long-term

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cultures and promote better cell interaction with the culture environment while minimising shear force (Wrzesinski & Fey, 2018; Zanoni et al., 2016). This gives the opportunity to investigate the metabolic processes of spheroids and evaluate their physiological functions (Ahn et al., 2019; Fey & Wrzesinski, 2012). This is beneficial because drug molecules are transported more rapidly through the microfluidic perfusion system, which mimics *in situ* conditions (Yildiz-Ozturk et al., 2021). The A549 spheroids previously cultured using different microgravity systems had limiting factors such as small sizes, variances in morphology, and a short viability window. This restricts timelines for experiments, especially drug efficacy, safety, and toxicity studies. The purpose of this study was to establish A549 spheroids using a clinostat-based rotating bioreactor system and characterise them in terms of to size, viability, morphology, and physiological relevance.

2. Methods

2.1. Three-dimensional cell culture

A549 human lung adenocarcinoma cells (ATCC® #CCL-185; Manassas, Virginia, USA) were maintained under standard two-dimensional culture conditions (Hołownia et al., 2015). Once the cells were confluent a cell suspension constituting 2.5×10^6 cell density was prepared and inserted in the ClinoReactors™ (CelVivo ApS, Odense, Denmark), and subsequently placed in a ClinoStar™ system (CelVivo ApS) where spheroids were formed in the microgravity environment (Van Niekerk et al., 2023; Wrzesinski et al., 2021). The culture media was supplemented with 3 μg/mL ascorbic acid for the first two days of culture (Van Niekerk et al., 2023). With excessive clumping, spheroid aggregates were gently disrupted by pipetting them with wide opening pipette tips, and gently coating them in foetal bovine serum (FBS).

2.2. Spheroid characterisation

After a nine-day stabilisation period, morphology, size, and viability were assessed every second day as described by Van der Merwe et al. (2022). Morphology was evaluated using light microscopy and histology. Photomicrographs were taken with a Nikon T100 inverted microscope and analysed for 25 days using ImageJ software version 1.8.0. Spheroid planar surface area and diameter were calculated to evaluate spheroid size and growth. Histological samples were processed according to standard procedures for haematoxylin-eosin (H&E) and Alcian blue staining (Merck Sigma-Aldrich). Soluble protein content was determined using the Bradford assay (Bradford, 1976) using Quick Start™ with Bradford protein (Lasec SA, Midrand, South Africa). The protein content of spheroids done as outlined by Van der Merwe et al. (2022) and Van Niekerk et al. (2023). Protein content was correlated to cell counts (data not shown). Intracellular adenosine triphosphate (ATP) (CellTiterGlo®) (Promega, Anatech Instruments, Johannesburg, South Africa), and approximate glucose consumption (OneTouch® glucometer; Life Scan; Switzerland) was used to monitor spheroid viability (Van Niekerk et al., 2023). For each sampling day, twelve spheroids ($n = 12$) were removed from the ClinoReactor™ and used to determine the soluble protein content, planimetry, and intracellular ATP. Approximate glucose consumption (relative to media glucose content) was determined using the spent media removed during media exchange.

2.3. Data analysis

The results are reported as mean ± SEM, and where applicable normalised in terms of protein content. Statistical differences were determined using Kruskal-Wallis ANOVA and Dunn's Multiple comparison tests (GraphPad Prism 9.4.1) and considered significant when $p < 0.05$.

3. Results

After nine days of establishment and stabilisation, A549 spheroids were present in the ClinoReactor™ growth chamber. However, severe clumping of spheroids was observed. This is a frequent problem experienced when culturing spheroids from mucin-producing cell lines in suspension. Clumping interrupts spheroid formation, reduces usable spheroid numbers, and weakens spheroid structures, which reduces the quality of the model. Initially, the spheroids clumped together from day 2 (Fig. 1A), and the clumping stopped upon intervention with ascorbic acid and FBS. More compact, single spheroids formed and were maintained until day 25 (Fig. 1B). The histological assessment of 9-day old spheroids showed clumping and the presence of acidic negative-charged mucin, visualised by the blue stained structures around the spheroids (Fig. 1C).

