JBACHS
Journal of Basic and Clinical Health Sciences

J Basic Clin Health Sci 2024; 8: 726-733
https://doi.org/10.30621/jbachs.1540783
RESEARCH ARTICLE

EFFECT OF COLLAGEN-COATING VARIATIONS ON THE MORPHOLOGY AND VIABILITY OF HUMAN VASCULAR SMOOTH MUSCLE CELLS

Ozge BAYRAK[1], Gizem CALIBASI KOCAL[2], Yasemin BASBINAR[2], Meltem ALPER[2], Serdar BAYRAK[2,3]

[1] Dokuz Eylul University, Institute of Health Sciences, Department of Oncology, Izmir, Turkey
[2] Dokuz Eylul University, Institute of Oncology, Department of Translational Oncology, Izmir, Turkey
[3] Dokuz Eylul University, Faculty of Medicine, Department of Cardiovascular Surgery, Izmir, Turkey

ORCID: O.B. 0000-0002-6223-1272; G.C.K. 0000-0002-3201-4752; Y.B. 0000-0001-9439-2217; M.A. 0000-0001-6359-9979; S.B. 0000-0003-1458-9023.

Corresponding author: Serdar BAYRAK, **E-mail:** serdar.bayrak@deu.edu.tr
Received: 29.08.2024; **Accepted:** 07.09.2024; **Available Online Date:** 30.09.2024

Cite this article as: Bayrak O, Calibasi-Kocal G, Basbınar Y, Alper M, Bayrak S. Effect of Collagen-Coating Variations on the Morphology and Viability of Human Vascular Smooth Muscle Cells. J Basic Clin Health Sci 2024; 8: 726-733.

ABSTRACT

Purpose: Collagen is a critical extracellular matrix (ECM) component that significantly influences cellular behaviors such as adhesion, migration, and proliferation. Optimizing collagen coating protocols is essential for developing accurate in vitro models, particularly for studying vascular smooth muscle cells (HVSMCs). The aim of this study was to optimize collagen coating protocols for in vitro models using HVSMCs by assessing cell morphology, adhesion potential, and viability under various collagen concentrations and incubation conditions.

Methods: HVSMCs were cultured on surfaces coated with different concentrations of Type 1 Rat Tail Collagen with different cell number (as 10^4 cells/well and 20^4 cells/well). The cells were incubated at various temperatures (4°C, 25°C, and 37°C). Morphological analysis was performed using phase-contrast microscopy to observe the alignment and phenotype of the cells. Cell adhesion was assessed using DAPI staining, and cell viability was evaluated using the Presto Blue assay after 96 hours of incubation.

Results: Collagen coating significantly influenced HVSMC behavior. The cells transitioned to a contractile phenotype, evidenced by tight, parallel bundle alignment, which is critical for maintaining vascular tone. Enhanced cell adhesion was observed in specific collagen-coated groups across different temperatures, particularly in the F, G, and H groups. Additionally, collagen coating did not significantly increase cell proliferation, making it suitable for in vitro vascular models. Optimal results were observed in groups seeded with 10^4 cells and incubated at 25°C and 37°C.

Conclusion: The study highlights the importance of optimizing extracellular matrix components like collagen in developing functional in vitro models. The identified optimal conditions for collagen coating will be valuable for future vascular modeling studies, providing a reliable foundation for in vitro research.

Keywords: Collagen-coating, in vitro vascular model, human vascular smooth muscle cells, morphology, viability

INTRODUCTION

Although diseases related to abnormal vascular systems generally exhibit common pathological features, they are highly heterogeneous, and each disease has its own unique characteristics. This necessitates the development of disease-specific vascular models (1). In this context, developing models specific to each disease is crucial for a better understanding of the pathogenesis and response to treatment. In modern medical research, it is essential to study the three-dimensional structures, mechanical properties, and biochemical microenvironments of tissues to understand how they form, function, become pathological, and behave as part of a living organism (1,2)

Traditional two-dimensional (2D) cell cultures are often unable to adequately replicate the complex dynamics of the in vivo cellular microenvironment, and therefore, they fail to fully sustain the differentiated functions that cells exhibit in their natural surroundings (4). In 2D cultures, cells may lose their natural morphology and fail to adequately replicate cell-cell or cell-extracellular matrix (ECM) interactions. These limitations hinder the accurate investigation of biological processes and disease mechanisms (5,6).

