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A Comparative Study of 3D Culture Methods in Lung Cancer Research: Finding the Optimal Path for Spheroid Formation

Akciğer Kanseri Araştırmalarıda 3 Boyutlu Kültür Yöntemlerinin Karşılaştırmalı İncelemesi: Sferoid Oluşumu için En Uygun Yöntemin Belirlenmesi

ABSTRACT

Objective

Cancer, characterized by uncontrolled cell proliferation and invasion into surrounding tissues, is a leading cause of global mortality. Traditional two-dimensional (2D) cell culture systems fail to adequately replicate the tumor microenvironment (TME). In contrast, three-dimensional (3D) culture systems, which better simulate cell–cell and cell–extracellular matrix (ECM) interactions, have become powerful tools in biomedical research. This study aims to compare the spheroid formation capacity of A549 lung cancer cells using three different 3D culture methods: ultra-low attachment (ULA) plates, agarose hydrogel, and the hanging drop technique. The primary objective is to identify the most effective spheroid formation method for A549 cells and to provide findings that can guide future biomedical research, particularly in cancer modeling, drug screening studies, and investigations of the tumor microenvironment.

Materials and Methods

A549 cells were cultured using three different 3D culture methods: ultra-low attachment plates, agarose hydrogel, and the hanging drop method. In the ultra-low attachment method, spheroid formation was observed at cell densities of 5,000, 10,000, and 30,000 cells/ml. In the agarose hydrogel method, agarose concentrations of 1%, 1.5%, and 2% were used to evaluate cell aggregation and spheroid stability. In the hanging drop method, cells aggregated under the influence of gravity. Spheroid diameter and area were analyzed using ImageJ software.

Results

In this study, the spheroid formation capacity of A549 lung cancer cells was evaluated using three different three-dimensional (3D) culture methods. The ultra-low attachment (ULA) plate method allowed cell aggregation; however, the resulting structures were not large or compact enough to be classified as spheroids. The hanging drop method showed that cells formed small clusters by day 3 but failed to develop a compact and stable spheroid structure by day 7. The agarose hydrogel method, particularly at a 2% agarose concentration, demonstrated the highest spheroid formation capacity compared to the other methods. In this method, spheroid for-

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mation began at 72 hours depending on cell density, with significant growth observed at a density of 30,000 cells/ml ($p < 0.0001$). Trypan Blue staining results indicated that 2% agarose and cell densities of 10,000–30,000 cells/ml provided the highest cell viability. Specifically, 4,800 viable cells were counted at a density of 30,000 cells/ml, while 3,600 viable cells were observed at 10,000 cells/ml. These findings suggest that the agarose hydrogel method, especially at 2% agarose concentration and higher cell densities, offers optimal spheroid formation and cell viability for A549 lung cancer cells.

Conclusion

This study demonstrated that the agarose hydrogel method effectively promoted stable and organized spheroid formation in A549 lung cancer cells. Notably, the 2% agarose concentration was identified as the most effective condition for maintaining cell viability and optimizing spheroid size. In contrast, the ultra-low attachment (ULA) plate and hanging drop methods exhibited limited spheroid formation capacity, resulting in less compact and disorganized structures. These findings emphasize the critical role of three-dimensional (3D) cell culture methods in biomedical research, particularly for experimental tumor modeling and drug screening studies. In this context, the agarose hydrogel method, with its high spheroid formation capacity and ability to support cell viability, emerges as a promising 3D culture model that warrants further exploration in cancer research.

Key Words

Lung Cancer, 3D Cell Culture, Spheroid

Amaç

Kanser, kontrolsüz hücre proliferasyonu ve çevre dokulara invazyon ile karakterize edilen, küresel ölçekte önemli bir mortalite nedenidir. Geleneksel iki boyutlu (2B) hücre kültürü sistemleri, tümör mikroçevresini (tumor microenvironment, TME) yeterince taklit edemezken, üç boyutlu (3B) kültür sistemleri, hücre-hücre ve hücre-ekstraselüler matriks (ECM) etkileşimlerini daha gerçekçi bir şekilde modelleyerek biyomedikal araştırmalarda önemli bir araç haline gelmiştir. Bu çalışmada, A549 akciğer kanseri hücrelerinin sferoid oluşturma kapasiteleri, üç farklı 3B kültür yöntemi - ultra düşük yapışma (ULA) yüzeyli kültür plakası, agaroz hidrojel ve asılı damla (hanging drop) teknikleri - kullanılarak karşılaştırılmıştır. Çalışmanın temel amacı, A549 hücreleri için en etkili sferoid oluşturma yöntemini belirleyerek elde edilen bulguların gelecek biyomedikal araştırmalara özellikle kanser modelleme, ilaç tarama çalışmaları ve tümör mikroçevresi araştırmaları gibi uygulamalara rehberlik etmesini sağlamaktır.

Gereç ve Yöntemler

A549 hücreleri ultra düşük tutunma plakası, agaroz hidrojel ve asılı damla yöntemleri kullanılarak kültüre edilmiştir. Ultra düşük tutunma plakası yönteminde 5×10^3, 1×10^4 ve 3×10^4 hücre/ml yoğunluklarında sferoid oluşumu gözlemlenmiştir. Agaroz hidrojel yönteminde 1%, 1.5% ve 2% agaroz konsantrasyonları kullanılarak hücre agregasyonu ve sferoid stabilitesi değerlendirilmiştir. Asılı damla yönteminde hücreler yerçekimi etkisiyle kümelenmiş, sferoid çapı ve alanı ImageJ yazılımı ile analiz edilmiştir.

Bulgular

Bu çalışmada, üç farklı üç boyutlu (3B) kültür yöntemi kullanılarak A549 akciğer kanseri hücrelerinin sferoid oluşturma kapasiteleri değerlendirilmiştir. Ultra düşük tutunma (ULA) plakası yöntemi, hücre agregasyonuna olanak sağlamış ancak oluşan yapılar sferoid olarak sınıflandırılacak kadar büyük ve kompakt bir yapı oluşturamamıştır. Asılı damla (hanging drop) yöntemi, hücrelerin 3. günde küçük kümeler oluşturduğunu, ancak 7. güne kadar kompakt ve stabil bir sferoid yapısı geliştiremediğini göstermiştir. Agaroz hidrojel yöntemi ise özellikle %2 agaroz konsantrasyonunda, diğer yöntemlere kıyasla en yüksek sferoid oluşturma kapasitesini sergilemiştir. Bu yöntemde sferoid oluşumu hücre yoğunluğuna bağlı olarak 72. saatte başlamış ve 30.000 hücre/ml yoğunluğunda anlamlı bir büyüme gözlenmiştir ($p < 0.0001$). Trypan Blue boyama sonuçları, %2 agaroz ve 1×10^4 – 3×10^4 hücre/ml yoğunluklarında en yüksek hücre canlılığının elde edildiğini ortaya koymuştur. Bu bağlamda, 30.000 hücre/ml yoğunluğunda 4.800 canlı hücre, 10.000 hücre/ml yoğunluğunda ise 3.600 canlı hücre sayılmıştır. Bu bulgular A549 akciğer kanseri hücreleri için agaroz hidrojel yönteminin, özellikle %2 agaroz konsantrasyonu ve yüksek hücre yoğunluğunda optimum sferoid oluşumu ve hücre canlılığı sağladığını ortaya koymaktadır.

