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Research Article

The Effects of Oil and Oil + Nicotine in Vaporizer Pens (E-Cigarettes) on the Cell Behavior of Undifferentiated Human Mesenchymal Stem, and Differentiated Human Foreskin Fibroblast

Hull B[1], Barreras A[1], Parris S[1] and Tawil B[1,2]*

[1]Biotechnology & Bioinformatics Master's Program, California State University, Channel Islands 1 University Dr., Camarillo CA, USA

[2]Department of Bioengineering, UCLA School of Engineering, Los Angeles, California, USA

***Corresponding author:** Bill Tawil, 420 Westwood Plaza, Room 5121, Engineering V. P.O. Box: 951600, Los Angeles, CA, USA

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Abstract

Electronic cigarettes, also known as vaporizer pens, have gained popularity in recent years as a safer alternative to smoking traditional tobacco cigarettes. However, little research has been conducted regarding the actual side effects of the smokeless vapor produced by vaporizer pens. In this study, we examined the effects of vaporizable oil, with and without nicotine, on Human Foreskin Fibroblast-1 Cells (HFF-1) and Human Mesenchymal Stem Cells (HuMSCs) cell proliferation, morphology and integrin expression. We investigated how HFF-1 and HuMSCs behave in the presence of varying concentrations of oil and oil + nicotine. Both HFF-1 cells and HuMSCs experienced a decrease in cell proliferation when seeded on fibronectin or collagen, as the concentration of oil was increased. A further slight decrease in cell proliferation was observed when nicotine was added to the oil. The cell morphology of both cell types gained more stretched forms in the presence of the oil or oil + nicotine. Furthermore, alpha5 and beta1 integrin subunits expression decreased when the cells were treated with oil or oil + nicotine. This study shows that the oil used in the E-cigarettes has a high negative effect on the cell proliferation and integrin expression for both undifferentiated and differentiated cells; the addition of nicotine slightly enhanced that negative effect. With these findings, we suggest that more research should be conducted to better understand the effects resulting from oil or oil + nicotine in vaporized form when inhaled using an electronic cigarette.

Keywords: Human foreskin fibroblasts; Human mesenchymal stem cells; Stem cells, Collagen; Fibronectin; Oil; Nicotine oils; E-liquid; Nicotine; Vaporizer; Vape; E-cigarette; Proliferation; Morphology; Extracellular matrix; Integrins; Cell behavior; Initial adhesion

Abbreviations

HuMSCs: Human Mesenchymal Stem Cells; MSCs: Mesenchymal Stem Cells; HFF-1: Human Foreskin Fibroblast-1; ECM: Extracellular Matrix; GF: Growth Factors; RGD: β1; α5; FA: Focal Adhesions

Introduction

Electronic cigarettes, also known as vaporizable pens, are a fairly new product on the market advertised as a safer alternative to smoking cigarettes or as a method to quit smoking entirely [1]. Unlike traditional cigarettes, vaporizable pens do not burn tobacco but instead employ a battery-powered heating element that vaporizes a liquid oil to emulate the sensation of smoking [1]. Various epidemiological studies reveal a considerable correlation between smoking or the use of smokeless tobacco substances and the prevalence of emphysema, heart disease, stroke and various cancers [2]. Despite their growing popularity, the biological consequences of the newly popular nicotine oils inhaled from the vaporizable pens on human health are not well known and have not been heavily researched [3,4].

Nicotine, the key constituent in cigarettes and nicotine containing vaporizable oil, has been shown to reduce cell viability and inhibit cellular proliferation of fibroblast cells *in vitro* [5]. Fibroblast cells are connective tissue cells that are responsible for secreting collagen proteins [6]. As a derivative of mesenchymal origin, they are inhabitants of the Extracellular Matrix (ECM) of connective tissues and are responsible for secreting proteins used to maintain tissue structure [7]. Fibroblasts play a vital role in the final stages of wound healing and use in many novel applications in tissue engineering [8]. Mesenchymal stem cells (MSCs) have been investigated for their potential use in cell-based therapies in clinical management of difficult wounds and therapeutic dermatology [9]. While fibroblasts cells are traditionally involved in wound healing, mesenchymal stem cells exhibit fibroblast like plastic-adherent morphology and have the capacity for multipotent differentiation *in vitro* and *in vivo* [10].

