Fibroblast Embedded 3D Collagen as a Potential Tool for Epithelial Wound Repair

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ABSTRACT
Collagen is a functional biomaterial with many applications, including wound healing. 3D collagen hydrogels mimic an *in vivo* cell culture experience in cell survival and growth studies. In experimentally examining human cells under contact with 3D collagen, it is possible to understand the role of collagen in human epithelial tissue repair. This study explored the growth and attachment response of human MCF-7 cells when exposed to 3D collagen by investigating if the presence of NIH/3T3 fibroblasts embedded within the collagen should produce an increased wound-healing response. 3D collagen and fibroblast presence were able to be analyzed in tandem with a "sandwich-like" configuration of the gels to determine how these variables impact or improve the tissue repair response in MCF-7 cells. Examinations in growth, attachment, viability, and migration patterns demonstrated that MCF-7 repair response may be increased when in contact with NIH/3T3 embedded 3D collagen without impairing viability. Most notably, results from the migration assay revealed that MCF-7 cells migrate the most when covered by and adhered to cellular 3D collagen. Fibroblast-embedded collagen on top of and below MCF-7 cells exceeded quantitative assessment to near confluency, whereas less than 50 counted cells per image migrated without any top collagen layering. The continuation of these methods could involve in vivo experiments that incorporate live animal models to determine if these results will continue to extend to live tissue.

KEYWORDS
Collagen; 3D Collagen; Fibroblasts; Wound Healing; Hydrogels; Tissue Repair; Migration

INTRODUCTION

Fibroblasts are structural mesenchymal cells in connective tissue that are fundamental to producing collagen fibers, proteoglycans, fibronectin, glycosaminoglycans, and other extracellular matrix (ECM) components.[1] In the wound healing process, the body's response phases are as follows: hemostasis, inflammation, migration, proliferation, and remodeling.[2] Fibroblast cells, during the proliferative phase, increase production of collagen, elastin, proteoglycans, and hyaluronic acid, and these molecules are reorganized into a new ECM during the final phase.[2] The presence of fibroblasts has shown increased keratinocyte migration in a 3D wound model compared to fibroblast absence, indicating the importance of these cells in tissue repair.[3] The NIH/3T3 fibroblast tetraploid cell line (ATCC® CCL-1658™) isolated from Swiss albino (*Mus musculus*) murine embryos is a widely used model system for their functionality as a transfection host.

Similarly, MCF-7 cells (ATCC® HTB-22™) are used in cell modeling for their unique wound-healing behavior. MCF-7 cells are epithelial human breast cancer cells isolated in 1973 from the pleural effusion of a 69-year-old woman.[4] Cancer cells hijack parts of the wound healing process to ensure survival.[5] 3D models can be used to identify invasive or migratory patterns compared with 2D models more clearly.[6] When using a 3D collagen model, cell migration and degeneration of the extracellular matrix can be observed, more accurately modeling cancer cell metastasis.[6] Because deregulated dynamics with the extracellular matrix are a main characteristic associated with cancers, treatments can be better directed because of this enhanced visualization of changes.[7] This

response can be paired with 3D matrices, which shows that when MCF-7 cells are cultured in hydrogels, there is up-regulation of breast-specific markers compared to a standard 2D culture.[8]

Collagen's triple helix structure, one of the most abundant in the ECM, provides an ideal scaffold for use with the above cell lines.[9, 10] Conventional 2D cell culture offers insight into cell pathology, physiology, and function. However, a 3D collagen matrix can more closely resemble *in vivo* interactions between cells.[11] Collagen's low cost and flexibility make it one of the most commonly used proteins in matrix creation. Additionally, pore size, ligand density, and stiffness are easily customizable when using collagen as a scaffold in 3D culture.

Different cell lines and biomaterials can be modified to deliver drugs or cell therapy, coat medical devices, and protect against infection.[12] The use of collagen in tandem with NIH/3T3 cells in medical applications is becoming more common for their excellent wound healing properties. Collagen is commonly used as a hydrogel material because of its biocompatibility and ability to promote cell attachment, proliferation, and migration. Cells have been cultured on and within collagen to mimic biological microenvironments and examine their potential for wound treatment.[13] One study seeded fibroblasts into a collagen-rich hydrogel; the hydrogel formulation was thought to be a candidate for diabetic wound healing.[14] Cell viability, proliferation, and migration were studied using *in vitro* models, which showed that fibroblast survival and proliferation increased with a higher collagen concentration.[14] Another study used a hydrogel made of recombinant human collagen as a skin graft overlay.[15] The hydrogels were subjected to various tests to determine their biological properties using cell lines and live rat subjects. A cytotoxicity study of HaCaTs, HUVECs, and primary human fibroblasts indicated that the collagen extract had slight cytotoxicity, particularly for the HUVECs.[15] A migration study indicated that the ability of each cell line to migrate was increased following six to 24 hours of culture.[15] Live animal experiments suggested that the hydrogel promoted wound healing, dermis formation, basement membrane formation, angiogenesis, and proliferation.[15] The researchers determined that this hydrogel has the potential to aid in wound healing as a graft overlay.[15] Collagen hydrogels with and without cells seeded within them have the potential to aid in the treatment of wounds, but further research and modeling are needed.