The mean planar surface area per spheroid increased over the sampling period, ending in significantly larger ($p < 0.01$) spheroids on day 23 and 25 *versus* day 9 (Fig. 2A). This increase is visualised in the difference in the size of the spheroids in Fig. 1A (day 2) and 1B (day 25). The soluble protein content per spheroid stayed constant for the first seven days of monitoring (days 9 to 15), whereafter there was a sharp increase in protein content to day 21 (Fig. 2B). Protein content then decreased to day 25, which was significantly lower ($p < 0.01$) compared to day 21. When the planar surface area was expressed as a function of the soluble protein content, similar results were seen as for the soluble protein content from day 9 to day 15 (figure not shown). Approximate glucose consumption per protein content was also stable from day 9 to 15 (Fig. 2C) but increased significantly on day 17 ($p < 0.05$), coinciding with the increase in surface area/soluble protein results. The glucose consumption then decreased over time but suddenly increased significantly on day 25 ($p < 0.05$), compared to days 9 and 23. Viability of the cells remained relatively unchanged throughout the study, and there were no significant differences in the intracellular ATP per protein ($p = 0.065$) between sampling days (figure not shown).

4. Discussion

After a 9-day stabilisation and maturation period, the spheroids grew to exhibit a homogenous round and compact morphology, as depicted in Fig. 1B. Sampling and characterisation were conducted exclusively during the metabolically stable spheroid phase, spanning from day 9 to 25. The clumping or aggregation illustrated in Fig. 1A, was successfully addressed by introducing a low concentration of ascorbic acid into the culture medium from day 0 up to day 2. Ascorbic acid, commonly known as vitamin C, enhances cell agglomeration and compactness by influencing collagen formation within the cells while developing into spheroids (Kapur et al., 2012). It therefore assists with spheroid structure and compactness. However, this was ceased from the second day of culturing to avoid the increased clumping, especially during the first week of culture (Rasouli & Tabrizian, 2021). Another technique used to

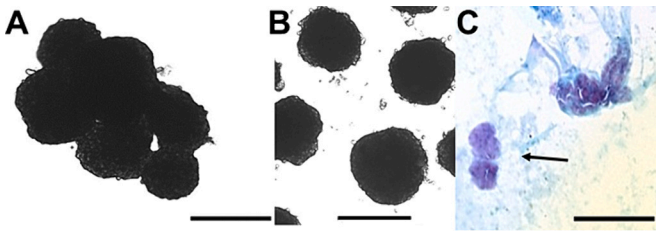

Fig. 1. Photomicrographs of A549 spheroids and their histological assessment on A) Day 2 presenting as aggregated clumps of spheroid structures, B) Day 25 showing rounded shape and compact density, and C) day 9 spheroids stained with H&E and Alcian blue, mucous indicated by arrow (Scale bars = 200 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