To overcome these shortcomings, three-dimensional (3D) cell cultures have been developed, which have the potential to better mimic the characteristics that cells exhibit in vivo. 3D culture systems can more accurately reflect important biological processes such as cell morphology, differentiation state, polarity, proliferation rate, gene expression, and genomic profiles by considering critical cell-cell and cell-ECM interactions. Cells grown in 3D cultures can adapt to the new microenvironment through changes in genetic and transcriptional activities, as well as in protein levels (5,6,7).

In this context, designing a three-dimensional human vascular system in vitro is of great importance for accurately mimicking vascular structures and functions (8). Such a model could significantly contribute to a better understanding of disease-specific vascular structures and processes, the development of new therapeutic strategies, and the advancement of personalized medicine approaches.

The aim of this study is to optimize the conditions that best support the physiological state of smooth muscle cells as a prerequisite for transitioning to a three-dimensional vascular model. In the development of three-dimensional (3D) cell culture models, accurate assessment of cell morphology and viability is critically important. Cell morphology reflects how well cells replicate their natural structures in vivo and whether biological processes are being correctly executed. Maintaining the correct phenotypic characteristics of cells, especially for vascular smooth muscle cells, is essential for preserving their functionality and accurately modeling diseases (5,8).

Moreover, monitoring cell viability is a fundamental criterion for evaluating the biological suitability and sustainability of the developed model. Ensuring that cells proliferate healthily but do not grow uncontrollably is necessary for disease models to accurately mimic biological reality under in vitro conditions. Therefore, optimizing basic parameters such as cell morphology and viability before beginning 3D modeling efforts should be prioritized to enhance the success and validity of the model (9,10).

For this reason, in the first phase of our study, we focused on the morphology and viability of smooth muscle cells to examine how these cells behave under physiological conditions and to what extent they meet the requirements of the 3D vascular model to be developed. Culturing cells under appropriate conditions is a critical step for maintaining their in vivo functions. The foundational data obtained will serve as a guide for the design and optimization of 3D structures that will be used in later stages of developing disease-specific models. In this way, it is aimed to preserve the correct phenotypic characteristics of the cells and contribute to the creation of models capable of mimicking the pathological processes of diseases. Ultimately, these studies will facilitate the development of more effective and specific therapeutic strategies for diseases.

MATERIAL AND METHODS

Cell Culture

In this study, primary human venous smooth muscle cells (CellBiologics, Cat No: H-6086) were cultured in vitro. The cells were maintained in a 5% CO_2 atmosphere at 37°C in an incubator, using vascular cell basal medium (ATCC, PCS-100-030) supplemented with a vascular smooth muscle cell growth kit (ATCC, PCS-100-042) and 1% Penicillin/Streptomycin. The cells were cultured in 75 cm^2 flasks.

Collagen Coating Methods

In this study, various coating protocols were developed using eight different collagen concentrations. The protocols were optimized by adjusting collagen concentrations and different culture medium conditions, and the most suitable coating method was determined. Type 1 Rat Tail Collagen (Corning 354236) was used for all groups and diluted in vascular cell basal medium and smooth muscle cell growth kit with 1% Penicillin/Streptomycin. The groups were prepared to contain different amounts of collagen per cm²; specifically, 0.2 µg (A), 2 µg (B), 5 µg (C), 10 µg (D), 156 µg (E), 42 µg (F), 93.75 µg (G), and 250 µg (H). Wells without collagen coating were used as the control group. The 96-well plates were prepared with 100 µl/well at the specified concentrations and incubated at +4°C, 25°C, and 37°C. Groups were formed using different cell densities of 10,000 cells/well and 20,000 cells/well to examine cell-cell interactions (11,12,13).

Morphological Observation

The 96-well plate surfaces were coated with the determined collagen coating protocols and allowed to dry. Human venous smooth muscle cells (HVSMC) were seeded at 10,000 (10k) and 20,000 (20k) cells per well for each group. The cells were incubated for 96 hours to assess cell morphology. After the incubation period, cell morphology was observed using phase-contrast microscopy.

DAPI Staining

To observe the attachment potential of cells to collagen-coated surfaces, DAPI staining was performed following a 2-hour incubation after applying the collagen coating protocols. The cells were washed twice with cold 1x PBS and fixed with 4% paraformaldehyde solution at room temperature for 10 minutes. The fixed cells were stained with DAPI (4',6-diamidino-2-phenylindole) diluted at a 1:1000 ratio (14).