Sonuç

Bu çalışma, agaroz hidrojel yönteminin A549 akciğer kanseri hücrelerinde stabil ve organize sferoid oluşumu sağladığını göstermiştir. Özellikle %2 agaroz konsantrasyonu, hem hücre canlılığını koruma hem de sferoid büyüklüğünü optimize etme açısından en etkili koşul olarak belirlenmiştir. Ultra düşük tutunma (ULA) plakası ve asılı damla (hanging drop) yöntemleri ise sferoid oluşumu açısından sınırlı bir kapasite göstermiş ve daha az kompakt yapılar üretmiştir. Elde edilen bulgular, üç boyutlu (3B) hücre kültürü yöntemlerinin, özellikle deneysel tümör modellemesi ve ilaç tarama çalışmaları gibi biyomedikal araştırmalarda kritik bir rol oynadığını vurgulamaktadır. Bu bağlamda, agaroz hidrojel yöntemi, yüksek sferoid oluşum kapasitesi ve hücre canlılığı sağlaması nedeniyle, kanser araştırmalarında etkili bir 3B kültür modeli olarak değerlendirilmeye açık bir yöntem olarak öne çıkmaktadır.

Anahtar Kelimeler

Akciğer Kanseri, 3B Hücre Kültürü, Sferoid

INTRODUCTION

Cancer is a group of diseases characterized by the uncontrolled proliferation of cells and their infiltration into surrounding tissues, remaining one of the leading causes of death worldwide (1). The etiology of cancer arises from a complex interplay between genetic and environmental factors, resulting in distinct clinical subtypes and biological behaviors. Lung cancer is a major cause of cancer-related mortality globally with millions of new cases diagnosed each year (2). This disease is particularly known for its poor prognosis, especially in advanced stages, posing a significant public health challenge in both developed and developing countries. Histopathologically, lung cancer is divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), with approximately 85% of cases classified as NSCLC (3). The major subtypes of NSCLC include adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Notably, the A549 cell line, derived from adenocarcinoma, represents human alveolar basal epithelial cells and was established by D.J. Giard and colleagues in 1972 cell lines are frequently utilized in cancer research due to their ability to mimic in vivo physiology within in vitro environments (4, 5).

Cancer cells exist within complex microenvironments where they are exposed to various physical and chemical stimuli that significantly influence their behavior, proliferation, and resistance to therapy (6). Accurately modeling these tumor microenvironments is crucial for understanding cancer biology and developing novel therapeutic approaches. The molecular pathogenesis of lung cancer involves the dysregulation of numerous oncogenes and tumor suppressor genes (7). Specifically, targetable molecular alterations, such as mutations in the epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK), have paved the way for personalized treatment strategies (8).

In vitro cell culture systems have been instrumental in cancer research, providing critical insights into cellular processes and responses to treatment. Traditional two-dimensional (2D) cell culture systems, which allow cells to grow in a monolayer, have limitations in accurately modeling cell-cell interactions and the tumor microenvironment (9, 10). These limitations mean that the behavior of cancer cells in natural settings is not fully replicated, restricting the translation of findings to clinical applications. To address these challenges, three-dimensional (3D) cell culture systems have been developed, allowing cells to grow in a more complex environment that better mimics the natural cellular architecture (11). In 3D cultures, cell-cell interactions, cell-matrix relationships, and cellular morphology are more faithfully preserved (12).

Spheroid formation in 3D cultures is particularly noteworthy, offering significant advantages in modeling the in vivo growth and metastatic potential of cancer cells (13, 14). Spheroids enable the examination of cell proliferation, differentiation, and apoptotic processes within a more natural microenvironment. Additionally, these structures serve as ideal models for investigating cellular resistance mechanisms to chemotherapy and radiotherapy (15). In 3D culture systems, various methods such as tumor spheroids, scaffold-based approaches, bioreactors, microfluidic devices, and organoids are employed to maintain and proliferate cancer cells. Among these, spheroids are recognized as one of the most compatible models, playing a crucial role in the identification of new drugs and biological agents across different cancer types (10).

Spheroid structures are particularly valuable in stem cell research due to their biophysical similarity to in vivo solid tumors, encompassing aspects such as cell morphology, proliferation, oxygenation, nutrient uptake, and drug delivery (16). Stem cell spheroid cultures have gained attention in recent years, particularly in the context of cancer, due to their enhanced anti-inflammatory, tissue regeneration, and repair potential. However, it is essential to select and optimize the most appropriate model for spheroid formation based on the study's objectives and the specific cancer type being investigated (17). In the case of lung cancer, findings suggest that stem cells contribute to increased tumor heterogeneity and accelerate the metastatic process, underscoring the need for a detailed examination of their biological behaviors. 3D culture systems provide a suitable model for understanding the role of stem cells in these complex processes and for developing potential therapeutic strategies (18).

In this study, we aim to compare the formation of three-dimensional spheroids in lung cancer using different 3D cell culture methods, evaluating their biological and therapeutic implications. By utilizing the adenocarcinoma-derived A549 cell line, our research compares three distinct 3D methods-agarose hydrogel, ultra-low attachment (ULA) plates, and hanging-drop techniques a platform that assesses the viability and size of the formed spheroids. This study adopts a multidisciplinary approach, integrating both engineering and molecular biology to contribute to the development of more effective strategies for lung cancer treatment. By evaluating the advantages and disadvantages of different 3D culture methods, we aim to identify more suitable models for lung cancer research and to foster the development of new therapeutic approaches.

MATERIALS and METHODS

Three-dimensional (3D) cell culture methods have gained significant attention in biomedical research due to their superior ability to mimic the in vivo environment compared to traditional two-dimensional (2D) cultures. Among these methods, agarose-based cultures, ultra-low attachment (ULA) plates, and hanging drop techniques are prominent for their unique advantages in promoting cell aggregation (19-21).