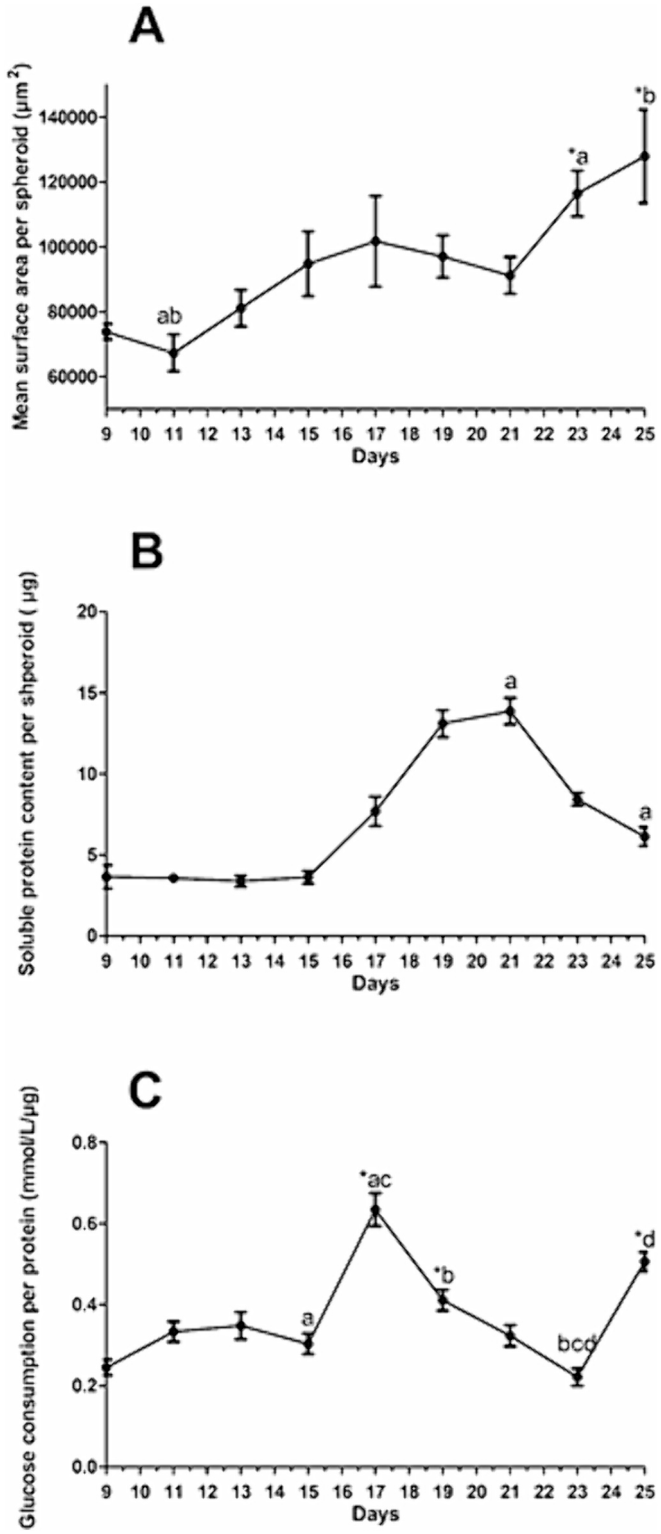

Fig. 2. Evaluation of A549 spheroid model growth and viability ($n = 12$) from day 9 to day 25, in terms of A) mean planar surface area per spheroid (µm^2), B) mean soluble protein content per spheroid (µg), and C) the mean approximate glucose consumption per protein content (mmol/L/µg). The asterisk (*) indicates significant differences ($p < 0.05$) compared to day 9 and common subscripts indicate significant differences ($p < 0.05$) between groups [Kruskal-Wallis ANOVA with Dunn's multiple comparison test].

minimize spheroid aggregation involved separating spheroid aggregates during media exchange by gently coating and pipetting them in FBS. The positive charges of FBS reduced spheroid adherence, leading to more round and individual spheroids, and promoted growth (Weeks et al., 2013). To mitigate the risk of mechanical disruption and damage to the spheroids during pipetting, tips with wide openings were used to prevent spheroid entrapment within the tip and reduce shear force (Zinn et al., 2023). To investigate the aggregation behaviour of the spheroids, Alcian blue staining was used to detect the presence of mucopolysaccharides in the A549 spheroids. The histological examination of the spheroids revealed the prominent presence of mucin. Mucin production is not generally observed in A549 monolayer cultures (Carterson et al., 2005; Saleh et al., 2020), but is observed in patient tumours (Lakshmanan et al., 2015). The pronounced clumping observed can be attributed to the secretion of mucin. This issue was partially mitigated by the mucolytic properties of ascorbic acid (Pillai et al., 2012), although complete removal of the mucin was not achieved. Mucin secretion has not been reported in other A549 3D models, either because it was not secreted or because clumping is not observed in single-well or hanging drop cultures where spheroids are kept separate from each other. The production of mucin in spheroids demonstrates their increased physiological relevance. In terms of morphology, fusiform structure and migratory cells were observed on the outer quiescent surface of the spheroid as shown in microphotographs Fig. 1B. The morphology of A549 spheroids can vary depending on the method of spheroid generation and the duration of cultivation, but this clinostat-based method generated spherical shaped spheroids from the second day of culture (Rozenberg et al., 2021).