Much of the work described previously utilized 2D culture models and animal models to test a material's wound healing properties. However, there is more to be done in developing 3D culture models. Because of the biochemical healing properties naturally expressed by fibroblasts, we proposed that a hydrogel constructed from 3D collagen embedded with NIH/3T3 cells can potentially increase the wound repair response in MCF-7 epithelial tissue. Repair response was measured using physical and quantitative values of cell attachment, growth, viability, and migration. Observing consistent cell viability and increased attachment and migration, cellular collagen hydrogels have the potential to be used as a tool for tissue repair.

METHODS AND PROCEDURES

Cell Maintenance

NIH/3T3 (ATCC® CCL-1658™) and MCF-7 (ATCC® HTB-22™) cell lines from the American Type Culture Collection (ATCC) were maintained in complete medium containing Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (PS). Cells were incubated at 37 °C and 5% CO_2 in T25 and T75 flasks. Cells were passaged every 48-72 hours as needed or when nearing confluence.

Cell Counting

Cells were trypsinized and diluted in complete medium. 100 µL of 0.4% Trypan Blue was added to a 100 µL aliquot of the cell suspension. 10 µL of the cell-dye mixture was loaded on a hemocytometer and analyzed using a 10x objective microscope. Cells within 1 mm^2 squares were manually counted, and the average of four squares was calculated. The total cell density was calculated using the following formula: average number of cells per square × 2 × 10^4.

Cell Suspension in 3D Collagen

Nearly confluent NIH/3T3 cells were trypsinized and resuspended in complete medium. For the attachment and proliferation and alamarBlue™ assays, a 3D collagen (Sigma-Aldrich® C4243™) mixture was prepared using 300 µL of cell suspension and 1.2 mL of collagen to create a final concentration of 2.02 × 10^3 cells per mL. For the migration experiment, the same method was used with different volumes: for the 300 µL and 200 µL aliquots, we used 360 µL and 240 µL of cell suspension with 1.44 mL and 960 µL of collagen mixture, respectively. The final concentrations were 3.9 × 10^3 cells per mL for the 300 µL aliquots and 4.6 × 10^3 cells per mL for the 200 µL aliquots. For the acellular collagen mixture, complete medium was added in place of a cell suspension. The collagen mixture was resuspended evenly. 300 µL and 200 µL aliquots were placed into the allotted collagen wells

for 12- and 24-well plates, respectively. The collagen was left to firm in a 37 °C incubator for two-to-three hours. Following gelation, MCF-7 cells were trypsinized to place on the set collagen gel. Medium was added to wells without cells seeded on top.

Attachment and Proliferation Assay

Attachment and proliferation were assessed by plating a 12-well plate of MCF-7 cells on acellular and cellular 3D collagen (**Table 1**). MCF-7 cells were seeded at equal concentrations on top of collagen either embedded with NIH/3T3 cells (Cell Col) or not embedded (Acell Col). Negative controls consisted of wells with collagen or on its surface. Positive controls consisted of wells with MCF-7 cells adhered and no collagen (No Col). 1 mL of complete medium was added to each well to ensure collagen hydration and cell maintenance. Observations were made after 24, 72, and 120 hours. At 120 hours, cell counts were performed and the remaining cells from representative conditions were transferred to a 96 well plate to perform an alamarBlue™ assay.

	1	2	3	4
A	MCF-7 Cells Only	MCF-7 Cells Only	Acellular Collagen with MCF-7s Seeded on Top	Cellular Collagen with MCF-7s Seeded on Top
B	Acellular Collagen	Acellular Collagen	Acellular Collagen with MCF-7s Seeded on Top	Cellular Collagen with MCF-7s Seeded on Top
C	Cellular Collagen	Cellular Collagen	Acellular Collagen with MCF-7s Seeded on Top	Cellular Collagen with MCF-7s Seeded on Top

Table 1. Design conditions for the attachment and proliferation assay in a 12-well plate.

AlamarBlue™ Assay

To assess viability of the MCF-7 cells in response to collagen exposure, an alamarBlue™ assay (BIOSOURCE® Cat. No. DAL1025) was performed using samples from a cellular control well, an acellular collagen well, and a cellular collagen well from the attachment assay. Each well was trypsinized and a cell count was performed with hemocytometry. The cell counts were found and used as a reference when setting up the assay. A 96-well plate was set up using three different samples: control MCF-7 cells grown in only medium (control), MCF-7 cells grown on acellular collagen, and MCF-7 cells grown on cellular collagen. A two-fold dilution series ranging from undiluted to 1:64 was performed, with a final cell solution volume in each well of 125 uL. 125 µL of medium was added to wells to serve as a blank. After incubating the plate for 72 hours, 12.5 µL alamarBlue™ Cell Viability Reagent was added to each well and incubated for two hours. A Biotek Synergy H1 Hybrid Multi-Mode Reader was used to read the fluorescence of the wells using excitation and emission values of 540 nm and 590 nm, respectively. The average of blank wells was calculated and used to find the blank-subtracted values of each well. Averages of the conditions (control, acellular collagen, cellular collagen) for each well dilution were calculated and used in statistical analyses using one-way ANOVA and two-sample t-tests ($p<0.05$).

Microscopy/Imaging

Each well was observed at 0, 24, 72, and 120 hours. Cells were assessed for attachment and growth through visual analysis. The shape of the cells, confluency, and other visually apparent changes were noted at each observation point. Images were taken through an Olympus IMT-2 Inverted Research Microscope at 100x magnification to record the qualitative data.