Cell Culture

This study was conducted on the non-human-derived A549 cell line. The A549 cell line used in this study was kindly gifted from Dr. Zuhal Hamurcu. The cell line was originally derived from human lung adenocarcinoma and is registered in the American Type Culture Collection (ATCC, CCL-185). Since no experiments were performed on living humans or animals, ethical approval is not required. Cryogenic vials containing A549 cells were retrieved from the -80°C storage or liquid nitrogen tank and quickly thawed in a 37°C water bath for 1-2 minutes. To prevent overheating, the vials were promptly removed from the water bath. Subsequently, 1 ml of pre-warmed Dulbecco's Modified Eagle Medium (DMEM; Gibco, #41966029), containing 10% Fetal Bovine Serum (FBS) (Thermo Fisher, #10270106), supplemented with 1% penicillin-streptomycin (Thermo Fisher Scientific, #15070063), 1% amphotericin B (Thermo Fisher Scientific, #15290026), and 1% L-glutamine (STEMCELL Technologies Inc., #7100), was slowly added to the vial. The cell suspension was gently mixed using a pipette to ensure homogeneous distribution. The suspension was centrifuged at 300 x g for 5 minutes, and the supernatant was carefully discarded. The resulting cell pellet was resuspended in 1 ml of fresh medium. The prepared cell suspension was transferred to a 25 cm² cell culture flask and incubated at 37°C in a humidified atmosphere containing 5% CO_2 to promote proliferation. Cell growth and morphology were monitored daily using a bright-field microscope, and the medium was replaced or cells were passaged when 80-90% confluency was reached.

Three-Dimensional Cell Culture

When the cells reached 80-90% confluence, the medium was removed, and the cells were detached with 0.25% Trypsin-ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher, #25200056). The trypsinization process was carried out in a 37°C, 5% CO_2 incubator for 3 minutes, followed by the addition of an equal volume of medium containing 10% fetal bovine serum (FBS)(Thermo Fisher, #10270106) to neutralize trypsin activity. The cell suspension was centrifuged at 400 x g for 5 minutes, the supernatant was discarded, and the cell pellet was resuspended in the appropriate medium for seeding. Cells were counted using a hemocytometer and allocated for three different 3D culture methods: ultra-low attachment plate, agarose hydrogel, and hanging drop. The cell densities for each culture method were determined based on the optimal cell numbers reported in the literature:

Ultra-low attachment plates: To support the formation of spherical cell aggregates, the minimum cell density was set at 5×10^2 cells/well, while the maximum cell density was 8×10^2 cells/well (22-24).

Agarose hydrogel method: To ensure homogeneous cell distribution and growth within the gel, cell densities were determined within the range of 5×10^3 to 8×10^3 cells/well (25, 26).

Hanging drop method: To maintain the optimal spheroid size and cell viability, cell densities of 5×10^2, 1×10^3, 3×10^3, and 5×10^3 cells/drop were used (27-29).

Ultra-Low Attachment Plate Method

ULA plates are designed to prevent cells from adhering to the culture surface, thereby promoting the formation of spheroids and organoids. These plates are coated with hydrophilic materials, which inhibit cell attachment and encourage cellular self-aggregation (30, 31). This method is particularly useful for studying cell-cell interactions and cancer cell behavior within a more physiologically relevant context (32).

A 96-well ultra-low attachment plate was used to culture A549 cells. Cells were seeded at densities of $5x10^2$, 1×10^3, $2{,}5 \times 10^3$, 5×10^3, 1×10^4, 2×10^4, 3×10^4, 4×10^4, and 8×10^4 cells per well in serum-free DMEM containing 1% penicillin-streptomycin, 1% amphotericin B, and 1% L-glutamine. The cells were incubated in a 37°C, 5% CO_2 environment and monitored daily for 15 days. Fresh medium (50 µl) was added every 2 days. Spheroid formation was observed and captured using an inverted microscope (Fıgure 1).

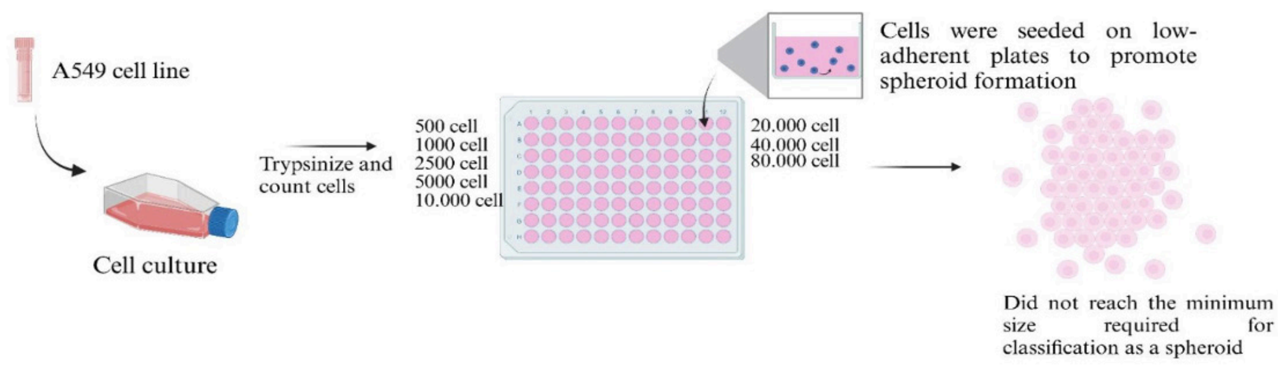

Figure 1. Experimental workflow and time-course evaluation of spheroid formation at various cell densities using ultra-low attachment plates in the A549 cell line. This figure illustrates the experimental workflow and the process of spheroid formation. A549 cells were trypsinized and counted, then seeded in ultra-low attachment plates with different cell densities (of $5x10^2$, $1x10^3$, $2{,}5x10^3$, $5x10^3$, $1x10^4$, $2x10^4$, $3x10^4$, $4x10^4$, and $8x10^4$ cells per well). Although cell aggregation occurred the clusters did not reach the minimum size required for classification as spheroids.

Agarose Hydrogel Method

Agarose, a versatile polysaccharide, serves as an excellent scaffold for 3D cell cultures. Its neutral charge minimizes non-specific interactions with biomolecules, making it suitable for various applications in tissue engineering and cell biology (33, 34). Studies have shown that agarose effectively encapsulates cells, facilitating the investigation of mechanotransduction and cellular responses within a controlled 3D microenvironment (35). Moreover, the biocompatibility of agarose and its ability to support cell viability make it a popular choice for generating spheroids and other multicellular aggregates (36).

Agarose (Sigma-Aldrich, #9012-36-6) was used to prepare hydrogels in 96-well plates for cell aggregation. Agarose solutions were prepared at concentrations of 1%, 1.5%, and 2% (w/v) by dissolving agarose in DMEM. The solutions were autoclaved at 120°C and 2 bar for 20 minutes, then distributed into wells (50 µl per well) and allowed to cool for 20 minutes. A549 cells were seeded at densities of 5 x 10^3, 1 x 10^4, 2 x 10^4, 3 x 10^4, 4 x 10^4, and 8 x 10^4 cells per well and incubated at 37°C in a 5% CO_2 incubator. Half of the culture medium was replaced with fresh medium every other day. Spheroid formation was monitored daily, and images were captured (Figure 2).