Cell Viability

Following the application of the collagen coating protocols, conditioned media were collected from the wells after 96 hours of incubation and transferred to new 96-well plates. Cell viability was assessed using the Presto Blue reagent (15).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software. Categorical variables were analyzed using the chi-square test and Fisher's exact test. Continuous variables were analyzed using the t-test under the assumption of normal distribution or the Mann-Whitney U test if the distribution was not normal. Statistical significance was accepted as $p \leq 0.05$. The significance levels were defined as follows: Ns ($p > 0.05$), * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$). All data are presented as mean ± standard deviation (mean ± SD).

This study was conducted as part of the project "Effects of Different Dietary Components on Metastasis/Angiogenesis Pathway in Cancer," supported by the Dokuz Eylül University Scientific Research Projects. The project was approved by the Dokuz Eylül University Non-Invasive Research Ethics Committee (Date: 22.02.2023, No: 2023/05-32).

RESULTS

Morphological Observations and Contraction of Vascular Smooth Muscle Cells

Morphological observations revealed that vascular smooth muscle cells (VSMCs) transitioned to a contractile phenotype in the collagen-coated groups (Figure 1). The alignment of cells into tight and parallel bundles is indicative of a contractile phenotype, suggesting that the collagen-coated groups are morphologically suitable for in vitro applications. The transition to a contractile phenotype reflects the proper functioning of actin-myosin interactions and the associated cellular signaling pathways within the cytoskeleton of VSMCs. Maintaining this phenotype indicates that the biological conditions in the in vitro environment are appropriately sustained, allowing for healthy interactions with the extracellular matrix.

Adhesion of Smooth Muscle Cells

The aim of this study was to determine the most effective method for enhancing the adhesion of smooth muscle cells. After 2 hours of incubation, cells were immediately fixed. Compared to control groups at different temperatures, groups that provided superior cell adhesion at all three temperatures were identified (Figure 2). In particular, the F, G, and H groups showed high success in cell adhesion. These findings highlight the biocompatibility of the collagen