Migration Assay

Two 12-well plates of MCF-7 cells on acellular and cellular 3D collagen (**Table 2**). Alongside the 12-well plates, a 24-well plate was used for 200 µL collagen gels to be transferred on top of the scratch once firmed. MCF-7 cells were seeded on top of collagen either embedded with NIH/3T3 cells or not embedded. Two wells with MCF-7 cells adhered to the well bottom without collagen on top were scratched for positive controls. 1 mL of complete medium was added to each well to ensure collagen hydration and cell maintenance. Each well was then lightly scratched with a 1000 µL pipette tip to simulate a wound. An hour after scratching, the collagen gels were transferred to the 12-well plates and placed on top of the scratch. Plates were incubated at 37 °C and 5% CO_2. Following 24 hours, observations were conducted of each well, and images were taken using a Biotek Cytation 5 Cell Imaging Multimode Reader at 10x brightfield. Image recoloring was performed in Google. Scratch images were divided into three fields of view (FOVs) acting as three technical replicates per condition, and the number of cells migrated within the scratch were counted per FOV. The average and standard deviation were calculated for each condition, and both one-way ANOVA and two-sample t-tests ($p<0.05$) were performed to determine significance between groups.

	1	2	3	4
A	MCF-7 Cells Only with Acellular Collagen on Top	MCF-7 Cells Only with Acellular Collagen on Top	MCF-7 Cells Only with Cellular Collagen on Top	MCF-7 Cells Only with Cellular Collagen on Top
B	Acellular Collagen with MCF-7s Seeded on Top (Acellular Collagen on Top)	Acellular Collagen with MCF-7s Seeded on Top (Acellular Collagen on Top)	Acellular Collagen with MCF-7s Seeded on Top (Cellular Collagen on Top)	Acellular Collagen with MCF-7s Seeded on Top (Cellular Collagen on Top)
C	Cellular Collagen with MCF-7s Seeded on Top (Acellular Collagen on Top)	Cellular Collagen with MCF-7s Seeded on Top (Acellular Collagen on Top)	Cellular Collagen with MCF-7s Seeded on Top (Cellular Collagen on Top)	Cellular Collagen with MCF-7s Seeded on Top (Cellular Collagen on Top)

	1	2	3	4
A	MCF-7 Cells Only With No Collagen on Top	MCF-7 Cells Only with No Collagen on Top	EMPTY	EMPTY
B	Acellular Collagen with MCF-7s Seeded on Top (No Collagen on Top)	Acellular Collagen with MCF-7s Seeded on Top (No Collagen on Top)	EMPTY	EMPTY
C	Cellular Collagen with MCF-7s Seeded on Top (No Collagen on Top)	Cellular Collagen with MCF-7s Seeded on Top (No Collagen on Top)	EMPTY	EMPTY

Table 2. Design conditions for the migration assay in two 12-well plates. Wells contained a bottom layer of collagen, top layer of collagen, both layers, or neither. MCF-7 cells were seeded in all experimental wells.

RESULTS

Cellular 3D Collagen increases MCF-7 attachment and does not decrease cell viability.

Visual confirmation of gel conformation and cell attachment was completed prior to and immediately following cell seeding. In some cases collagen did not set evenly through the entirety of the well, or had air pockets (independent of the presence of cells). Every effort was made to acquire images in properly set and relatively unform collagen gels. **Figure 1** depicts the visual confirmation of cells being present following seeding. Immediately following seeding, cells maintained a rounded shape, indicating potential lack of attachment and spreading on the bottom of the well. This assay ran for 120 hours total. At the 24-hour time point of the assay, the MCF-7 cells seeded on top of the collagen had become partially attached. In all wells, a significant number of rounded cells remained, indicating a lack of attachment and spreading on the collagen gel. When examining for visual confirmation of attachment, cells appeared to be viable with typical conformation and growth patterns. The attempted monolayer creation of MCF-7 cells on the top of the collagen gel was successful, as all of the cells were visually in one layer and had not migrated or fallen into the gel layer. When observing the wells with NIH/3T3 cells embedded in the collagen, they were not easily visible, leaving only the MCF-7 cells to be viewed with a high level of certainty. A significant number of cells in every well lacked spreading and remained unattached or partially attached. Although medium was changed through pipette aspiration followed by addition of new media, many of the rounded cells remained. This suggests partial attachment rather than complete unattachment as aspiration would have removed fully unattached cells.

At the 72-hour time point (**Figure 2B**), each well was observed for cell conformation, attachment, viability, and growth. Based on visual observations, cells in the cell-only (No Col) well and on top of the acellular collagen (Acell Col) and cellular collagen (Cell Col), were all growing at an expected rate, reaching 40% confluency (**Figure 2B**). Some cells in every well continued to maintain a rounded shape indicative of lack of attachment or partial attachment and spreading. At the end of observations, each well was given fresh media.