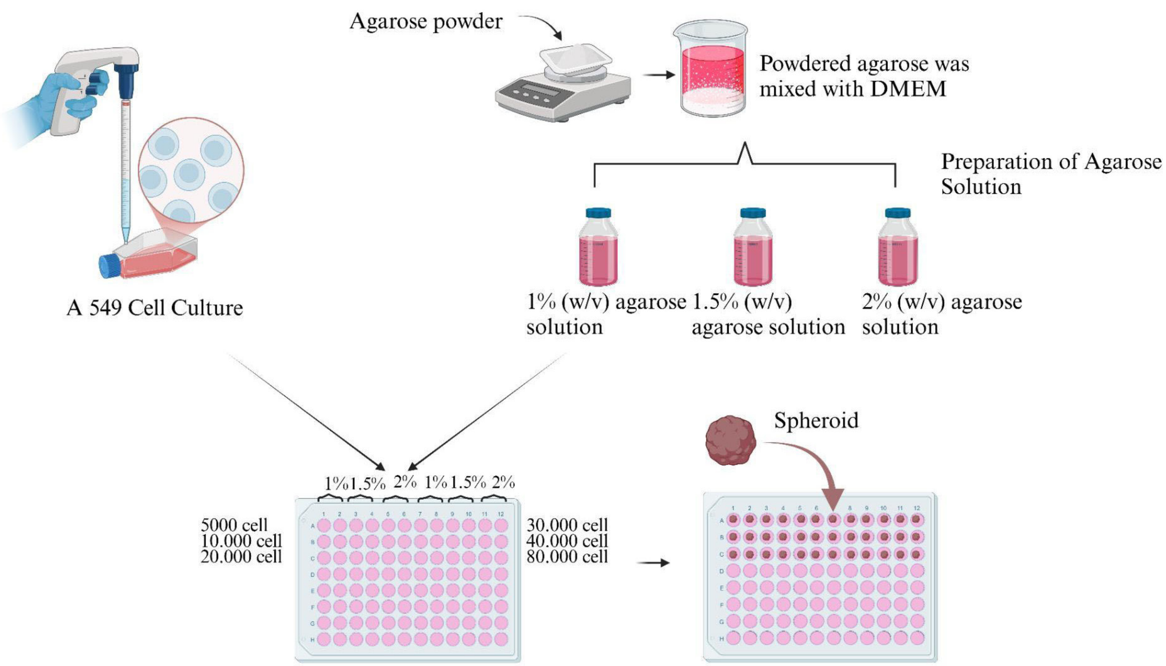

Figure 2. Assessment of A549 spheroid formation and growth using the agarose hydrogel method, highlighting changes in cell aggregation, spheroid size, and diameter across different cell densities and time points. Agarose hydrogel method for spheroid formation. A549 cell culture was prepared and powdered agarose was mixed with DMEM to create agarose solutions of varying concentrations (1%, 1.5%, and 2%). Cells were seeded in 96-well plates with different agarose concentrations and cell densities (5x10^3, 1x10^4, 2x10^4, 3x10^4, 4x10^4, and 8x104 cells per well). Spheroid formation was successfully observed in specific conditions.

Hanging Drop Method

The hanging drop technique is another effective 3D culture method that utilizes gravity to facilitate cell aggregation and spheroid formation. In this method, droplets of cell suspension are placed on the underside of a culture plate lid, allowing cells to aggregate without adhering to any surface (31, 37). This technique is widely employed for creating tumor spheroids and studying their responses to various therapeutic agents (37). The hanging drop method is particularly advantageous due to its simplicity and ability to produce uniform spheroids, which are essential for reproducibility in experimental setups (31, 38).

For the hanging drop technique, a 100 mm culture dish was utilized. In this method, cell aggregation is facilitated by gravitational forces. Droplets of 15 µl, 17 µl, and 20 µl were placed on the lid of the culture dish, each containing 5 x 10^2, 1 x 10^3, 3 x 10^3, 5 x 10^3cells. The dish was inverted and incubated at 37°C in a 5% CO_2 environment. To prevent droplet evaporation and changes in osmolality, 5 ml of DPBS was added to the culture dish. Cell aggregation and spheroid formation were monitored daily, and images were captured. Fresh medium (2 µl per droplet) was added every 2 days (Fıgure 3).

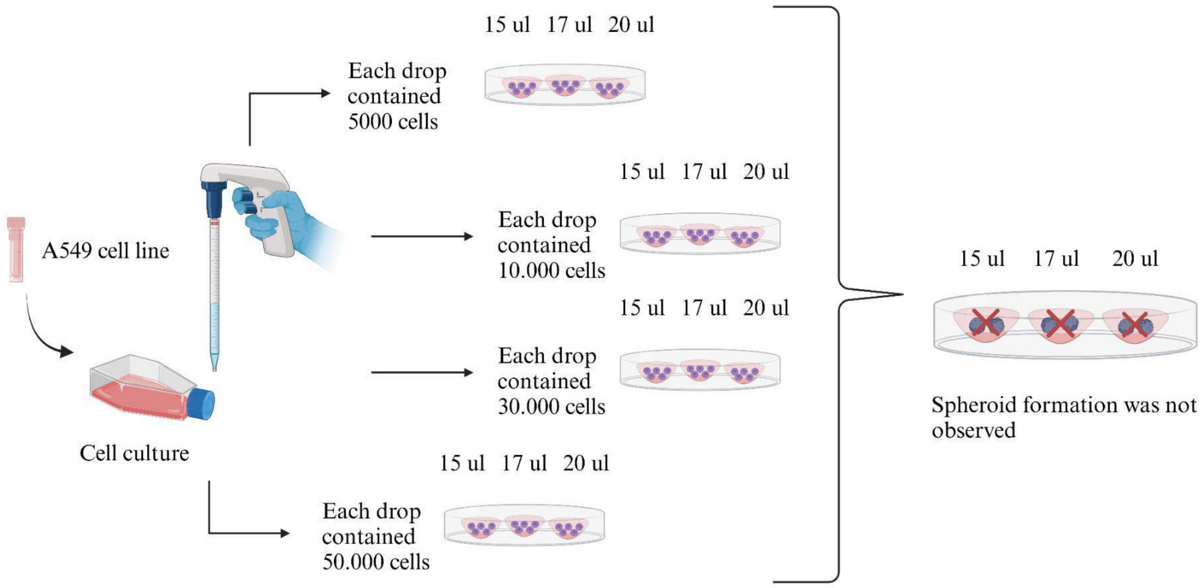

Figure 3. Experimental workflow and time-course evaluation of spheroid formation using the hanging drop method at different cell densities and volumes in the A549 cell line. Hanging drop method for spheroid formation. A549 cells were cultured and dispensed into drops containing different cell densities ($5x10^2$, $1x10^3$, $3x10^3$, or $5x10^3$ cells per drop) and volumes (15 µL, 17 µL, and 20 µL). Despite varying cell densities and volumes, no spheroid formation was observed under these conditions.