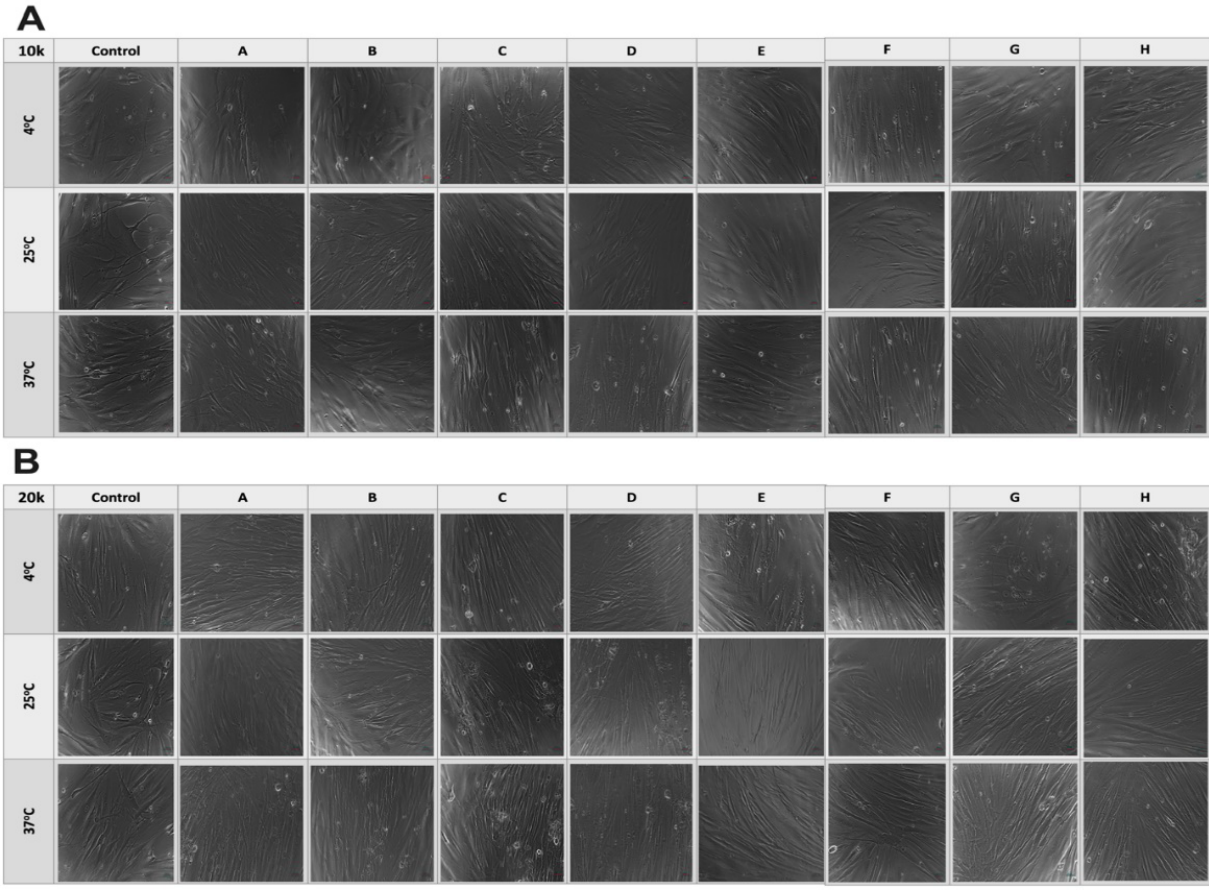

Figure 1. Morphological analysis of human primary vein smooth muscle cells (HVSMCs) on different collagen-coated surfaces. HVSMCs were cultured on plates coated with various collagen dilutions (A, B, C, D, E, F, G, and H) and visualized after 96 hours using phase-contrast microscopy. The images demonstrate successful collagen coating, as evidenced by tightly packed parallel bundles.

coating methods used and their positive impact on cell behavior.

Proliferation of Smooth Muscle Cells

To analyze the proliferation of smooth muscle cells, they were incubated for 96 hours, which is sufficient for a full cell cycle. In vascular pathologies, smooth muscle cells typically transition from a contractile phenotype to a synthetic one, leading to increased proliferation. In this study, it was crucial to select the collagen coating methods that did not promote excessive proliferation.

Among the groups seeded with 10k cells, no significant increase in cell viability was observed compared to the control group at 4°C, 25°C, and 37°C. These results suggest that this model is appropriate for in vitro modeling of vascular pathologies, as it does not promote excessive cell proliferation and accurately mimics physiological conditions (Figure 3). However, a statistically significant effect of collagen coating on cell proliferation was observed in the group of 10k cells incubated at 4°C and in all temperature groups seeded with 20k cells. Given that the aim of the study is to develop a healthy vascular model and that cell proliferation can indicate endothelial damage, it is preferable to select experimental conditions that do not affect cell proliferation. Since the groups seeded with 20k cells are completely excluded from consideration, it appears more appropriate to use 10k cells for cell seeding. As proliferation differences were observed in the 4°C group of 10k cells, the 25°C and 37°C conditions are deemed more suitable.

DISCUSSION

In our study, we aimed to optimize collagen coating protocols for in vitro vascular models by considering the morphology, adhesion potential, and viability of HVSMCs. The findings of this study demonstrated significant differences in cell behavior depending on the collagen concentration and incubation conditions, highlighting the importance of protocol optimization in the biocompatibility of biological materials.