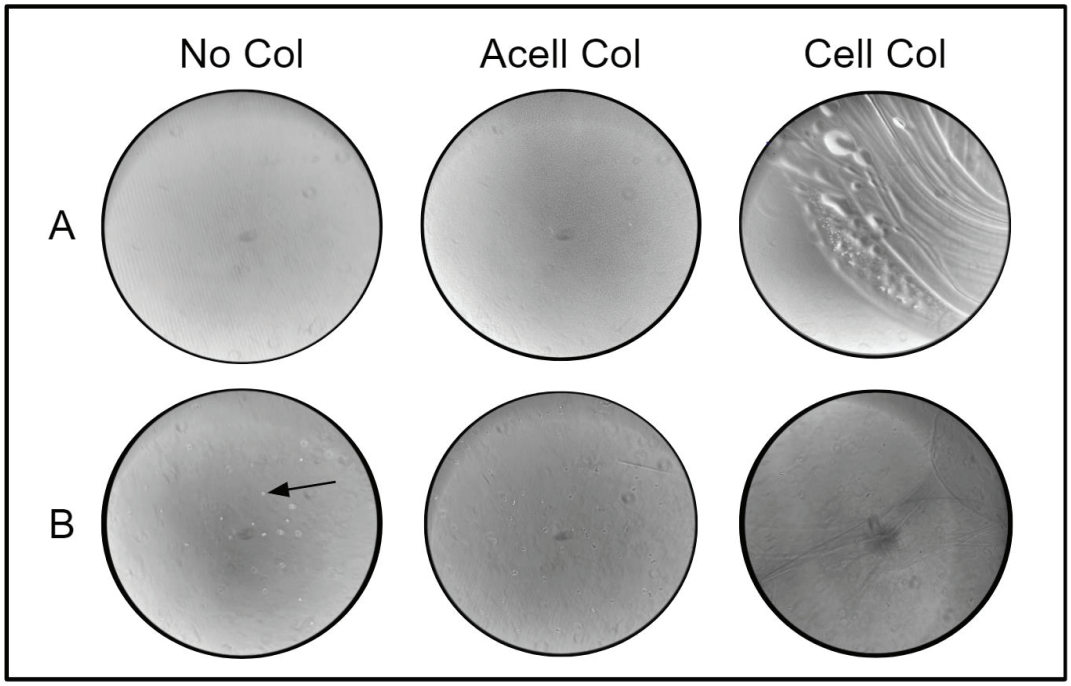

Figure 1. Attachment and Proliferation Assay, before **(A)** and after **(B)** cell seeding. From left to right: No Collagen (No Col), Acellular Collagen (Acell Col), and Cellular Collagen (Cell Col). Arrow indicates a rounded, not-yet or partially attached cell.

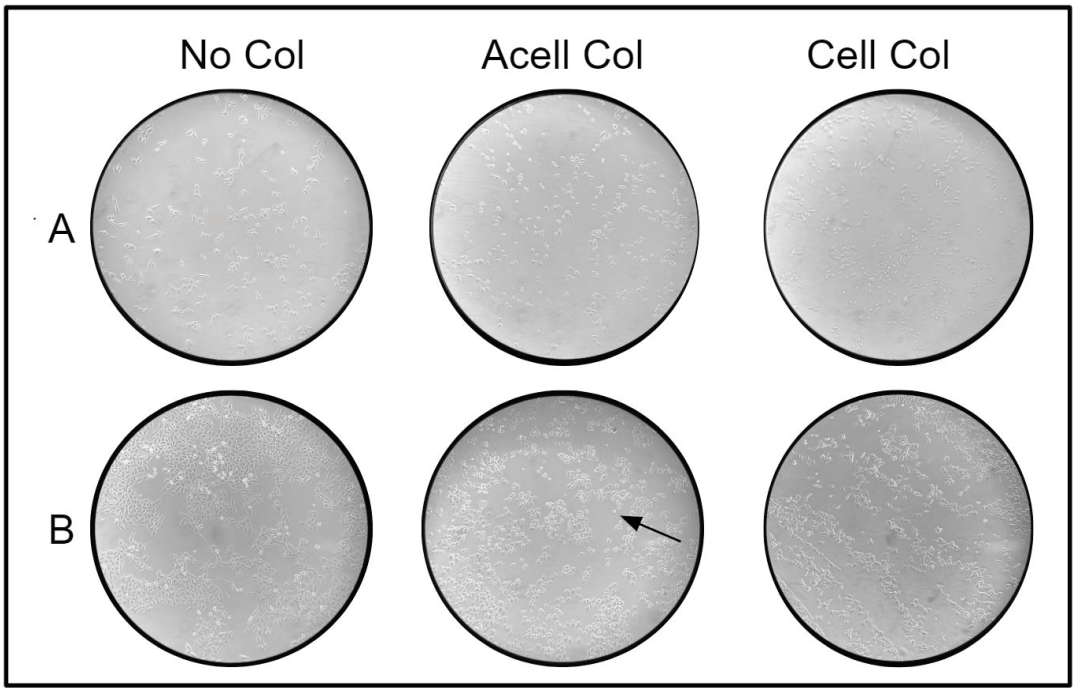

Figure 2. Attachment and Proliferation Assay, 24 **(A)** and 72 **(B)** hour time point. From left to right: No Collagen (No Col), Acellular Collagen (Acell Col), and Cellular Collagen (Cell Col). Arrow indicates a rounded, not-yet or partially attached cell.

Cells were again observed at the 120-hour time point for cell conformation, attachment, viability, and growth. Cells in all wells appeared to be more attached and spread than at the 72-hour time point. In wells with collagen that had MCF-7 cells seeded on top, distinct clusters of cells had been created. These areas had visual separations between highly confluent areas of cells and those with less confluence. This was noted on the wells with unseeded collagen and MCF-7 cells on top of the gel (**Figure 3A**).

