Effect of Retinoic Acid on HaCaT and NIH-3T3 cells in an *in vitro* 3D Collagen Cell Culture Skin Model

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ABSTRACT
Human skin aging is characterized by epidermal and dermal thinning, loss of elasticity, and wrinkles. Keratinocytes, the most common type of skin cell and fibroblasts, present in the cellular stroma beneath the skin's surface, each play a role in aging. Using these cell types in *in vitro* research can reveal a deeper understanding of the dermatological function and cellular changes in aged skin. 3D cell culture techniques provide an opportunity to use these cell types in a model that can more accurately mimic human skin. Treatment of aging skin is of interest to both medical and consumer communities. Retinoic acid (RA) is a metabolite of vitamin A and retinol that assists in cell proliferation, differentiation, and immune functions. Over the counter (OTC) and prescription retinoids are common topical products used for anti-aging and acne treatments. This study seeks to determine the impact of topical retinoid creams on keratinocyte proliferation and morphology in 3D cell culture models of aged and unaged human skin. NIH-3T3 fibroblasts were embedded in a 3D collagen matrix of varying thickness, and HaCaT keratinocytes were seeded on top of the matrix at varying seeding densities to mimic aging and youthful skin. 0.1 μM 0.025% tretinoin and 0.1 μM 0.1% adapalene topical creams were prepared in culture medium and used to treat cells daily, on alternate days, or just once during a week-long period. Alamar Blue assays and microscopy showed that tretinoin treatment was cytotoxic at this concentration, with a single treatment reducing cell viability by ~43% compared to the untreated control. Adapalene treatment, while showing significantly greater cell proliferation than tretinoin, did not exceed the proliferation of the untreated control. It is understood that retinol increases cell turnover by killing cells rapidly, so it is proposed that in our model, the rate of proliferation does not overcome the rate of cell death. Cell viability trends remain similar between young and old skin model treatments. Future studies should focus on creating a 3D model more accurately representing the aging *in vivo* skin environment where keratinocytes can be readily differentiated from epidermal stem cells.

KEYWORDS
HaCaTs; NIH-3T3; Retinoids; Retinoic Acid; Tretinoin; Adapalene; 3D Collagen Gel; Proliferation

INTRODUCTION

Epithelial cells are polygonal-shaped cells with regular, consistent dimensions. In two-dimensional culture, they grow in patches, form a monolayer, and tend not to migrate. Keratinocytes are the most common type of skin cell and function as the structural and barrier components of the epidermis, the outermost layer of the skin. Keratinocytes differentiate from epidermal stem cells and originate from the stratum basale, the deepest layer of the epidermis. As they age, grow, and differentiate into keratinocytes, they make their way to the epidermis, where they stay until they reach the final layer of the stratum corneum as corneocytes, where they die and are eventually shed by the body. Through differentiation, they produce keratin, the protein that strengthens the skin, hair, and nails.[1] These cells can be used to study the release of inflammatory and repair mediators. These cells can be used as a method to study the molecular mechanisms of epidermal regulation, homeostasis, pathophysiology, wound healing, and dermatologic diseases in *in vitro* models.[3] HaCaT cells are a nontumorigenic monoclonal cell line of spontaneously immortalized keratinocytes derived from human skin. HaCaTs are epithelial in nature and fully differentiate under culture conditions.[3] After stimulation from different calcium concentrations in the medium, HaCaT cells can form stratified layers, differentiate, and express differentiation markers. In addition, they can revert back and forth between differentiated and basal states.[2]

NIH-3T3 cells are fibroblasts derived from mouse embryos.[4] Fibroblasts are a key component of the stroma and, more specifically, the extracellular matrix (ECM). In connective tissue stroma, fibroblasts make several important products, including collagen, glycans, and prostaglandins. Commonly characterized by their plasticity, fibroblasts can differentiate to have many distinct functions. In the skin, for example, superficial fibroblasts hold a role in follicle formation and wound healing, while deeper cells are involved in ECM reorganization.[5] These cells are in constant communication with their surroundings and respond to both autocrine and paracrine signaling. Fibroblasts can interpret these signals and synthesize or remodel the ECM with regulation from various growth factors and pathways. Fibroblast structure is characterized by a star-like shape with several cytoplasmic projections.[5] In culture, the live cells are adherent and migratory. This specific 3T3 cell line was established in the 1960s, and since then, it has advanced the scientific understanding of fibroblast biology and lineage.[6] Fibroblasts are a valuable component of many cell culture models. In 3D models specifically, fibroblast-collagen matrices provide cell cultures that more closely represent the *in vivo* environment than traditional 2D cultures. Aspects such as cell-to-cell signaling and cell migration in a 3D fibroblast-collagen matrix more closely mimic those physiological conditions in the extracellular matrix.[7]

Collagen has a highly complex and hierarchical conformation with a recognizable triple helix secondary structure of amino acids.[8] Collagen is the most abundant protein in animals and is the primary component of the extracellular matrix, with 90% of the 29 identified types of collagens in humans being fibrillar. [9] Collagen I is the most common fibrillar collagen for cell culture, makes up the major structural component of many tissues, and can be found in skin, bone, tendons, and other connective tissues.[9] Collagen is the most used naturally derived hydrogel and coating for tissue engineering.[10] Collagen I is used in 2D cell culture as a coating or base and in 3D cell culture as a neutralized hydrogel to replicate the in-vivo environment of the body. 3D collagen hydrogels are used widely in cell culture, biotechnology, and medicine due to their polymer network and high-water content. As defined by Andersen et al., a three-dimensional cell culture involves cells embedded in a 3D matrix where cell and ECM signaling can be sent and received in all directions.[11] Cells can be embedded in the 3D collagen hydrogels or seeded on top of the hydrogel, serving as a 2D surface.[6] For example, a commonly used concentration of collagen is 2 mg/mL, a low concentration, specifically used for embedding cells as the hydrogel is less stiff and gives cells higher motility and higher cell migration velocity.[12] Collagen is advantageous for cell culture because of its biometric cytocompatibility, ability to assist cell adhesion, promote cell growth and differentiation, and can provide a similar viscoelastic environment.[7]

Retinoic acid (RA) is a metabolite of vitamin A that assists in the vitamin's related biological activities. RA is also a natural metabolite of retinol that assists in cell proliferation, differentiation, and immune functions.[13] Researchers have studied the growth and differentiation of human keratinocytes (HaCaTs) in response to RA *in vivo* and *in vitro* by evaluating differentiation through keratin synthesis and production; they found that RA was correlated to cell proliferation in vitro.[14] In addition, they discovered that HaCaT cells expressed specific keratins dependent on RA and calcium levels in the media.[14] When compared to untreated keratinocytes, HaCaTs treated with retinoic acid showed a mild increase in cell proliferation at certain doses.[14]

When skin ages, it undergoes structural changes such as thinning of its epidermal and dermal layers as well as weakening of the junction between these layers.[15] In a study with women aged 18-69, it was found that there is progressive and significant thinning, 6.4% per decade, of the epidermis beginning at age 30.[12] Mimicking these changes *in vitro* can be achieved by obtaining skin explants from elderly donors or treating cells with age-inducing chemicals. Researchers have developed a 3D model of aging skin to replicate the phenotypic changes observed in aging human skin using a collagen-based matrix seeded with normal human fibroblasts and then seeded healthy human skin cells from young subjects on top.[16] They found that in these prolonged cultures, epidermal thickness decreased over time.[16] Further analysis also indicated similarities to naturally aging skin, such as the thickening of the lamina densa, a part of the skin between the epidermis and dermis.[16] Another study used human skin models, to represent aging by manipulating the seeding concentration of senescent and normal fibroblasts in the matrix while monitoring keratinocyte proliferation and differentiation.[17] In their aged models they used specific keratinocyte seeding densities and fibroblast densities to more accurately represent *in vivo* skin.