Cell Viability Determination

Cell viability was assessed using the Trypan Blue exclusion method, which differentiates viable cells from non-viable ones. A 0.4% Trypan Blue solution was prepared, and 10 µl of cell suspension was mixed with 10 µl of the dye. The mixture was applied to a hemocytometer, and cells were counted under an inverted microscope. The following formulas were used to determine cell viability:

- Cell count/ml = (Average cell count in four squares) × 10^4 × (Dilution ratio) × 2
- % Viable cells = [1.00−(Number of blue cells /Total number of cells)] × 100

Statistical Analysis

Microscopic images of spheroids were analyzed using ImageJ software. The analysis settings were optimized for the specific cell type to achieve accurate image segmentation. Parameters such as size, area, volume, and diameter of the spheroids were measured and recorded. Statistical significance across the compared cell densities and agarose concentrations was assessed using Two-Way ANOVA. All analyses were conducted using GraphPad Prism 9.0.0 (GraphPad Software Inc., USA) statistical software, and a p-value <0.05 was considered statistically significant.

RESULTS

In the hanging drop and ULA 3D cell culture methods, spheroid images were obtained on days 3, 5, and 7. However, due to cell death at day 7, no further images were captured beyond this time point. In contrast, in the agarose-based 3D culture, spheroid formation was observed, and images were recorded on days 3, 5, 7, 9, and 11. The findings obtained are consistent with the existing literature.

1. Cell Aggregation Profiles in Ultra-Low Attachment Plates

The results indicated that the ultra-low attachment method allowed some degree of cell aggregation but the structures formed were not large enough to be classified as spheroids. At higher seeding densities, cell aggregation increased however the resulting structures still did not reach the required size for spheroid classification (Figure 4).

2. Effectiveness of the Hanging Drop Method in Spheroid Formation

The hanging-drop method failed to induce aggregation or spheroid formation in any of the droplets. On Day 3, small cell groups started to aggregate but the organization remained loose and incomplete. By Day 5, the clusters grew larger but they still lacked the compact structure typically associated with mature spheroids. On Day 7, the cells continued to form dispersed clusters with no cohesive or stable spheroid formation. These observations indicate that under the current experimental conditions, the hanging drop method was insufficient to promote the development of compact and mature spheroids (Figure 5).

3. Optimization of Spheroid Formation with Agarose Hydrogel

The agarose hydrogel method successfully facilitated spheroid formation. Cells aggregated over time, forming stable spheroid structures. Various agarose concentrations were tested, and the optimal concentration was determined to be 2%, which supported consistent spheroid formation. Spheroids were first detected 72 hours after cell seeding (Figure 6).

Figür 7 A and 7 B illustrate the changes in spheroid areas of A549 cells at different cell densities (5 x 10^3, 1x 10^4 and 3 x 10^4cells) and time points (Days 3, 5, 7, 9, and 11). The spheroid areas increased significantly over time and with higher cell densities (Figür 7 A; ** $p < 0.0001$, * $p < 0.001$). This increase was particularly pronounced at higher cell densities (e.g., 3 x 10^4 cells/ml). However, no statistically significant differences were observed between time points at the same cell density (Figure 7 B; $p > 0.0005$). Similarly, the changes in spheroid diameters at different time points and cell densities are shown in Figür 7 C and Figüre 7 D. The spheroid diameter exhibited a significant increase with higher cell densities (Figure 7 C; ** $p < 0.0001$, * $p < 0.001$). Nonetheless, no significant differences were observed between time points at the same cell density (Figure 7 D; $p > 0.0005$).

These findings indicate that cell density is the primary determinant of spheroid size, while the agarose hydrogel matrix provides a stable microenvironment for consistent spheroid growth over time.

4. Cell Viability Analysis: Results from the Trypan Blue Assay

The Trypan Blue assay was used to assess the viability of spheroids. Trypan Blue is a dye that distinguishes between living and dead cells. The dye penetrates only the membranes of dead cells and does not affect living cells. Thus, living cells appear bright, allowing them to be distinguished from dead cells (Table I).

Table I. Effect of Agarose Concentration (1-2%) and Cell Density (10^4 and 3x10^4 cells) on Viable Cell Count (Mean ± Standard Deviation, n = 3).

Agarose Concentration	Live Cell Count (3x10^4 Density)	Live Cell Count (10^4 Density)
1%	2000 ± 100	1500 ± 50
1.5%	1100 ± 50	1000 ± 20
2%	4800 ± 100	3600 ± 100

All data are expressed as mean ± standard deviation (SD). Statistical analyses were performed using two-way analysis of variance (Two-Way ANOVA) followed by the Post Hoc Tukey test. A p-value of $p<0.05$ was considered statistically significant.

Table I demonstrates that 2% agarose is the most effective concentration for maintaining cell viability at both 1 x 10^4 and 3 x 10^4 cell densities. In the 3 x 10^4 -cell condition, 2% agarose resulted in the highest live cell count (4800 cells), indicating that higher agarose concentrations provide a stable and protective matrix to preserve cell integrity. In contrast, 1.5% agarose was less effective, particularly under high-density conditions, where metabolic stress and nutrient competition are more pronounced. Similarly, at 1 x 10^4 cell density, 2% agarose yielded the best outcome, with 3600 live cells, reinforcing the idea that this concentration provides an optimal environment for cellular growth and stability. The drop in live cell counts at 1.5% agarose highlights the importance of carefully balancing agarose concentration with cell density to reduce metabolic stress and promote viability.

In summary, the Trypan Blue assay confirms that 2% agarose offers the best support for maintaining cell viability (Table I). While higher cell densities (3 x 10^4 cells) impose greater metabolic demands and stress, optimizing the agarose concentration can mitigate these effects. Therefore, using 1 x 10^4 cells with 2% agarose represents the most favorable combination for achieving stable and viable 3D cell models, particularly for applications such as tumor modeling (Figure 8 A).

Figure 8 A and 8 B illustrate the effects of agarose concentration and cell density on cell count. The number of A549 cells significantly varied depending on both the agarose concentration (1%, 1.5%, 2%) and the initial cell density (3 x 10^4 and 1 x 10^4 cells per well). As shown in Figure 8 A, an increase in cell density within the same agarose concentration resulted in a significant rise in cell count (** $p < 0.0001$). Similarly, Figure 8 B compares the combined effects of agarose concentration and cell density on cell count. Notably, higher agarose concentrations (e.g., 2%) and cell densities (3 x 10^4 cells/well) led to significantly higher cell counts (** $p < 0.0001$). These findings suggest that both agarose concentration and cell density have a synergistic effect on cell viability and proliferation, with higher concentrations providing a more supportive environment for cell stability and growth.