The wells that appeared to have the highest confluency of MCF-7 cells were of the Cell Col condition. All cells appeared to be more attached than at previous time points and had less rounded conformation. Throughout each well, conformation and shape of cells was consistent with each other and with what is expected of the cell line. The average number of cells was 3.4×10^4 cells/mL in No Col control wells, 3.2×10^4 cells/mL in Cell Col wells, and 1.0×10^4 cells/mL in Acell Col wells. Cell counts revealed that there was no significant increase nor decrease in growth from the control condition compared to cellular collagen attachment. Interestingly, the decrease in cell concentration for acellular collagen conditions was remarkable. It appeared that cellular attachment and, most notably, confluency increased in collagen wells. In contrast, MCF-7 proliferation rates did not seem to improve as supported by the cell counts.

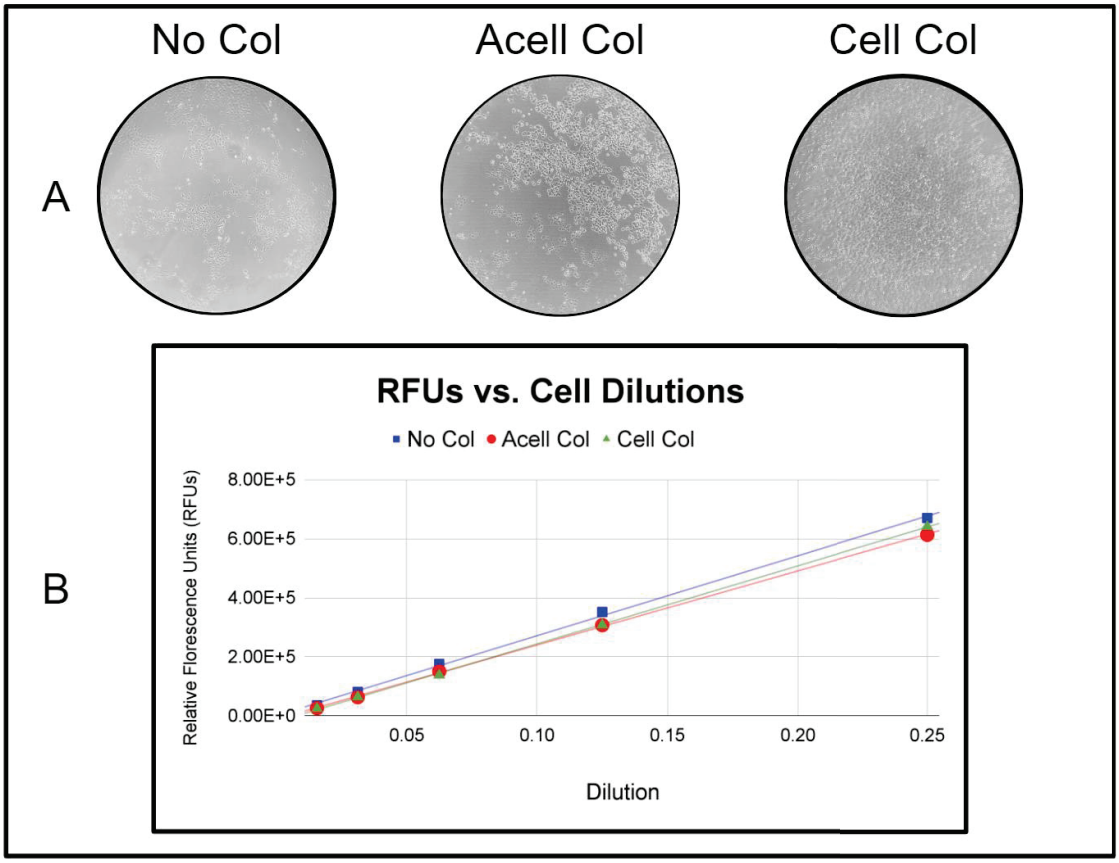

Figure 3. (A) Attachment and Proliferation Assay, 120 hour time point. From left to right: No Collagen (No Col, blue square), Acellular Collagen (Acell Col, red circle), and Cellular Collagen (Cell Col, green triangle). **(B)** Graph of fluorescence plotted against dilution for each condition of the previous attachment and proliferation assay.

The alamarBlue™ assay using a resazurin solution measured MCF-7 viability after no collagen exposure, exposure to 3D collagen, and exposure to fibroblast-embedded 3D collagen. When relative fluorescence units (RFUs) were graphed over dilutions, all three conditions showed a consistent linear growth across the dilutions 0.02 to 0.25 with R^2 values approaching 1 (**Figure 3B**). More concentrated dilutions (1.0 and 0.5) decreased the linear trend for each category, indicating that the concentration of cells in these wells exceeded detectable fluorescence levels. Therefore, data points from dilutions up to 0.25 were analyzed. Statistical analysis using both one-way ANOVA and two-sample t-tests comparing conditions in the 0.25 diluted wells revealed no significant differences ($p<0.05$), suggesting that contact with collagen does not affect cell viability.

MCF-7 cells migrate the most when covered by and adhered to cellular 3D collagen.

The migration assay began with plating MCF-7 cells onto either acellular or cellular collagen and incubating for 24 hours. At this time, cells not adhered to collagen were 30% confluent, cells adhered to acellular collagen were 75% confluent, and cells adhered to cellular collagen were 95% confluent (**Figure 4A**). After 24 hours of incubation, each well was scratched with a 1000 µL pipette tip (**Figure 4B**). Each scratch was made in the center of a well and did not puncture through the collagen gel, if present. After the scratches were made, collagen gels were added on top of designated scratches to represent a wound repair aid.