Human skin needs to be examined experimentally to understand how different types of retinoic acid help its rejuvenation. Investigating this topic using a 3D cell culture more accurately models the *in vivo* skin environment. Topical Vitamin A treatments, such as retinol, retinoid, tretinoin, and adapalene, are used to treat acne by unclogging pores and providing anti-aging effects by increasing collagen production and cell proliferation while exfoliating and removing dead skin cells.[18] Tretinoin is a prescription retinoid whereas adapalene is sold over the counter. Since tretinoin is more concentrated it is used for different purposes like treatment of cystic acne and to assist in the healing of acne scars. Adapalene is used for skin rejuvenation, prevention and removal of wrinkles and fine lines.[19] The concentration of each drug, adapalene and tretinoin, is determined by several factors such as potency, effectiveness, and safety profile. Tretinoin and adapalene's concentration are also related to their mechanisms of action. Tretinoin is a highly potent retinoid that binds to nuclear retinoic acid receptors and regulates gene expression and leads to changes in cellular differentiation, proliferation, and apoptosis.[20] The effectiveness of tretinoin is dose-dependent and higher concentrations of the drug can lead to greater therapeutic effects. However, tretinoin can also be highly irritating and cause side

effects such as redness, peeling, and dryness of the skin.[20] On the other hand, adapalene is a less potent retinoid that acts by binding to specific retinoic acid receptors in the skin and regulating sebocyte differentiation and proliferation.[19] Adapalene is generally less irritating and handled better by users than tretinoin.[19] Therefore, lower concentrations of adapalene can still achieve similar therapeutic effects while minimizing the harmful side effects.

Previous studies have investigated the molecular basis of retinoids *in vivo* for cosmetic purposes.[21] Tretinoin and other FDA-approved retinoids are more thoroughly studied, with the mechanisms of action, genomic and nongenomic, well defined.[22] They also have clinical evidence to support their efficacy.[22] More must be understood about the comparative efficacy of over-the-counter (OTC) retinoids, like adapalene, which are readily available and popular for cosmetic use. As previously mentioned, retinoids show anti-aging effects, however, there need to be more studies surrounding these effects *in vitro*, specifically in 3D skin models. Our study begins to address this gap by creating a 3D model of human aging skin to investigate the proliferation rate of human epidermal tissue cells after treatment with prescription tretinoin and OTC adapalene creams. In this study, we show how HaCaTs (human epithelial cells), proliferate and behave in the presence of topical vitamin A treatments in *in vitro* 3D cell culture.

METHODS AND PROCEDURES

Cells and Culture Conditions

NIH-3T3 (ATCC, Catalog No. CRL-1658) and HaCaT (AddexBio, Catalog No. T0020001) cells were obtained. These cell lines were cultured at 37 °C and 5% CO_2 in complete medium that contains Dulbecco's Modified Eagle Medium (DMEM) (Corning, Catalog No. 10-013-CV) with 1x penicillin-streptomycin and 10% fetal bovine serum (FBS). Dulbecco's Modified Eagle Medium (DMEM) has a calcium concentration of 1.8 mM.[23] Cells were routinely passaged with 5% trypsin every two days prior to seeding in the 3D model.

3D Gel Model

To embed NIH-3T3 fibroblasts in a 3D collagen gel with a layer of HaCaTs on top of the gel a multi-step procedure was performed; a schematic can be found in **Figure 1** below. To mimic aged skin, the layer of fibroblasts embedded in collagen was 1 mm thick, as this was the smallest thickness that would cover a 24-well plate and be correlated to skin age. Since there is a 6.4% decrease in epidermis thickness every 10 years, younger skin was chosen to be 2 mm thick.[24] To seed 2 mm thick collagen gel, 0.2 mL of the fibroblast embedded collagen gel solution was needed to create this thickness, and 1 mm thick collagen gel required 0.1 mL of the fibroblast embedded collagen gel solution. The fibroblast-embedded collagen gel solution was 20% cell suspension and 80% 3D collagen. The gels were seeded with a fibroblast density of 0.55 x 10^6 cells/mL. So, a T75 flask of confluent NIH3T3 cells, at a concentration of approximately 0.7x10^6 cells/mL, was trypsinized with 1 mL of 5% trypsin and centrifuged for 5 minutes in a clinical centrifuge at 3000 rpm. The cell pellet was resuspended into 1 mL into complete media, counted, and resuspended again in 0.9 mL of complete media to create the 20% of 4.5 mL needed for the cell suspension. To make the fibroblast-embedded collagen gel solution, 3.6 mL of 3 mg/mL 3D collagen gel (Sigma Aldrich Catalog No. C4243) was mixed with 0.9 mL of the cell suspension was added (see Appendix). To add the control gels to the plate, 0.2 mL, or 0.1 mL of 3D collagen gel without fibroblast cells was added to non-fibroblast control wells. To add the remaining control and treatment gels to the plate, 0.2 mL or 0.1 mL of the fibroblast-embedded collagen gel solution was added to the wells. The plate was incubated for 1 hour for the gel to harden. A schematic of the plate can be found in **Table 1** below.

To mimic skin, a layer of HaCaTs were seeded on top of the fibroblast-embedded collagen gel with a density of 3500 cells/cm^2 to mimic aged skin and 7000 cells/cm^2 to mimic younger skin.[17] The surface area of a 24-well plate is 1.9 cm^2 and it has a volume capacity 0.75 mL of complete media. The cell density needed for the aged skin model was 6650 cells per well with 0.75 mL of media, and 10 mL of this cell suspension was needed to add to the top of 13 wells; the math can be seen in **Equation 1** below. Therefore, the cell density for the younger skin model was 13,300 cells per well in 0.75 mL media. To seed this layer of HaCaTs on top of the fibroblast collagen gel, a confluent T75 flask of HaCaT cells was trypsinized with 1 mL of trypsin, resuspended in 1 mL of complete media, counted, and resuspended in the correct amount of media to make the needed cell densities for each treatment group. After the fibroblast-embedded collagen gel solidified, 0.75 mL of the correct cell suspensions were added to the respective wells according to the schematic in **Table 1**. Finally, the plate was incubated at 37 °C with 5% CO_2 in a humid environment.