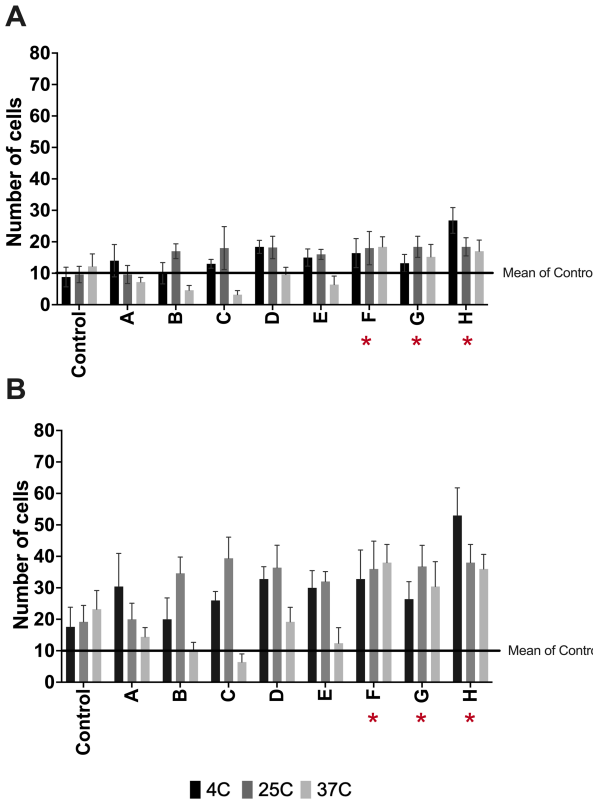

Figure 2. Adhesion efficiency of HVSMCs on collagen-coated surfaces at different temperatures. The number of adhered HVSMCs was quantified for each collagen coating method (A, B, C, D, E, F, G, and H) at 4°C, 25°C, and 37°C. DAPI staining was used to visualize the nuclei of adhered cells. The data indicate that F, G, and H coatings provided superior adhesion across all tested temperatures.

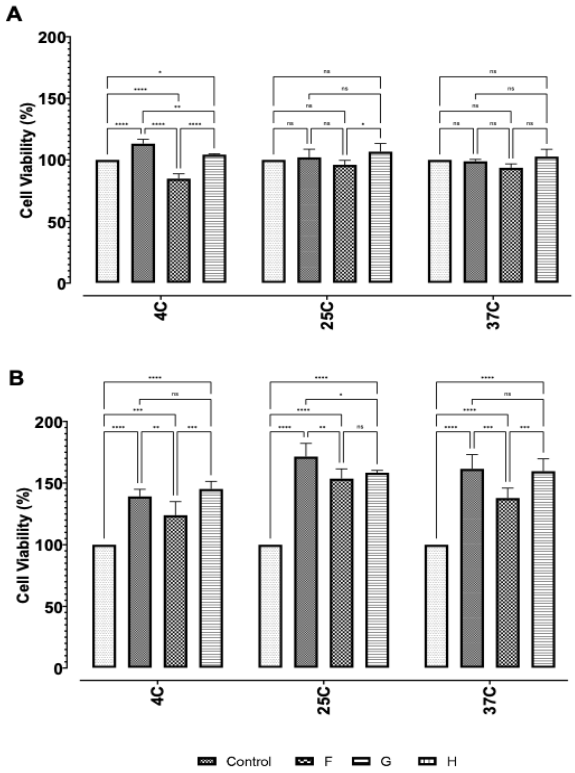

Figure 3. Cell viability of HVSMCs on different collagen-coated surfaces. HVSMCs were seeded at 10k and 20k cells per well on collagen-coated plates and incubated for 96 hours. Cell viability was assessed using the Presto Blue assay, and the results were normalized to control (uncoated) conditions. The analysis revealed that F, G, and H coatings maintained HVSMC viability, particularly at 4°C, 25°C, and 37°C, without promoting excessive proliferation.

Collagen is an abundant fibrous protein that constitutes the majority of the extracellular matrix (ECM) in all animals. This biological polymer is the main component of connective tissues, accounting for more than 30% of total protein in mammals. Generally, collagen provides structural integrity to all tissues, including blood vessels, skin, and bones. It is well established that collagen possesses biological properties that significantly influence cellular adhesion, migration, and proliferation (16, 17,18).

In this study, morphological observations revealed that vascular smooth muscle cells (HVSMCs) cultured on collagen-coated surfaces transitioned distinctly to a contractile phenotype (Figure 1). The alignment of cells into tight and parallel bundles is indicative of a contractile phenotype, which is critical for maintaining vascular tone and function in vivo. The contractile phenotype is characterized by the upregulation of contractile proteins such as α-smooth muscle actin (α-SMA) and smooth muscle myosin heavy chain (SM-MHC), which are necessary for actin-myosin interactions and cellular contractility (19).

The ability of HVSMCs to maintain this phenotype in vitro indicates that collagen coating not only provides an appropriate extracellular matrix for cell adhesion but also supports the biochemical signals necessary for the effective functioning of the contractile mechanism. This observation is consistent with previous studies that have shown the critical role of ECM composition in HVSMC phenotype (20,21). Additionally, the contractile phenotype observed and maintained in the collagen-coated groups suggests that the cellular signaling pathways remain intact and functional (22). These findings indicate that collagen

coating protocols are crucial for developing in vitro vascular models that aim to mimic the physiological conditions of blood vessels.