After a 24-hour incubation period, representative images of each scratched artificial wound were taken to assess the proliferation and migration of MCF-7 cells in response to 3D collagen treatment (**Figure 5**). The averages for each condition were calculated using three fields of view along the scratch (Figure 6A). Cell counts varied between conditions depending on the presence of collagen on top or bottom and whether fibroblasts had been embedded. Wells with acellular collagen on the bottom produced the highest attainable MCF-7 count (**Figure 6B**) when acellular collagen was placed on top, with an average of 78 migrated cells. Other wells contained more than 78 migrated cells but could not be counted accurately due to an overabundance of cells. All fields of view with cellular collagen on the bottom and top were too confluent to count the number of migrated cells.

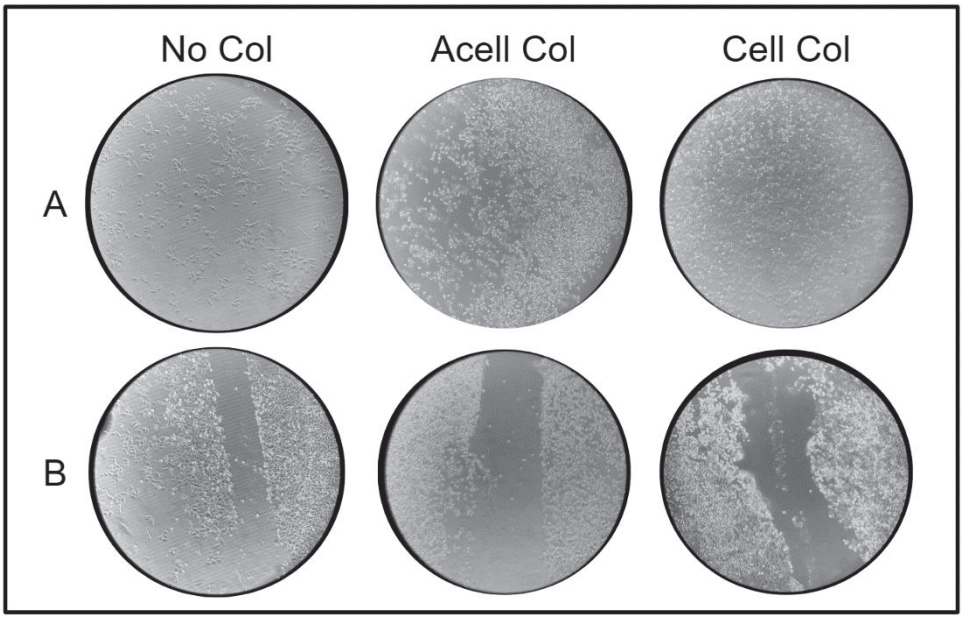

Figure 4. (A) NIH/3T3 Collagen base #2 MCF-7 cells before scratching. From left to right: Cells on No Collagen (No Col), MCF-7 cells on Acellular Collagen (Acell Col), and MCF-7 cells on Cellular Collagen (Cell Col). **(B)** Initial scratches of MCF-7 cells. From left to right: No Collagen (No Col), Acellular Collagen (Acell Col), and Cellular Collagen (Cell Col).

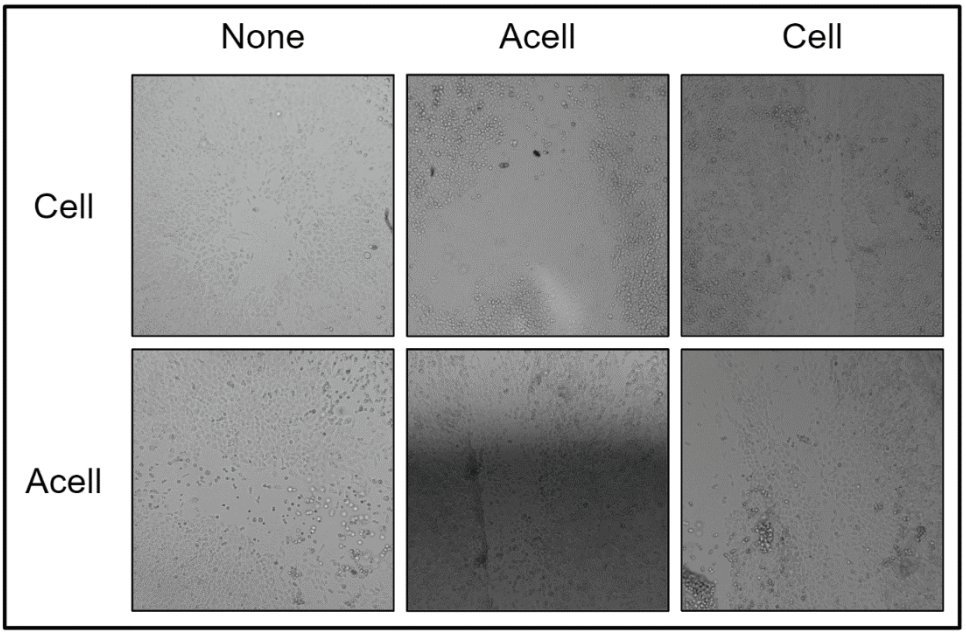

Figure 5. Scratches of MCF-7 cells following 24 hours (None - No Collagen on Bottom, Acell - Acellular Collagen on Bottom/Top, Cell - Cellular Collagen on Bottom/Top)