3500 cells/cm^2 with 0.75 mL media
24 well plate = 1.9 cm^2 surface area

$$\frac{3500\ cells}{cm^2} \times 1.92\ cm^2 = 6.650\ cells\ per\ well\ with\ 0.75\ mL\ media$$

$$\frac{6.650\ cells}{0.75\ mL\ media} \times \frac{cells\ per\ 1\ mL}{1\ mL\ media} = 8867\ cells/mL$$

Equation 1.

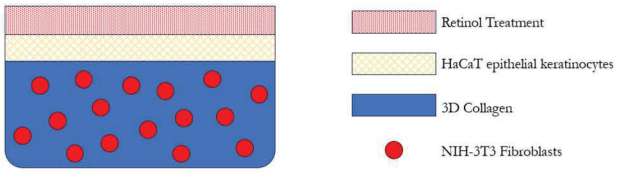

Figure 1. This Figure shows the collagen-cell matrix design. NIH-3T3 fibroblasts are embedded in a 3D collagen gel with a thickness of either 2 mm (0.2 mL) or 1 mm (0.1 mL) and HaCaT epithelial cells are seeded on top of the gel.

	1	2	3	4	5	6
A	2 mm CG + H + M	2 mm CG(F) + H + M	1 mm CG(F) + H + M	2 mm CG + M	1 mm CG + M	M only
B	2 mm CG(F) + H + T daily treatment	2 mm CG(F) + H + T alternating treatment	2 mm CG(F) + H + T single treatment	1 mm CG(F) + H + T daily treatment	1 mm CG(F) + H + T alternating treatment	1 mm CG(F) + H + T single treatment
C	2 mm CG(F) + H + A daily treatment			1 mm CG(F) + H + A daily treatment	1 mm CG(F) + H + A alternating treatment	1 mm CG(F) + H + A single treatment
D		2 mm CG(F) + H + A alternating treatment	2 mm CG(F) + H + A single treatment			

M = Complete DMEM
CG = Collagen Gel
CG(F) = Collagen Gel with NIH-3T3 fibroblast cells embedded
H = HaCaT cells
A = Adapalene treatment
T = Tretinoin treatment

Table 1. Retinoid Treatment Plate Set up.

Retinoid-Media Preparation

Perrigo Tretinoin Cream, USP 0.025% and Differin Gel Adapalene 0.1% were used. Due to retinoid sensitivity to both light and heat, these sterilization methods were not possible without altering the potency of the creams. Tretinoin and adapalene creams were streaked on LB agar plates and incubated for two days at 37°C to assess sterility. No growth was visible on any of the plates. 1 μM retinoid-media solutions were prepared by first massing specified amounts of 0.025% tretinoin cream and 0.1% adapalene cream according to the calculations in **Equations 2, 3, and 4** below for a final media volume of 20 mL. **Equation 2** shows the calculation of the desired 1 μM concentration in moles. **Equation 3** calculates the mass required for 1 μM 0.025% Tretinoin in 20 mL media. **Equation 4** calculates the mass required for 1 μM 0.1% Adapalene in 20 mL media. After measuring the appropriate retinoid weight, the cream was resuspended in 20 mL complete DMEM, 10% FBS as used in culturing described above. The solutions were filter sterilized using a 50 mL Steriflip filter unit (Millipore Sigma, Catalog No. C3238). Once prepared, the retinoid-media solutions were stored at 4 °C and equilibrated to room temperature prior to cell treatment.

$$1\ \mu M \times 20\ mL = 20 \times 10^{-9}\ mol$$

Equation 2.

1 μM 0.025% Tretinoin

$$(300.44\ g/mol(20 \times 10^{-9}\ mol))/0.00025 = 2.4 \times 10^{-2}\ g \text{ in } 20\ mL \text{ media}$$

Equation 3.

1 μM 0.1% Adapalene

$$(412.52\ g/mol(20 \times 10^{-9}\ mol))/0.001 = 8.25 \times 10^{-3}\ g \text{ in } 20\ mL \text{ media}$$

Equation 4.

Retinoid Treatments

After the plate was prepared and incubated for the cells to reach the correct confluence, the wells were treated with the 1 μM adapalene and tretinoin media prepared above. DMEM was removed from the wells and replaced with the respective treatment media. In the original treatment plate, control models were treated with complete DMEM every other day. For testing the impact

of tretinoin on cells, young and old skin models were treated with either consecutive 5 days (days 1, 2, 3, 4, and 5), 3 alternating days (days 1, 3, and 5), or a single first day (day 1) treatment of 0.1 μM 0.025% tretinoin. Young and old skin models were treated with 0.1 μM 0.1% adapalene in the same manner. Each well was observed daily, with a final imaging and proliferation assay readout on the 9th day after treatment.

Microscopy

During treatment, each well was imaged every day the media was changed on days 1, 2, 3, 4, 7, and 9 with the Olympus IMT-2 inverted microscope at 100X magnification, under 10X objective with a 10X eyepiece. After treatment concluded, HaCaT cells were imaged using a Cytation Gen 5 microscope under brightfield at 10X objective magnification.

Alamar Blue Resazurin Proliferation Assay

To assess cellular activity dependent on retinoid treatment conditions, a cell proliferation assay was completed with Alamar Blue Cell Viability Reagent (Invitrogen, Catalog No. DAL1025). For T=0 data, wells with 2 mm collagen-cell model, 1 mm collagen-cell model, and media only were treated 72 hours after seeding with Alamar Blue reagent at 10% of the gel and media volume in the well. For example, the wells with 0.2 mL collagen gel and 0.75 mL of media had 95 μL of Alamar Blue added. The plate was incubated at 37 °C and 5% CO_2 for 40 hours. The fluorescence of each well was measured using an excitation wavelength of 540 nm and an emission wavelength of 590 nm. This fluorescence intensity was measured in relative fluorescence units (RFU). Each measurement had an associated blank measurement without cells which was used to determine the baseline fluorescence of the media and collagen gel. The blank measurement value was subtracted from the cell dilution measurements to obtain fluorescence values from the cellular activity. This procedure was repeated 72 hours after completion of the treatment plate.

RESULTS

Proliferation Imaging Results

The wells were imaged every time the media were changed to observe HaCaT confluency and cell morphology. Models without cells were not imaged. Through visual observations, the HaCaTs proliferated normally in control models, treated with complete media. Controls included HaCaTs seeded on top of 2 mm thick 3D collagen gel with no fibroblasts embedded inside the gel and HaCaTs seeded on top of 2 mm and 1 mm thick 3D collagen gel with fibroblasts embedded inside the gel. Throughout the experiment, these models maintained a healthy cell morphology, with the cells being very confluent by the end of the experiment on day 9, as shown in **Figure 2**. As the experiment goes on the cells become more confluent as expected with very few floaters every day. The few floaters were removed with media changes every other day. The first day after seeding the HaCaTs seeded on top of the gel with fibroblasts embedded were more confluent than the HaCaTs seeded without fibroblasts embedded.