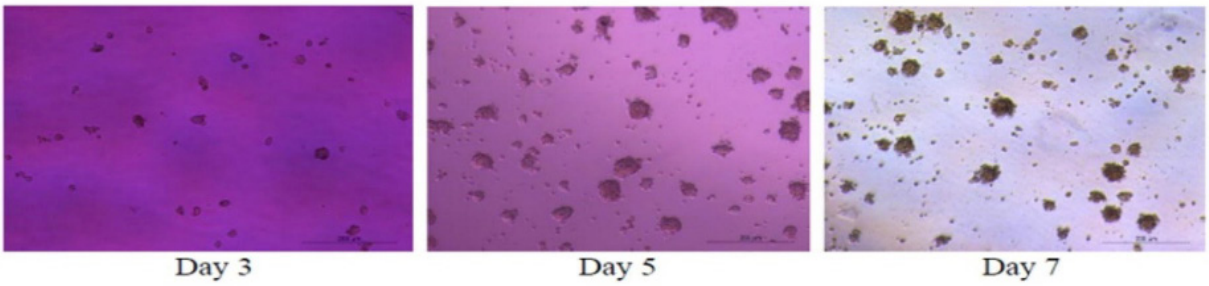

Figure 4. Spheroid formation of A549 cells over time. Images show cell aggregation and spheroid development on Days 3, 5, and 7. On Day 3, small and loosely organized cell clusters are visible. By Day 5, cell aggregation has progressed, resulting in denser structures. On Day 7, more compact and organized spheroid structures are observed, though full maturity is not yet reached (10X, size bar = 200 μm).

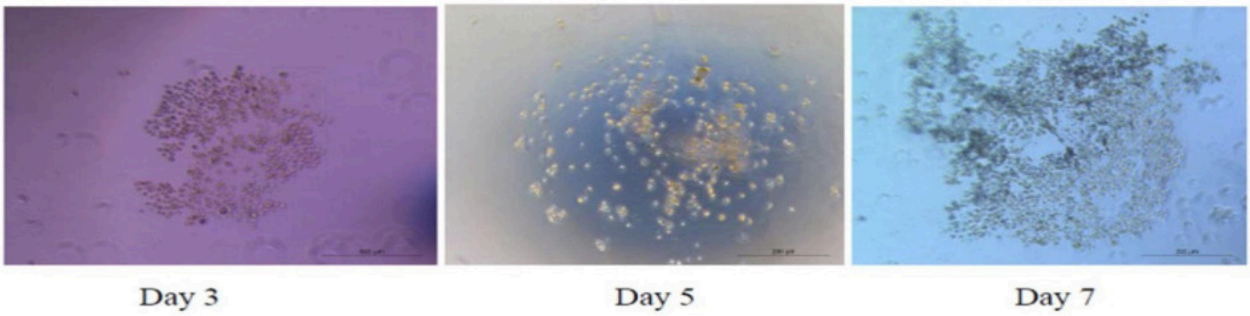

Figure 5. Spheroid formation process of A549 cells on Days 3, 5, and 7. On Day 3, cells started to cluster loosely. By Day 5, cell aggregation increased resulting in a denser structure. On Day 7, the cells spread over a larger area, but a fully compact spheroid structure had not yet formed. Day 3 (4X, size bar 500 μm.) Day 5 and Day 7 (4X, size bar = 200 μm)

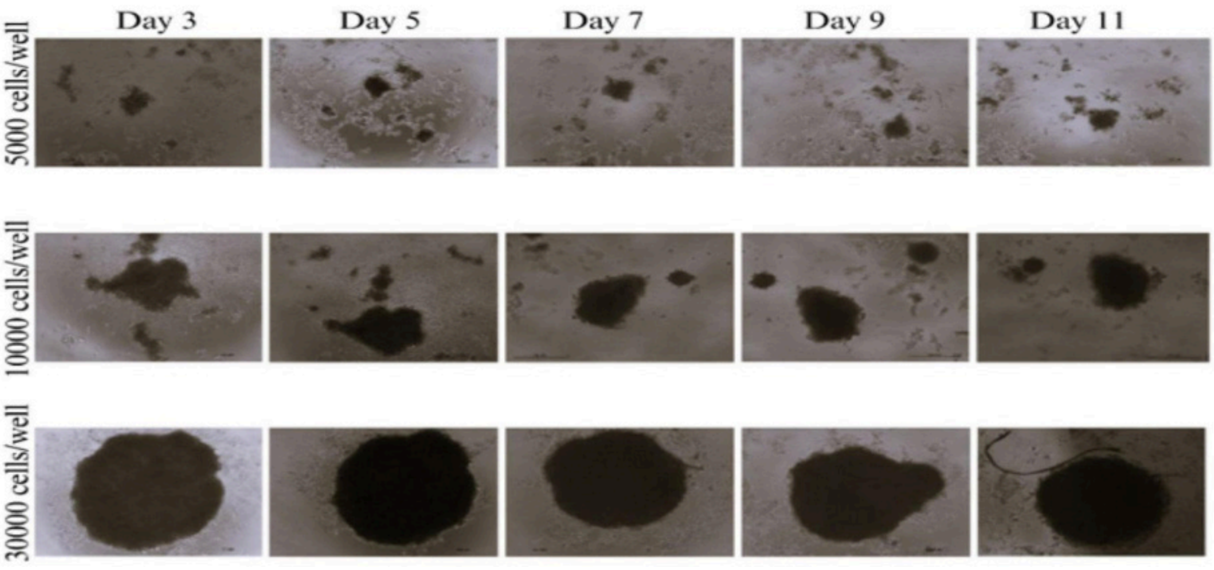

Figure 6. Progressive development of A549 cell spheroids over time. Images show cell aggregation and spheroid formation on Days 3, 5, 7, 9, and 11. Initial days exhibit dispersed cell clusters, while more compact and mature spheroids are observed in later days. (10X, size bar = 200 μm).