Our results showed that groups F, G, and H exhibited significantly higher cell adhesion compared to control groups across different incubation temperatures (Figure 2). These findings suggest that the collagen concentrations used in these groups enhanced the biocompatibility of the surfaces, positively impacting cell behavior. The observed effect on cell adhesion in these groups is likely due to the ECM properties provided by collagen. Previous studies have reported that the biochemical composition and structural properties of ECM proteins, such as collagen, are critical for cell adhesion, migration, and survival (23,24). The increased adhesion observed in groups F, G, and H suggests that collagen coating not only facilitates initial cell adhesion but also supports integrin-mediated signaling pathways necessary for stable adhesion (6,25). The consistent cell adhesion observed at different temperatures indicates that the bioactive properties of collagen coatings are maintained despite environmental changes, aligning with studies on physiological compatibility under various conditions (26).

In our study, HVSMCs were incubated for 96 hours with collagen coating to analyze cell proliferation. This duration is sufficient not only for the completion of a cell cycle but also for observing the transition from a contractile phenotype to a synthetic phenotype, which is associated with vascular pathologies (19). Selecting a collagen coating concentration that does not increase cell proliferation is important for the potential to mimic physiological conditions in vitro. It was observed that collagen coating had a statistically significant effect on cell proliferation in the group seeded with 10k cells and incubated at 4°C, as well as in all groups seeded with 20k cells. This suggests that cell density and specific temperatures can alter cell behavior. This finding supports the idea that these variables can affect cell proliferation and phenotypic stability in *in vitro* models.

The lack of significant increase in cell viability compared to the control group in groups incubated at 4°C, 25°C, and 37°C indicates that these coating methods do not trigger cell proliferation. This suggests that this model is suitable for in vitro vascular pathology modeling. Additionally, although no statistically significant changes were observed, detailed examination of cell viability compared to the control group (where cell viability was considered 100%) revealed that the G group at 25°C with a viability rate of 100.1% and the F group at 37°C with a viability rate of 99.7% were selected as the most applicable groups (Figure 3).

However, a statistically significant effect of collagen coating on cell proliferation was observed in the 4°C group seeded with 10k cells and in all temperature groups seeded with 20k cells. Given that the goal of the study is to develop a healthy vascular model and that cell proliferation can be an indicator of endothelial damage, experimental groups that do not affect cell proliferation were selected as the most appropriate conditions. Since the groups seeded with 20k cells were completely excluded, it appears more appropriate to use 10k cells for seeding. Furthermore, since proliferation differences were observed in the 4°C group of 10k cells, the 25°C and 37°C conditions are deemed more suitable.

In both temperature conditions, groups F (42 µg), G (93.75 µg), and H (250 µg) represented different collagen concentrations. To determine the optimal temperature, proliferation data among the F-G-H groups were compared, and given the lack of differences between the groups, the 37°C condition was deemed more appropriate. Among the F-G-H groups, the F group was preferred due to the lower collagen concentration. Therefore, for the subsequent studies required for vascular modeling in our project, it was determined that experiments would continue with 10k cell seeding and 42 µg collagen coating at 37°C.

Acknowledgements: None.

Author Contributions: OB: Conception, design, data collection, analysis interpretation, literature review, writing, critical review. GCK: Design, data collection, analysis-interpretation, literature review, writing, critical review. YB: Conception, design, data collection, analysis-interpretation, critical review. MA: Data collection, literature review, critical review. SB: Conception, design, supervision, funding, materials, data collection, analysis-interpretation, literature review, writing, critical review.

Conflict of Interest: There is no conflict of interest.

Ethical Approval: This study was conducted as part of the project "Effects of Different Dietary Components on Metastasis/Angiogenesis Pathway in Cancer," supported by the Dokuz Eylul University Scientific Research Projects. The project was approved by the Dokuz Eylul University Non-Invasive Research Ethics Committee (Date: 22.02.2023, No: 2023/05-32).

Funding: The study is funded by Dokuz Eylul University Scientific Research Projects Coordination Unit with project number TSG-2022- 2576. O.B. was supported by TUBITAK 2211C Domestic Priority Doctoral Scholarship Program, and Council of Higher Education 100/2000 scholarship in priority field program.