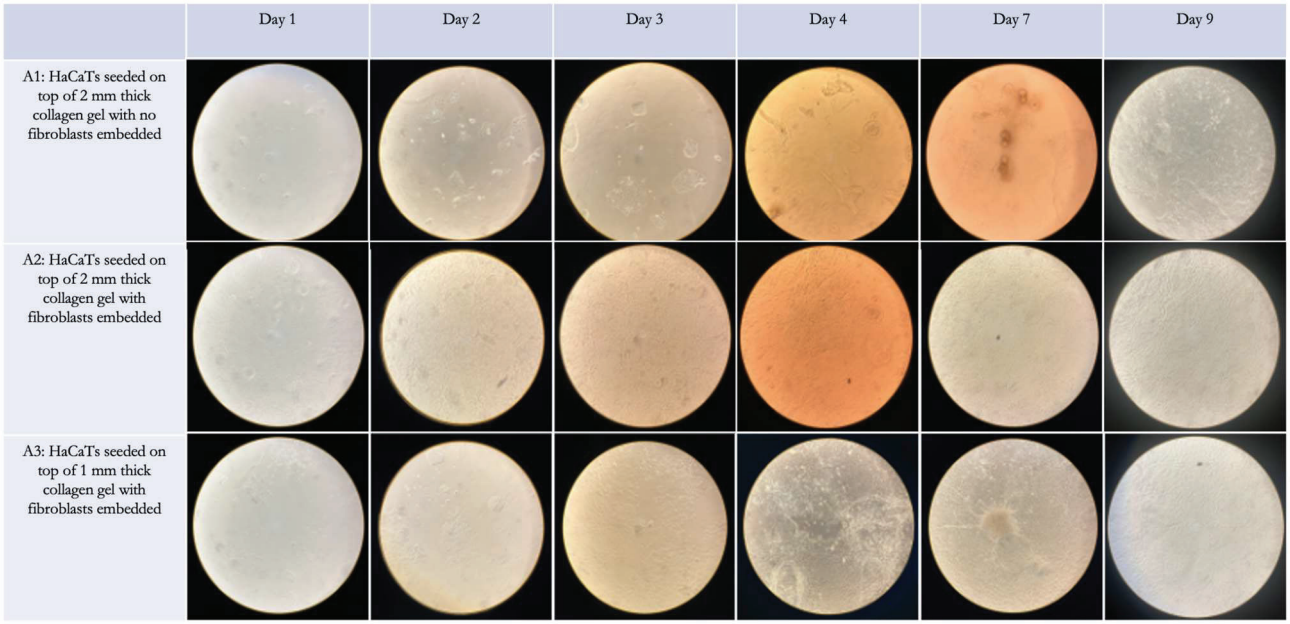

Figure 2. Daily imaging of HaCaT control wells A1 (2 mm collagen gel, no fibroblasts) and A2/A3 (2 mm and 1 mm collagen gel with fibroblasts) shows increasing confluency, reaching full confluence by day 9 with healthy cells.

The tretinoin treatments produced cells that balled up, detached from the gels, and floated. Images correlating to tretinoin treatment wells can be found in **Figures 3** and **Figure 4** for the younger and aged treatments respectively. Throughout the course

of the tretinoin treatment, the wells with less treatment frequency had healthier-looking and more confluent cells. The wells treated with tretinoin every day were losing cells when the media was replaced; the remaining cells were circular and not attached to the gel and by day 9 the well had lost most of its cells and was not very confluent; this may be due to tretinoin's mechanisms of action.

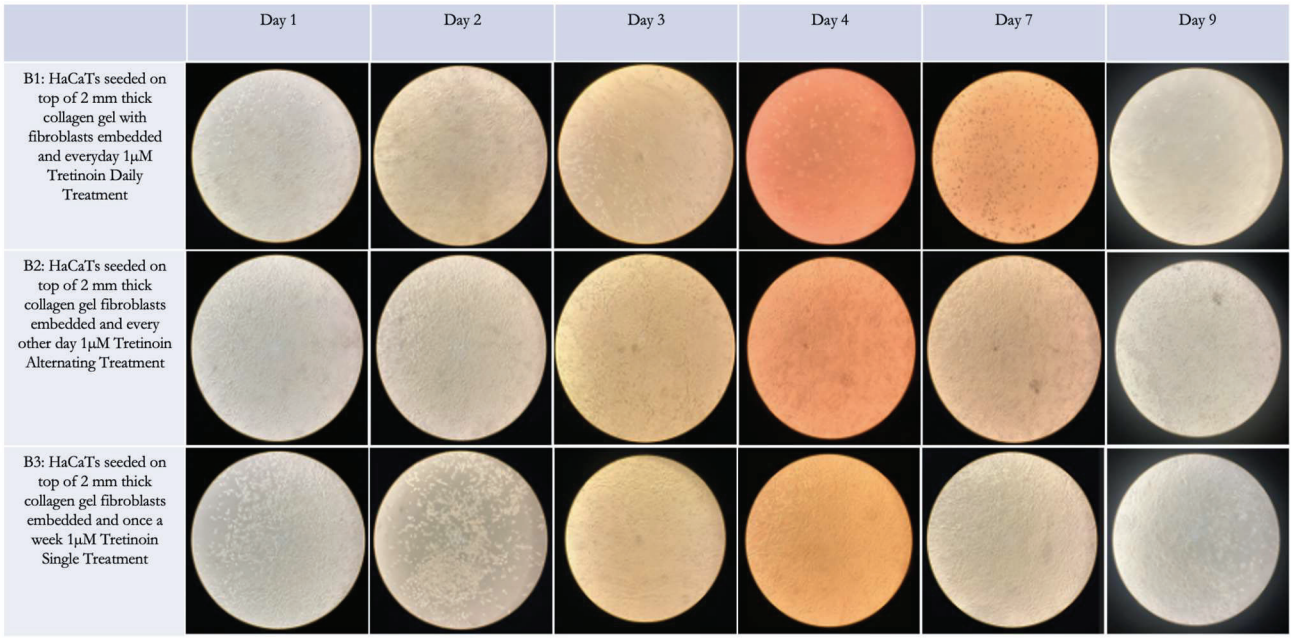

Figure 3. Daily imaging of HaCaT cells on 2 mm collagen gel with embedded fibroblasts. B1 received daily 1 μM tretinoin, B2 every other day, and B3 once a week with media changes. B1 confluency decreased, while B2 and B3 increased daily, except for B2 by day 9.