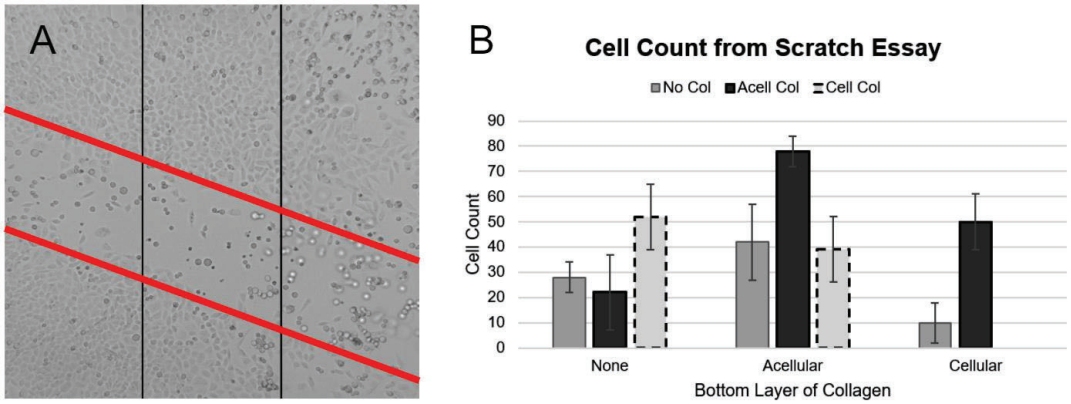

Figure 6. (A) Fields of View (FOV) for Cell Count, the black lines indicate the field of view and between red lines indicate the scratch location. Image taken at 100x magnification. **(B)** Results of Scratch Assay Cell Count Averages. Legend represents the condition of collagen on top. Error bars represent standard deviations of the technical replicates. The cell count for Cellular Collagen on Top/Bottom was not attainable due to an overabundance of cells to count.

The cells were microscopically examined, and images were taken after 24 hours (**Figure 5**) to perform a cell count of the scratched region. The wells with no collagen on the bottom had average cell counts of 28 ± 6, 22 ± 15, and 52 ±13 cells for the following conditions: no collagen on top, acellular collagen on top, and cellular collagen on top (**Figure 6B**). Of the conditions with no collagen on the bottom, the cellular collagen on top resulted in the largest number of migratory cells. The wells that had acellular collagen on the bottom had average cell counts of 42 ± 15, 78 ± 6, and 39 ± 13 cells for the following conditions: no collagen on top, acellular collagen on top, and cellular collagen on top, respectively (**Figure 6B**). The largest cell count was found in the well with acellular collagen on top and bottom. Finally, in the wells with cellular collagen on the bottom, the average cell counts were 10 ± 8 and 50 ± 11 cells for the conditions with no collagen on top and acellular collagen on top, respectively (**Figure 6B**). The condition where cellular collagen was present on the bottom and top of a cell monolayer was unable to be counted due to the density within the scratch.

Overall, conditions in which no collagen was placed on top of the scratch wound were associated with the least amount of cell migration. The conditions with the highest visible migration and wound healing rate were wells that had collagen placed on both the top and bottom with matched fibroblast variables (e.g., cellular collagen on top with cellular collagen on bottom). The wells with cellular collagen on both top and bottom or wells with acellular collagen on both top and bottom had the most growth success when compared to wells with no collagen placed on top of the wound or collagen that had mismatched cellular states. It is important to note that mere presence of collagen tended to increase migration rate, as the average counts for conditions without collagen on top were below 50 cells per FOV.

We performed one-way ANOVA statistical analyses to assess the significance of our results when greater than two conditions were being directly compared. The results of ANOVA did not meet the standard of significance; having data from more experimental replicates would likely increase the statistical resolving power. After performing a two-sample t-test ($p<0.05$) on the cell counts from the scratch assay in **Figure 6B**, the wells were compared to determine how significant cell migration was across scratch wounds. The migration of MCF-7 cells was larger and statistically significant for wells with acellular collagen on top and collagen on bottom, regardless of the presence of fibroblast cells within that bottom layer, when compared to wells that did not have any collagen layer on bottom (control). Similarly, wells with acellular collagen on top and collagen on bottom, regardless of the cellular status of that bottom layer, had a larger and statistically significant migration compared to wells with cellular collagen on top. Finally, cell migration was larger and statistically significant for wells with a top layer of cellular collagen, regardless of the presence of cells within the bottom layer, when compared to wells entirely without collagen and wells with only a top layer of acellular collagen.

DISCUSSION

This study aimed to explore the growth and attachment response of human MCF-7 cells when exposed to 3D collagen by investigating if the presence of NIH/3T3 fibroblasts embedded within the collagen should produce an increased response in cell repair. 3D collagen and fibroblast presence were analyzed in tandem with a "sandwich-like" configuration of the gels to determine how these variables impact or improve the tissue repair response in MCF-7 cells. Through the use of physical and quantitative values of cell attachment, growth, viability, and migration assay techniques, the wound repair response was measured and assessed. Examinations in growth, attachment, viability, and migration patterns demonstrated that MCF-7 repair response may be increased when in contact with NIH/3T3 embedded 3D collagen without impairing viability.