REFERENCES

1. Bhat SM, Badiger VA, Vasishta S, Chakraborty J, Prasad S, Ghosh S, Joshi MB. 3D tumor angiogenesis models: recent advances and challenges. J Cancer Res Clin Oncol 2021;147(12):3477-3494.
2. Baker BM, Chen CS. Deconstructing the third dimension: how 3D culture microenvironments alter cellular cues. J Cell Sci 2012;125(Pt 13):3015-3024.
3. Knight E, Przyborski S. Advances in 3D cell culture technologies enabling tissue-like structures to be created in vitro. J Anat 2015;227(6):746-756.
4. Huh D, Hamilton GA, Ingber DE. From 3D cell culture to organs-on-chips. Trends Cell Biol 2011;21(12):745-754.
5. Pampaloni F, Reynaud EG, Stelzer EH. The third dimension bridges the gap between cell culture and live tissue. Nat Rev Mol Cell Biol 2007;8(10):839-845.
6. Yamada KM, Cukierman E. Modeling tissue morphogenesis and cancer in 3D. Cell 2007;130(4):601-610.
7. Birgersdotter A, Sandberg R, Ernberg I. Gene expression perturbation in vitro--a growing case for three-dimensional (3D) culture systems. Semin Cancer Biol 2005;15(5):405-412.
8. Rensen SS, Doevendans PA, van Eys GJ. Regulation and characteristics of vascular smooth muscle cell phenotypic diversity. Neth Heart J 2007;15(3):100-108.
9. Cukierman E, Pankov R, Stevens DR, Yamada KM. Taking cell-matrix adhesions to the third dimension. Science 2001;294(5547):1708-1712.
10. Edmondson R, Broglie JJ, Adcock AF, Yang L. Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. Assay Drug Dev Technol 2014;12(4):207-218.
11. Hashimoto K, Yamashita K, Enoyoshi K, et al. The effects of coating culture dishes with collagen on fibroblast cell shape and swirling pattern formation. J Biol Phys 2020;46(4):351-369.
12. O'Sullivan D, O'Neill L, Bourke P. Direct Plasma Deposition of Collagen on 96-Well Polystyrene Plates for Cell Culture. ACS Omega 2020;5(39):25069-25076.
13. Chua P, Lim WK. The strategic uses of collagen in adherent cell cultures. Cell Biol Int 2023;47(2):367-373.
14. Karimi M, Mosaddad SA, Aghili SS, Dortaj H, Hashemi SS, Kiany F. Attachment and proliferation of human gingival fibroblasts seeded on barrier membranes using Wharton's jelly-derived stem cells conditioned medium: An in vitro study. J Biomed Mater Res B Appl Biomater 2024;112(1):e35368.
15. Ziaei Amiri F, Pashandi Z, Lotfibakhshaiesh N, Mirzaei-Parsa MJ, Ghanbari H, Faridi-Majidi R. Cell attachment effects of collagen nanoparticles on crosslinked electrospun nanofibers. Int J Artif Organs 2021;44(3):199-207.
16. Sorushanova A, Delgado LM, Wu Z, et al. The Collagen Suprafamily: From Biosynthesis to Advanced Biomaterial Development. Adv Mater 2019;31(1):e1801651.
17. Provenzano PP, Eliceiri KW, Inman DR, Keely PJ. Engineering three-dimensional collagen matrices to provide contact guidance during 3D cell migration. Curr Protoc Cell Biol 2010;Chapter 10:Unit-10.17.
18. Rezvani Ghomi E, Nourbakhsh N, Akbari Kenari M, Zare M, Ramakrishna S. Collagen-based biomaterials for biomedical applications. J Biomed Mater Res B Appl Biomater 2021;109(12):1986-1999.
19. Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol Rev 2004;84(3):767-801.
20. Kural MH, Billiar KL. Regulating tension in three-dimensional culture environments. Exp Cell Res. 2013;319(16):2447-2459.
21. Tracqui, P. Biophysical models of tumour growth. Reports on Progress in Physics 2009;72:056701.
22. Mack CP. Signaling mechanisms that regulate smooth muscle cell differentiation. Arterioscler Thromb Vasc Biol 2011;31(7):1495-1505.
23. Hynes RO. The extracellular matrix: not just pretty fibrils. Science 2009;326(5957):1216-1219.
24. Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. J Cell Sci 2010;123(Pt 24):4195-4200.
25. Geiger B, Yamada KM. Molecular architecture and function of matrix adhesions. Cold Spring

Harb Perspect Biol 2011;3(5):a005033.
26. Cummings CL, Gawlitta D, Nerem RM, Stegemann JP. Properties of engineered vascular constructs made from collagen, fibrin, and collagen-fibrin mixtures. Biomaterials 2004;25(17):3699-3706.