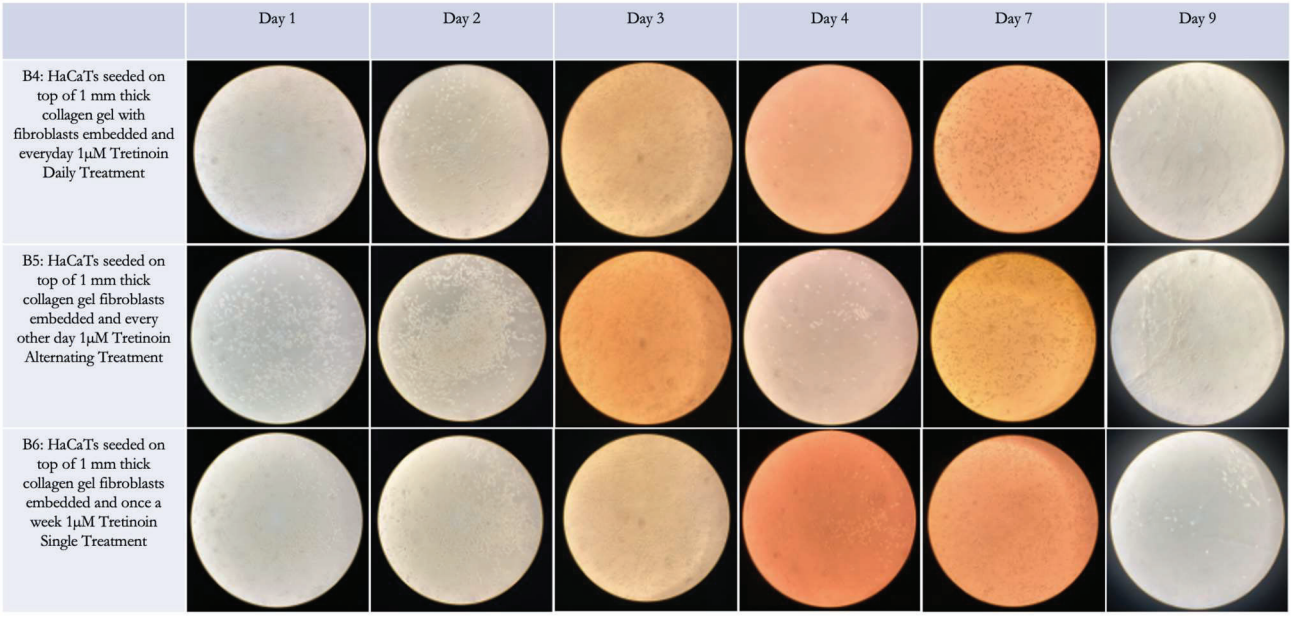

Figure 4. This figure shows daily imaging of HaCaT cells on 1 mm collagen gel with embedded fibroblasts. B4 received daily 1 μM tretinoin, B5 every other day, and B6 once a week with media changes. B4 and B6 confluency decreased, while B5 increased daily. The cells showed unhealthy morphology and detachment by the end of the experiment.

The adapalene treatments produced much healthier cells with better morphology and higher proliferation rates. The images that correlate to the adapalene treatment wells can be found in **Figure 5** and **Figure 6** for the younger and aged treatments respectively. Again, the wells treated less frequently with adapalene were more confluent and healthier. The confluence of younger

treatments is significantly more confluent than the older treatments, but the seeding density of the older treatments was lower than the younger treatments. This may be because there was more room for the cells to proliferate and grow since the seeding density was lower.

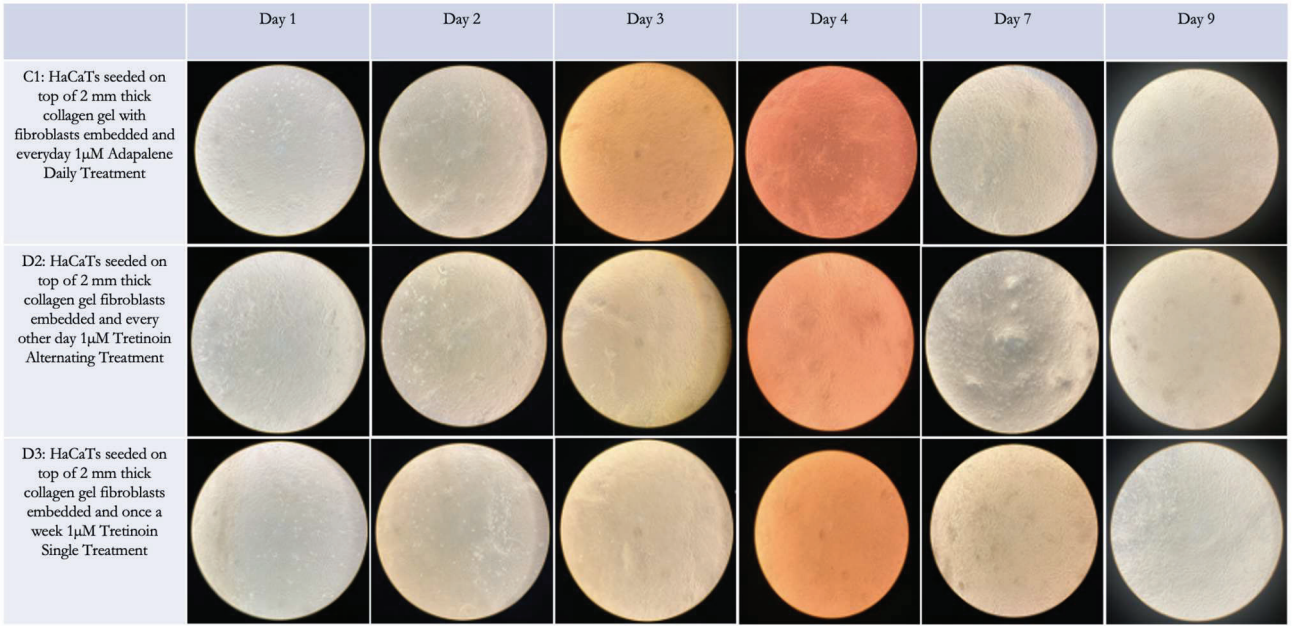

Figure 5. Daily imaging of HaCaTs on 2 mm collagen gel with embedded fibroblasts. C1 received daily 1 μM adapalene, D2 every other day, and D3 once a week with alternate-day complete media replacements. Wells remained consistently healthy and confluent throughout the experiment.