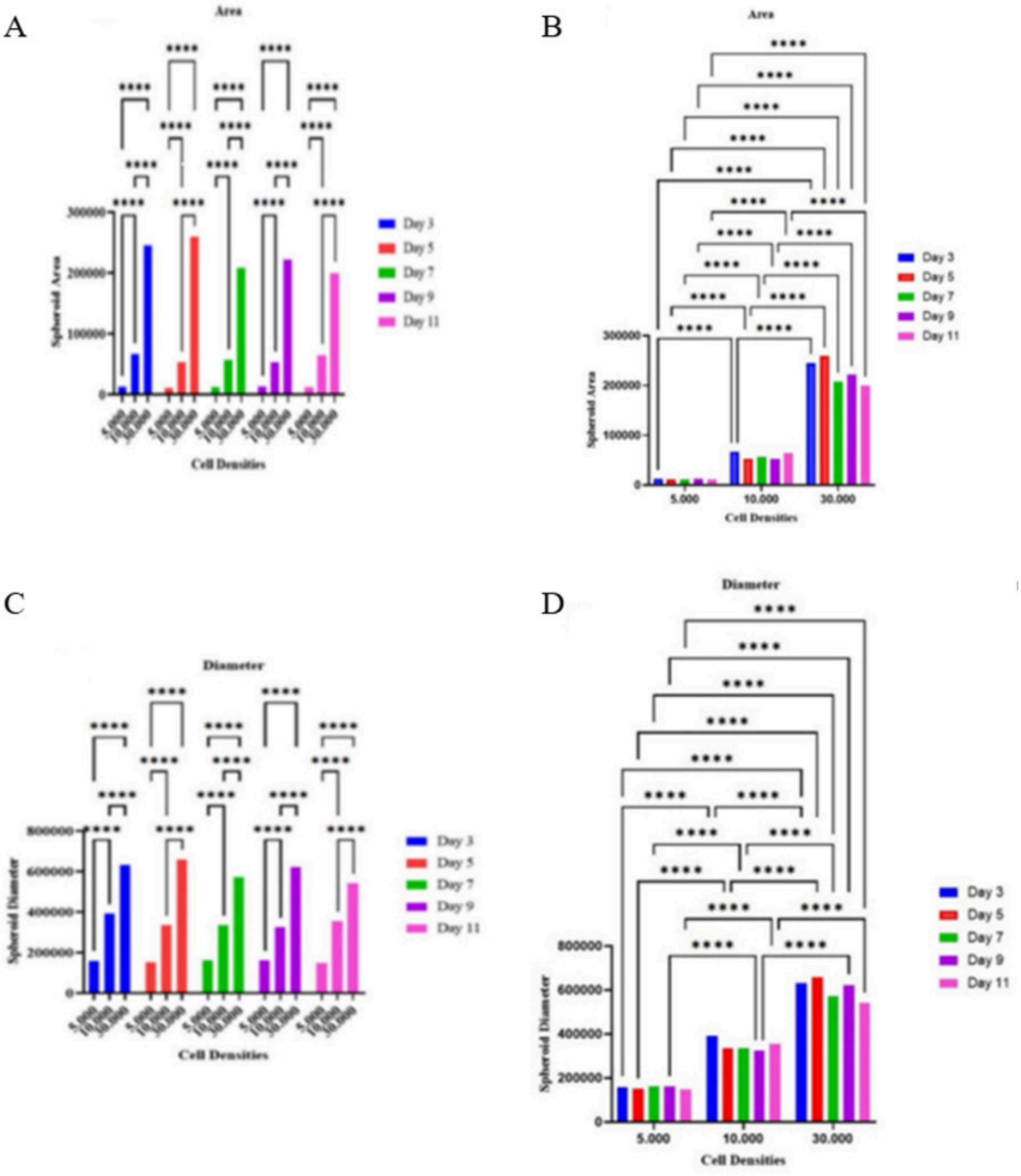

Figure 7. A) Spheroid area measurements of A549 cells at different cell densities ($5x10^3$, $1x10^4$, $3x10^4$ cells) and time points (Days 3, 5, 7, 9, and 11) using the agarose hydrogel method. Statistically significant differences in spheroid area are indicated (** $p < 0.0001$, * $p < 0.001$). The data show an increase in spheroid area with time, particularly at higher cell densities. B) Spheroid area measurements of A549 cells at different cell densities ($5x10^4$, $1x10^3$, and 3.10^4cells) and time points (Days 3, 5, 7, 9, and 11) using the agarose hydrogel method. Each color represents a specific time point: blue for Day 3, red for Day 5, green for Day 7, purple for Day 9, and orange for Day 11. No statistically significant differences were observed across time points at the same cell density ($p > 0.0005$). C) Spheroid diameter measurements of A549 cells at different cell densities ($5x10^3$, $1x10^4$, $3x10^4$ cells) and time points (Days 3, 5, 7, 9, and 11) using the agarose hydrogel method. Statistically significant differences in spheroid diameter are indicated (** $p < 0.0001$, * $p < 0.001$). The data show an increase in spheroid diameter with time, particularly at higher cell densities. D) Spheroid diameter measurements of A549 cells at different cell densities ($5x10^3$, $1x10^4$, $3x10^4$ cells) and time points (Days 3, 5, 7, 9, and 11) using the agarose hydrogel method. Each color represents a specific time point: blue for Day 3, red for Day 5, green for Day 7, purple for Day 9, and orange for Day 11. No statistically significant differences were observed across time points at the same cell density ($p > 0.0005$).

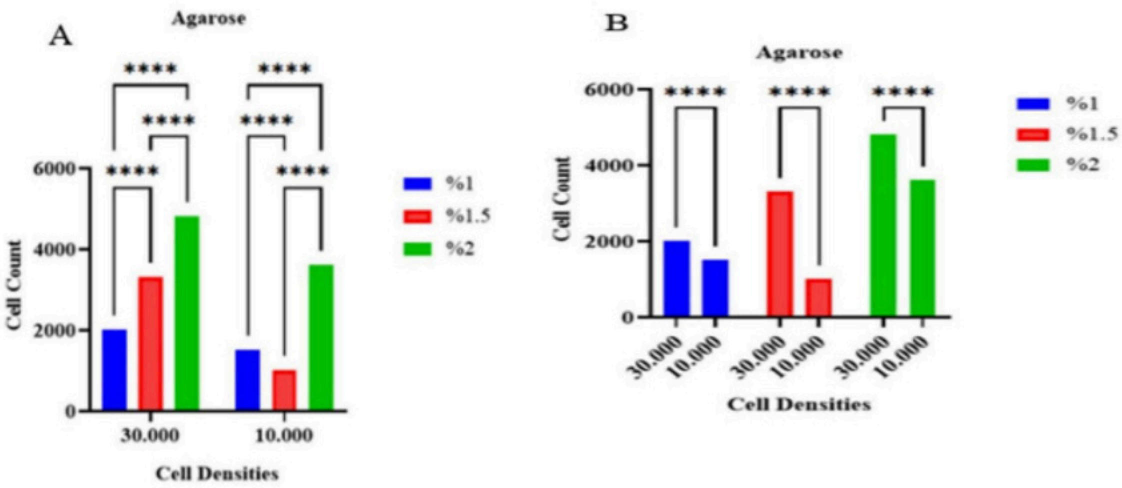

Figure 8. Effect of Different Agarose Concentrations and Cell Densities on Cell Proliferation. A) Effect of agarose concentration and cell density on cell count. Bars represent cell counts at different agarose concentrations (1%, 1.5%, and 2%) and cell densities (3x10^4 and 1x10^4 cells per well). Each color represents a different agarose concentration, with blue for 1%, red for 1.5%, and green for 2%. Significant differences between cell densities within each agarose concentration group are indicated with **** ($p < 0.0001$). This demonstrates that both agarose concentration and cell density. B) Comparison of cell counts across different agarose concentrations (1%, 1.5%, and 2%) and cell densities (3x10^4 and 1x10^4 cells per well). Each color represents a specific agarose concentration: blue for 1%, red for 1.5%, and green for 2%. Significant differences between each agarose concentration and cell density combination are indicated with **** ($p < 0.0001$). The data reveal a statistically significant effect of both agarose concentration and initial cell density on cell viability, with higher cell counts generally observed at increased agarose concentrations and higher cell densities.