The A549 spheroids exhibited diameters ranging from approximately 300 to 460 µm between day 9 and day 25. These growth dimensions are approximately 2 folds smaller when compared to the 500 to 1100 µm diameter spheroids, reported by Zanoni et al. (2016) (Table 1). Contrary, the growth rates were satisfactory considering that Zanoni et al. initiated these A549 spheroids from a cell suspension of 40 $\times$ 10^6 cells compared to the 2.5 $\times$ 10^6 used in this study. Furthermore, the ClinoStar™ formed metabolically stable spheroids after only 9 days, compared to the Rotary Wall Vessel system (RWV) which formed spheroids only after 14 days (Carterson et al., 2005b) (Table 1). Nevertheless, the spheroids generated in this study are suitable for pharmacological and toxicological studies, as A549 spheroids with sizes as small as 200 µm have been shown to be metabolically active (Honeder et al., 2021). The development of a necrotic core typically commences at a diameter of 500 µm, as reported by Zanoni et al. (2016), which was not seen in the current study's spheroids.

The soluble protein content of the spheroids remained constant for 15 days and then increased rapidly. The soluble protein content of a spheroid refers to the amount of protein that is present in the extracellular space of a spheroid and provides insight on the growth of the spheroid (Fey & Wrzesinski, 2012). The observed increase in protein content could be due to changes in the synthesis and release of proteins

Table 1
Summary of morphology, diameter (µm), days to spheroid formation, and days of spheroid viability of A549 spheroids from microgravity systems in literature compared to this study.

Morphology	Diameter (µm)	Days to form spheroid	Viability (Days)	References
Heterogenous (spherical, ellipsoidal and irregular)	500–1100	15 days	25 days	Zanoni et al., 2016
Not reported	Not reported	14 days	28 days	Carterson et al., 2005
Spherical	300–460	9 days	25 days	Current study

involved in 3D growth (Ellero et al., 2021). A549 spheroids have cadherin transmembrane proteins on the cell surfaces (Liu et al., 2011), which gradually increases as the spheroid becomes more compact (Han et al., 2021). Cadherins play a vital role in homeostasis and cell morphogenesis, due to its mediation of cell-to-cell adhesion, contact and stability (Maître & Heisenberg, 2013), increasing the similarities to that of *in vivo* tissue. Spheroids become compact as cells aggregate to each other from the initial step of spheroid establishment. After 21 days the protein content steadily declined. The decrease in protein content from Day 21 to Day 25, despite spheroid size increasing, may be a result of the spheroids maturing, causing the cells to adjust in metabolism and decreased proteins produced (Martinez et al., 2022; Wrzesinski & Fey, 2018). Approximate glucose consumption was determined through measuring glucose disappearance from the culture medium, and then normalizing it relative to the biomass present at the time, *e.g.* the protein content. The graph therefore shows metabolic activity of the cells in terms of glucose use. Approximate glucose consumption per protein increased steadily to accommodate the spheroid growth and shows that the A549 spheroids were metabolically active (Honeder et al., 2021). As the spheroids grow, they require more energy to support their growth and cell function.

The viability of the A549 cultivated spheroids remained constant throughout the 25 days of the study. Although there are numerous reports on the culture of other lung cancer cell lines using various microgravity bioreactor systems (Massai et al., 2016), there is relatively little information available regarding A549 spheroids. Further investigation such as activity screening can be conducted to further evaluate the effectiveness of the culture techniques and the physiological relevance of A549 spheroids when treated with drugs. Based on our findings, the optimal experimental window for this model seems to be between days 15 to 21, when the spheroids are growing and healthy. However, further viability assessment studies are needed to confirm.

5. Conclusion

Culturing A549 spheroids using the clinostat-based system resulted in a longer experimental window compared to other techniques from literature. The system also allows researchers opportunity to produce a large quantity of biological material for downstream analysis. We successfully established a stable and viable A549 spheroid model in the ClinoStar™ system from a single cell suspension, and managed to overcome challenges that may be encountered when working with a mucous-secreting cell line.

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CRediT authorship contribution statement

Charity M. Mabela: Writing – original draft, Investigation, Formal analysis, Data curation. **Chrisna Gouws:** Writing – review & editing, Supervision, Project administration, Formal analysis, Conceptualization. **Wihan Pheiffer:** Writing – review & editing, Supervision, Project administration, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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