Based on the experimental plate setup with the collagen unevenly distributed, there could have been growth patterns of cells that were distributed or organized in a different orientation. This could have caused some of the stark lines or grouping of cell growth that we observed or the slower rate of attachment. Additionally, this environment and transfer likely caused a significant amount of cell stress which could have influenced the slowed attachment rate, more rounded epithelial shape, and decrease in proliferation for Acell Col wells. Overall, based on the repair pattern of the MCF-7 cells over time, the wells with the highest attachment rates were those with collagen gels seeded with NIH/3T3 cells. Despite this, conditions with cellular collagen were expected to have increased MCF-7 growth and replication due to NIH/3T3 cell wound healing capabilities. Fibroblast cells increase the production of collagen, elastin, proteoglycans, and hyaluronic acid, which promote new extracellular matrices to be developed. Further investigation should be done to confirm the absence of improved growth response from the cellular collagen-attached cells.

It is important that wound treatments do not impact cell viability nor cause increased cell death. Because collagen conditions did not impact viability compared to the control, we can infer that a collagen hydrogel does not produce any cytotoxic effects on live tissue cells. A majority of the dilution wells had linear trends with R^2 high values for the three conditions, indicating consistency in the viabilities. An ideal healing model would not only be safe and non-toxic to cells but also increase tissue regeneration. While these results did not show improved proliferation using a constant cellular concentration, further exploration of varying NIH/3T3 density within collagen may address this aspect.

When setting up the attachment and viability assay, multiple wells had collagen bases that did not set evenly through the entirety of the well. This was a factor that had to be considered through the whole assay as the inconsistency of the gel width could cause data discrepancies. Additionally, the collagen appeared to have pockets of air when observed with a microscope before seeding cells (**Figure 1A - Cell Col**). These bubble pockets existed in both cellular and acellular collagen. This was also seen in the migration/scratch assay. When the wells were scratched with the 1000 µL pipette tip, some of the cells were lifted from the collagen, and there was additional movement and folds added to the collagen.

The scratch-migration assay demonstrated that the most successful wound healing occurs when the cellular state of the top and bottom collagen agreed. The highest growth rate for all conditions was seen in the well with cellular collagen gels on both the top and bottom of the MCF-7 cells. However, the cellular state of the collagen only benefited the wound healing progression when both of the gels were cellular. When one collagen gel was cellular and one was acellular, this resulted in worse wound healing than the condition which had matching acellular collagen gels on the top and bottom of the scratch. This data shows that it is important to prioritize matching the collagen gel states to generate the most successful wound healing. Because cellular collagen is able to be used as a representative model of human skin, this makes the well conditions with cellular collagen on the bottom-most relevant to human application.

When cellular collagen was added to the top of scratch wounds in MCF-7 cells, the growth of the cells was too confluent in the scratch fields to count accurately. This result suggests that the addition of the cellular collagen layer on top of the wound rendered the scratch completely healed after 24 hours from a visual assessment standpoint. Additionally, it is important to recognize that even though when this condition had acellular collagen gels added to the top of the scratch wound, the healing was not as significant when compared to the cellular condition; however, the cell growth with the acellular gel was higher than the condition in which no collagen was added to the top of the scratch. Based on this, an acellular gel for wound healing may be more beneficial than not having one at all in situations in which a cellular collagen gel is not available. Overall, these results support the hypothesis that cell migration increases when exposed to cellular collagen, indicating a potential for applications of fibroblast-based wound healing mechanisms.

CONCLUSIONS

The cell attachment and viability assays showed that cellular collagen gels that are seeded with NIH/3T3 cells are able to promote higher levels of growth of cells than acellular collagen. To add to this finding, both cellular and acellular collagen gels resulted in more successful wound healing when collagen was added to the top of the scratch wound. Specifically, wound healing based on migration occurred most successfully for the cellular collagen conditions, but it was also noted that matching cellular states of the gels was important as well. This data supports our hypothesis that cellular collagen will promote wound healing because of its ability to model human epithelial cells and wound healing mechanisms. Our work provides a novel approach to explore the applications of collagen gels in wound healing. By creating a nontoxic model that placed collagen on both top and bottom of a scratch, we were able to more closely resemble a model of human epithelial tissue. This work is significant as it provides data on

the benefits of using cellular collagen as a model for epithelial tissue and as a method for wound healing, as well as developing a novel method of testing layered collagen gels for cell growth. Future directions to continue these methods would be to expand the number of biological and experimental replicates to increase the statistical resolving power, vary cellular concentrations within this experiment, and to conduct *in vivo* experiments that incorporate live animal models to determine if the results would continue to extend to live tissue.

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PRESS SUMMARY

Fibroblasts are a cell type that helps to form connective tissue, which acts as the glue to support organs in the body and play a role in wound healing. Similarly, 3D collagen hydrogels have been studied as wound-healing systems for skin cells. In this study, MCF-7 skin cells were exposed to 3D collagen embedded with NIH/3T3 fibroblasts to explore the growth and attachment response as a representation of cell repair. The results showed that the most wound-healing effects were seen when MCF-7 cells were covered by and adhered to cellular 3D collagen in a "sandwich-like" configuration or if collagen was embedded with fibroblast cells. These methods can be continued *in vivo* to determine if the results would extend to live tissue.