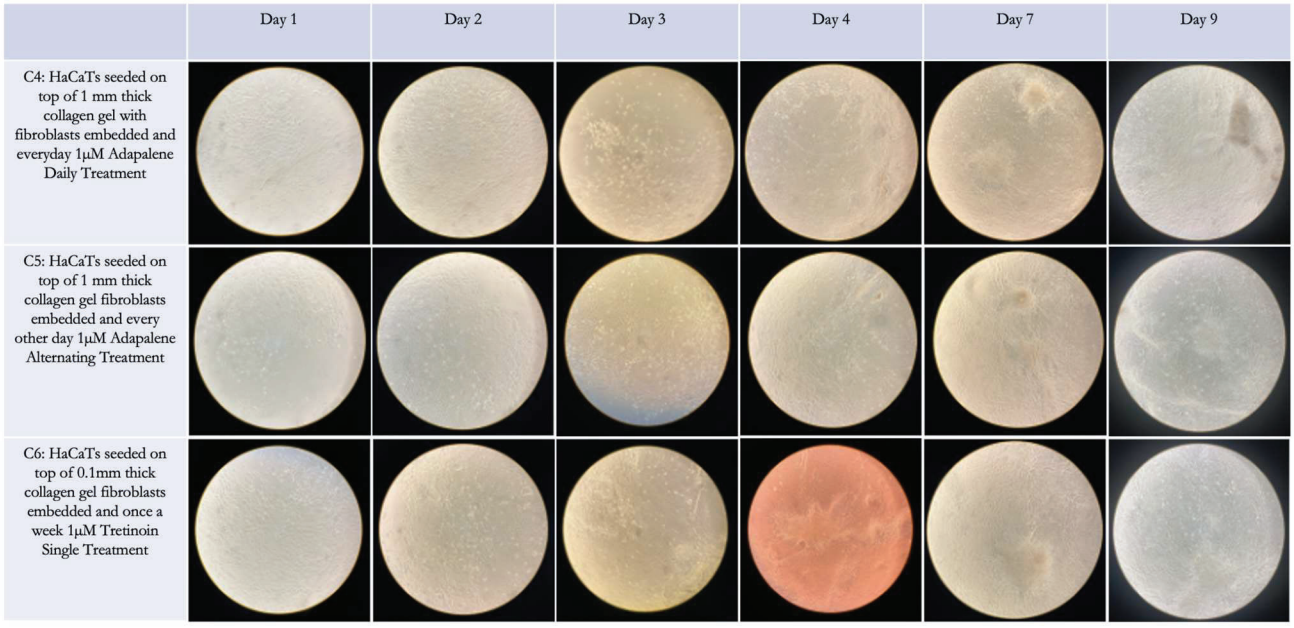

Figure 6. Daily imaging of HaCaTs on 2 mm collagen gel with embedded fibroblasts. C4 received daily 1 μM adapalene, C5 every other day, and C6 once a week with alternate-day complete media replacements. Despite lower initial cell density, all wells showed healthy and confluent cells by the experiment's end.

On the final day, the wells were imaged to assess the health of the cells, and the images can be found in **Figure 7**. Overall, the cells from the control model of HaCaTs with no retinoid treatments, were healthy and confluent. Control models with fibroblasts embedded were more confluent than those without, indicating a possible relationship between fibroblast and HaCaT proliferation. The models treated with tretinoin had cells that were less confluent than those treated with adapalene. Tretinoin is more concentrated than adapalene, so the effects are stronger. Overall, the adapalene treatment, no matter the treatment frequency or younger or aged model, had healthy and very confluent cells. For the younger adapalene treatments, as the

treatment frequency decreased, the cell confluency increased. However, for the aged adapalene treatments, as the treatment frequency decreased, the cell confluency decreased.

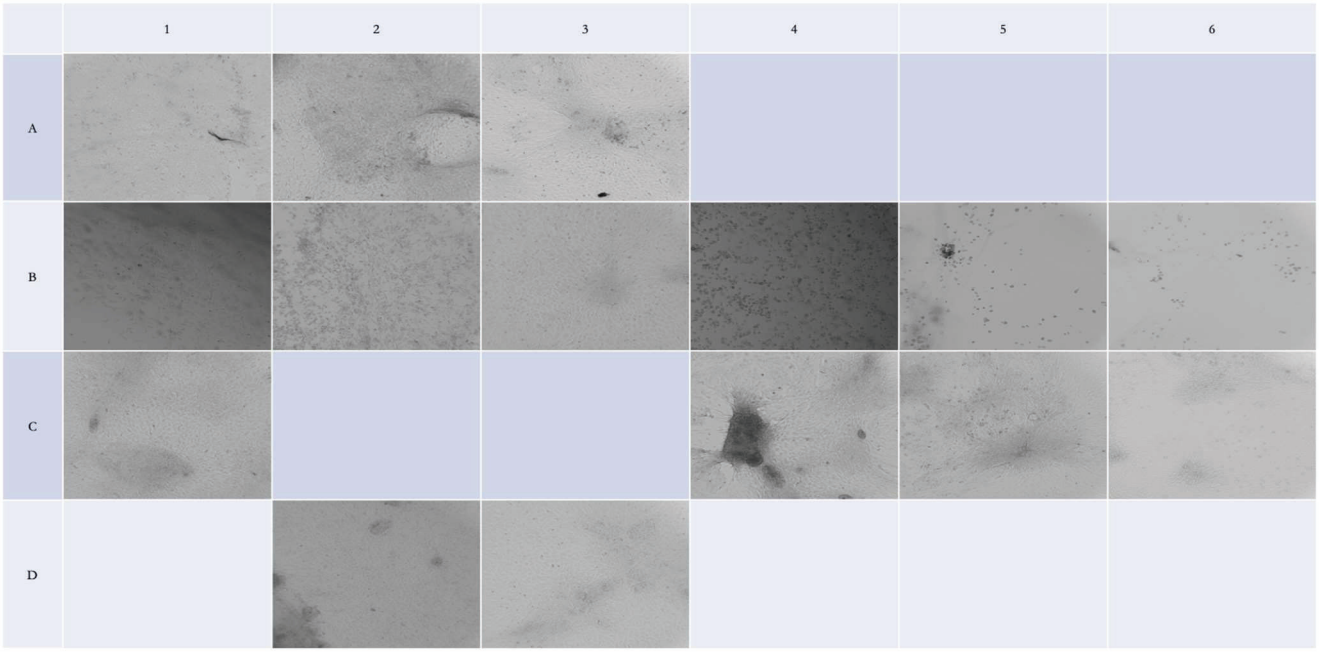

Figure 7. Final day images of HaCaTs on 3D collagen gel wells. A1, A2, and A3 show healthy and confluent cells. In the young tretinoin models (B1, B2, B3), cell density varies, with B2 showing unhealthy morphology. Aged tretinoin models (B4, B5, B6) exhibit different cell densities and morphologies, with B5 and B6 showing unhealthy cells.