DISCUSSION

Cancer remains a leading cause of mortality worldwide, characterized by uncontrolled cell proliferation and invasion into surrounding tissues. However, cancer is not merely an accumulation of unregulated proliferating cells; rather, it consists of highly organized and interconnected structures. According to the latest GLOBOCAN 2022 data, approximately 20 million new cancer cases were diagnosed worldwide, leading to 9.7 million cancer-related deaths. Lung cancer remains the most frequently diagnosed malignancy, with 2.48 million new cases (12.4%) and 1.82 million deaths, making it the leading cause of cancer-related mortality globally (39). Similarly, in Turkey, 41,032 new lung cancer cases were reported, representing 17.1% of all cancer cases, with 38,505 lung cancer-related deaths, accounting for 29.7% of total cancer-related deaths (39). Among lung cancer subtypes, non-small cell lung cancer (NSCLC) accounts for approximately 80–85% of cases, with adenocarcinoma being the most common histological variant (40). The A549 cell line, derived from human lung adenocarcinoma, was established by D.J. Giard et al. in 1972 and has since become a widely used in vitro model for lung cancer research (LGC, CCL-185). Due to their ability to mimic in vivo conditions, cell lines are an essential tool in cancer research, particularly for investigating cancer biology, drug responses, and therapeutic strategies (41). For decades, two-dimensional (2D) cell culture systems have been the standard model in cancer research. However, these models fail to accurately replicate the biological behavior of cells within the tumor microenvironment. Since 2D cultures grow on flat surfaces, they lack critical cell-cell and cell-extracellular matrix (ECM) interactions, leading to discrepancies in cell proliferation, gene expression, and drug response when compared to in vivo tumors (42). Consequently, three-dimensional (3D) culture techniques have gained significant attention as they enable cells to self-organize into structures that more closely resemble natural tumor architecture (11, 19). Studies have demonstrated that tumor cells cultured in 3D environments exhibit gene expression profiles more closely aligned with clinical tumor samples than those cultured in 2D systems (43).

Thus, 3D culture models provide a more predictive and physiologically relevant platform for cancer research and the development of novel therapeutic strategies. This study compared three distinct 3D culture techniques—ultra-low attachment plates (ULA), the hanging drop method, and the agarose hydrogel method—to evaluate their effectiveness in generating A549 lung cancer spheroids. The results revealed substantial differences between the methods, with the agarose hydrogel method emerging as the most efficient and reliable approach for producing stable and compact spheroids. The ULA method facilitated limited cell aggregation, as shown in Figure 1 and Figure 4. At higher cell densities (e.g., 4 × 10^4 and 8 × 10^4 cells/ml), clusters were observed; however, these structures lacked sufficient compactness for classification as spheroids. Over time, small and loosely organized clusters formed by day 3 progressed to denser structures by day 7, yet these structures remained immature. These findings align with previous research, which has shown that while ULA plates promote initial aggregation, they lack the structural support required for the development of stable spheroids (44, 45).

Similarly, the hanging drop method failed to produce cohesive spheroids under the tested conditions. As depicted in Figür 2 and Figüre 5, cell clusters remained loose and disorganized throughout the experiment, and by day 7, although larger aggregates had formed, they lacked compact spheroid structure. These findings are consistent with previous studies indicating that hanging drop cultures are less effective for cell lines lacking strong intrinsic adhesion properties or requiring scaffold-like support for proper organization (46).

In contrast, the agarose hydrogel method successfully promoted robust spheroid formation, particularly at a 2% agarose concentration and cell densities of 1×10^4 to 3×10^4 cells/ml. As shown in Figür 3 and Figür 6, spheroids were detectable by day 3 and became progressively more compact and organized by day 11. Quantitative analyses revealed significant increases in spheroid area and diameter over time, particularly at higher cell densities (e.g., 3×10^4 cells; Figür 7 A- Figür 7 D, $p < 0.0001$). These findings suggest that the semi-solid agarose matrix provides mechanical support while promoting cell-cell interactions, which is consistent with previous studies (47).

The cell viability analysis further highlighted the advantages of the agarose hydrogel system. As shown in Table I and Figure 8 A and 8 B, 2% agarose supported the highest viable cell counts at both 1×10^4 and 3×10^4 cell densities, with statistically significant differences across conditions ($p < 0.0001$). These findings emphasize the importance of optimizing agarose concentration and cell density to balance structural support with nutrient diffusion, both of which are crucial for maintaining cell viability and proliferation in 3D cultures.

Overall, the agarose hydrogel method outperformed the ULA plate and hanging drop techniques in generating consistent, compact, and viable spheroids. The semi-solid agarose matrix mimics in vivo tumor conditions, making it a suitable model for cancer biology and drug response studies. However, further studies incorporating additional lung cancer cell lines and investigating hypoxia and nutrient gradients are recommended to enhance the physiological relevance of these findings.

This study contributes to the growing body of evidence supporting 3D spheroid culture as a robust tool for cancer research. By tailoring culture conditions to cell-specific requirements, researchers can develop more predictive tumor models, ultimately improving translational outcomes in drug discovery and therapeutic testing.

Ethics Committee Approval

This study does not involve human participants or animal experiments. Therefore, ethics committee approval was not required.

Informed Consent

As this research was conducted using the A549 cell line and does not include human participants, informed consent is not applicable.

Author Contributions

Concept – Z.V., S.Y.; Design – Z.V., E.A.B., M.S.K.; Supervision - S.Y., M.D.S.; Resources - Z.V., E.A.B., M.S.K.; Materials - Z.V., E.A.B., M.S.K., N.M.Ö.; Data Collection and/or Processing - Z.V., E.A.B., M.S.K., N.M.Ö.; Analysis and/or Interpretation –M.S.K., M.D.S.; Literature Search - Z.V.; Writing Manuscript - Z.V., E.A.B., M.S.K, M.D.S,; Critical Review - Z.V., E.A.B., M.S.K., M.D.S., S.Y.

Conflict of Interest

The authors declare no conflict of interest.

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