Effects on Cell Proliferation

The Alamar Blue resazurin assay provided a quantitative reflection of cell metabolism for each condition, which correlates to proliferation of live cells. **Table 2** shows the contents of the Alamar Blue plate along with the associated reference-subtracted fluorescence readouts. Numbers under each description represent these reference-subtracted fluorescence values obtained for the well. 2 mm gel models used the 2 mm controls for reference subtraction, while 1 mm gel wells used the controls with 1 mm gels. Media only wells were self-subtracted. Wells are color coded according to fluorescence values, with the brightest red color representing the lowest reference-subtracted fluorescence, associated with the least number of live cells, while the brightest green color representing the highest reference-subtracted fluorescence, associated with the greatest number of live cells. A detailed key for the color code is included to the right of the table. Control wells present in both the T=0 and treatment plate included gels with and without embedded fibroblasts. The presence of fibroblasts in the collagen gel matrix correlated to greater proliferation and subsequently greater HaCaT cell proliferation. A fluorescence of 649,675 RFU was recorded with embedded fibroblasts in the 2 mm gel, and only 176,057 RFU for the same gel thickness without fibroblasts (A1).

Highest Fluorescence

Lowest Fluorescence

M = Complete DMEM
CG = Collagen Gel
CG(F) = Collagen Gel with NIH-3T3 fibroblast cells embedded
H = HaCaT cells
A = Adapalene treatment
T = Tretinoin treatment

	1	2	3	4	5	6
A	2mm CG + H +M 176057	2mm CG(F) + H +M 649675	1mm CG(F) + H + M 477158	2mm CG + M 0	1mm CG + M 0	M only 0
B	x	x	x	x	x	x
C	2mm CG + H +M 1132518	2mm CG(F) + H +M 2083577	1mm CG(F) + H +M 2055823	2mm CG + M 0	1mm CG + M 0	M only 0
D	2mm CG(F) + H + T daily 197042	2mm CG(F) + H + T alternating 740699	2mm CG(F) + H + T single 1180820	1mm CG(F) + H + T daily 98911	1mm CG(F) + H + T alternating 631870	1mm CG(F) + H + T single 1186139
E	2mm CG(F) + H + A daily 1983226	2mm CG(F) + H + A alternating 8319	2mm CG(F) + H + A single 1780495	1mm CG(F) + H + A daily 864618	1mm CG(F) + H + A alternating 2053607	1mm CG(F) + H + A single 1910548

Table 2. Alamar Blue well contents and associated reference-subtracted fluorescence values.

Table 2 shows the well contents for the Alamar Blue assay from the T=0 control plate, in row A, and the original treatment plate, in rows C through E. Numbers under each description represent the reference-subtracted fluorescence values obtained for the well. 2 mm gel wells used the controls in wells A4 and C4 for reference subtraction, while 1 mm gel wells used the controls in wells A5 and C5. Wells are color coded according to fluorescence values, with the brightest red color representing the lowest reference-subtracted fluorescence, associated with the least number of live cells, while the brightest green color representing the highest reference-subtracted fluorescence, associated with the greatest number of live cells. A detailed key for the color code and abbreviations is included to the right of the Table.

To better visualize and compare fluorescence values for the different control and treatment conditions, data was graphed in **Figures 8 and 9**. The graphed values are referenced subtracted in the same manner as previously described. **Figure 8** depicts the primary T=0 controls, 2 mm (young) and 1 mm (old) collagen gels embedded with fibroblasts, seeded with HaCaTs on top, and DMEM media treatment. This assay was completed prior to treatment and after 3 days of seeding. The young skin model was seeded with higher HaCaT density and shows a significantly higher fluorescence, correlating with higher cell viability. Conversely the old skin model shows lower fluorescence, and less cells. These controls act as a basis for the following young and old skin models that were treated. **Figure 9** includes data for the treated models with HaCaT proliferation based on an Alamar Blue assay fluorescence readout plotted against frequency of tretinoin or adapalene treatment for young (2 mm) skin in the left panel and old (1 mm) skin in the right panel. 5 days indicates 5 consecutive days of treatment, 3 days indicates alternate days of treatment, and 1 day indicates a single first day of treatment during the course of 5 days. The well for alternate day adapalene treatment for the young skin dried causing cell death and an inaccurate readout, so this data point is not representative of the true effects of the treatment, so it was removed from **Figure 9a**; however, these data correlate with the microscopy results which show very few viable cells after the well dried. Ignoring this data point, the adapalene appears to increase proliferation with increased treatment frequency for young skin, but it does not meet or surpass the fluorescence measured for the untreated control. Adapalene with the old skin model shows alternate day treatment to have the greatest fluorescence with a value nearly equivalent to the untreated control (2,053,607 RFU and 2,085,823 RFU, respectively). Tretinoin treatment in both young and old skin shows an interesting linear trend. When a simple linear regression is made for these points, there is a nearly perfect fit in both the young and old skin, with R^2 values of 0.9963 and 0.9999, respectively. Tretinoin performed better when only treated once a week, but in both models this single treatment reduces cell viability by about 43% compared to the untreated control. More significant toxicity was observed if treated more than once per week.

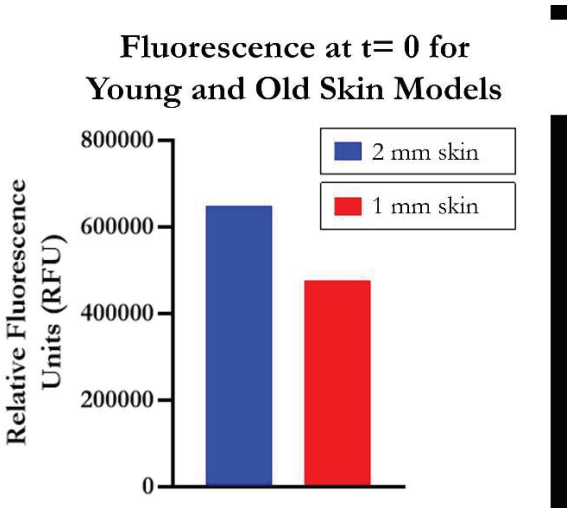

Figure 8. This Figure shows the fluorescence detected using an Alamar Blue assay for the young (2 mm) and old (1 mm) skin models at T=0, prior to treatment and 3 days after seeding. Cell metabolism was more active at the higher seeding density, as expected.

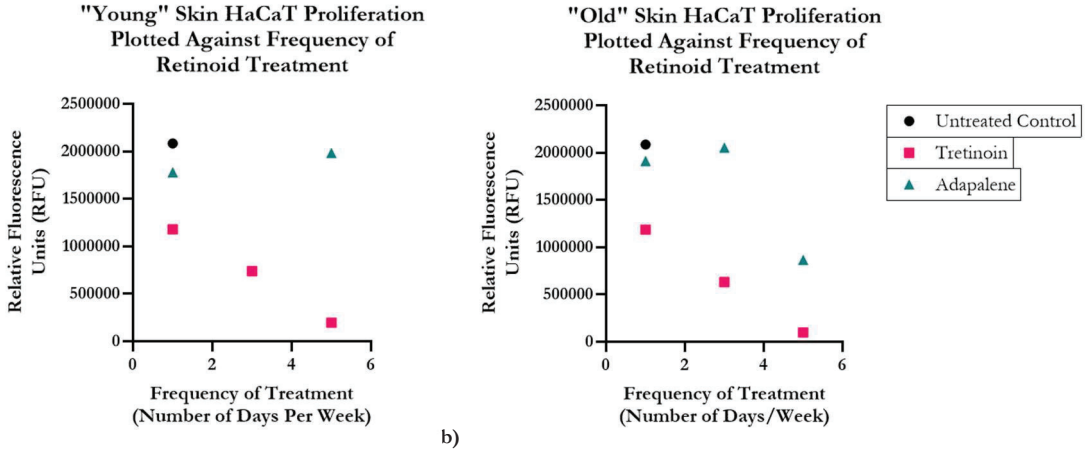

Figure 9. These Figures depict HaCaT proliferation measured by Alamar Blue assay fluorescence, plotted against tretinoin or adapalene treatment frequency for a) young (2 mm) and b) old (1 mm) skin. Note: Alternate-day adapalene data for young skin is missing due to well drying out. When a simple linear regression model is fitted for the tretinoin points on each graph, the R^2 values are 0.9963 and 0.9999, respectively.

DISCUSSION

As human skin ages, it has been shown the epidermal and dermal layers thin due to the weakening of the junction between those layers.[15] It is also known that different types of retinoic acid help human skin rejuvenation and providing anti-aging effects by increasing collagen production and cell proliferation while exfoliating and removing dead skin cells. It is necessary to understand the proliferation rate provided by differing treatment frequencies with retinol products in 3D cell culture that mimics human skin.[18] The data gathered for this paper shows the proliferation rates of different treatment frequencies of retinol products through an Alamar Blue proliferation assay and visual observations during and after treatment on HaCaT cells.

The results show the frequency of treatment, and the type of treatment affects the proliferation rate of the HaCaT cells. Retinoids like adapalene and tretinoin are known to increase cell turnover by promoting differentiation of skin cells, which can lead to shedding of dead skin cells and the growth of new ones.[18] Adapalene and tretinoin are known to cause differentiation in HaCaT cells, differentiation of cells can lead to a slower rate of cell division and an overall decrease in cell proliferation; however, the treatment may also have induced apoptosis. When looking at the data of the untreated control wells, they have a much higher proliferation rate than those of the adapalene or tretinoin treated wells. Throughout the daily images, it can also be seen how the tretinoin treatment causes the cells to change their morphology. This may be because tretinoin is inherently more concentrated and thus more toxic to the cells than adapalene.

In addition, the side effects and safety profiles of each treatment should be considered when assessing the results. Tretinoin is known to cause skin irritation and sensitivity, while adapalene is generally better tolerated.[18] The effects of retinoids on cell turnover and proliferation depend on the concentration of the treatment and duration of treatment. When the cells were treated with adapalene they had a healthier, more confluent appearance than when treated with tretinoin. Again, since tretinoin is more concentrated, it probably leads to higher rates of cell turnover, resulting in more dead cells that were lifted from the gel and were removed, without new cells being turned over in the skin cycle. The higher number of dead cells through more concentrated and more frequent treatments can be seen quantitatively in **Figure 9a** and **Figure 9b**. In this figure, tretinoin-treated models are significantly less confluent than the adapalene and control counterparts. When treated once a week and supplemented with a complete medium, more nutrients and growth factors were introduced to the cells to help the surviving cells heal and proliferate. This led to an increase in cell number over time in the once-a-week treatment.

Further studies need to be done to obtain proliferation data that incorporates HaCaT production instead of purely HaCaT growth from a set cell density. Recommendations for future experiments would be to perform a viability assay to determine the percentage of live and dead cells as well as immunostaining to assess the effects on cell differentiation between the two treatments. For future experiments, observing the healthy proliferation rate of HaCaTs and supplementing additional HaCaTs into the treatment wells to mimic cell turnover during treatment would help make the experiment and skin model more accurate. Another aspect that can be considered is the elasticity of the model since loss in elasticity is a sign of aging skin.

CONCLUSIONS

When looked at in tandem, the data from the Alamar Blue proliferation assay and the daily visual observations indicate the proliferation of HaCaTs under retinoic acid conditions is not improved. However, this is due to the set-up of the model, the

model fails to account for the loss of elasticity associated with ageing skin. It has also been shown that retinoic acid does increase the proliferation of cells in *in vivo* conditions because the body is producing more HaCaT cells underneath the layer that retinoic acid removes. When used on human skin, retinol products exfoliate and remove the top layer of skin to bring the bottom layers of skin to the surface faster to increase cell turnover. Since there is nothing in the model that allows new HaCaTs to be brought up from below the surface, proliferation could not be measured precisely. From this experiment design and preliminary data, the optimal frequency and duration of retinoid treatment cannot be concluded as there were errors in the treatment that were not consistent between each treatment well. Regardless, this study addressed the gap by creating a 3D model of human aging skin while investigating the proliferation rate of human epidermal tissue cells and the retinoic acid effects.

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Samantha Lopez and Olivia Atkins graduated in May 2023 with a Bachelor of Science in Biomedical Engineering and Biology and Biotechnology, respectively. They both plan on furthering their education and pursuing a master's degree in their respective fields.

PRESS SUMMARY

Human skin aging is characterized by thinning, loss of elasticity, and wrinkles. Keratinocytes, the most common type of skin cell, and fibroblasts, cells present in the stroma beneath the skin's surface, both play a role in aging. Using these cell lines in research can reveal a deeper understanding of skin function and changes in aging. 3D cell culture techniques provide an opportunity to use these cell lines in a model that can more accurately mimic human skin compared to 2D models. Treatment of aging skin is of interest to both medical and consumer communities. Retinoic acid (RA) is a derivative of both vitamin A and retinol that assists in cell growth and immune functions. Over the counter (OTC) and prescription retinoids are common topical products used for anti-aging and acne treatments. This study seeks to determine the impact of topical retinoid creams on skin cell growth and characteristics in 3D cell culture models mimicking aged and unaged human skin. NIH-3T3 fibroblasts were embedded in a 3D collagen matrix of varying thickness, and HaCaT keratinocytes were seeded on top of the matrix at varying seeding densities. Equivalent concentrations of 0.025% tretinoin and 0.1% adapalene topical creams were prepared in cell medium and used to treat cells daily, on alternate days, or just once during a week-long period. Alamar Blue proliferation assays and microscopy provided quantitative and qualitative data regarding the effects of the retinoid products on the skin models. Tretinoin treatment killed the cells, and adapalene treatment, while resulting in significantly more live cells than tretinoin, did not exceed the proliferation of the untreated control. It is understood that retinol increases cell turnover by killing cells rapidly, so it is proposed that in this model, the rate of proliferation does not overcome the rate of cell death. Considering the data is only preliminary, future studies should focus on creating a 3D model that can more accurately represent the skin environment in the human body where cells can be readily grown.