Data currently suggests that MSC differentiation pathways are regulated through dynamic interactions with the Extracellular Matrix (ECM) [11]. The ECM is a dynamic network of macromolecules present within all mammalian tissues that provides not only the physical scaffolding between cells but also essential biochemical cues to initiate differentiation and promote cell communication amongst

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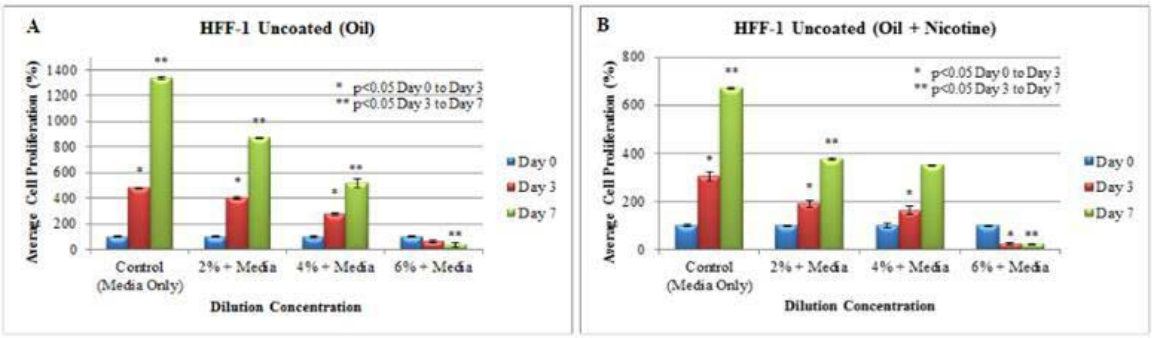

Figure 1: HFF-1 2D cell proliferation on uncoated wells treated with oil and oil + nicotine at various dilutions on day 0, 3 and 7. (A) HFF-1 cell proliferation effected by 0%, 2%, 4% and 6% oil on day 0, 3 and 7 in uncoated conditions. (B) HFF-1 cell proliferation effected by 0%, 2%, 4% and 6% oil + nicotine on day 0, 3 and 7 on uncoated conditions.

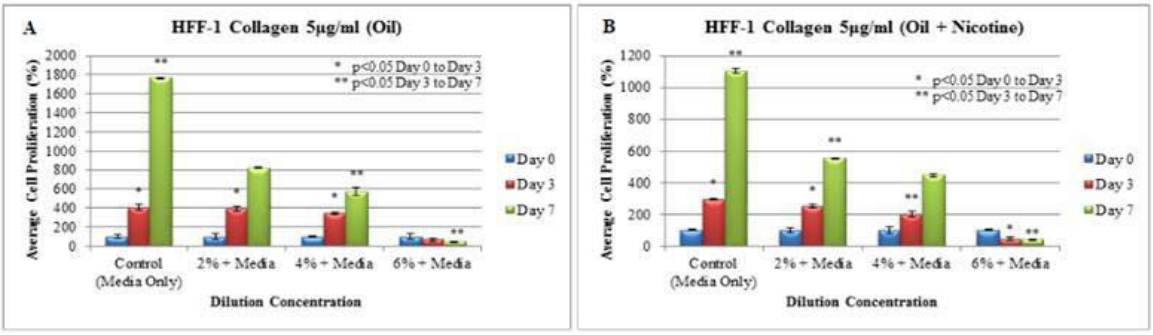

Figure 2: HFF-1 2D cell proliferation on collagen 5µg/ml coated wells treated with oil and oil + nicotine at various dilutions on day 0, 3 and 7. (A) HFF-1 cell proliferation effected by 0%, 2%, 4% and 6% oil on day 0, 3 and 7 on collagen 5µg/ml coated conditions. (B) HFF-1 cell proliferation effected by 0%, 2%, 4% and 6% oil + nicotine on day 0, 3 and 7 in collagen 5µg/ml coated conditions.

adjacent cells [12]. Cells incorporate these cues via integrin signal transduction pathways and eventually translate this information into regulation of gene expression and cell fate decisions [13]. Integrins are a superfamily of αβ heterodimeric transmembrane receptors that bind to ECM proteins such as fibronectin and collagen to regulate cellular function [14]. Integrins are heterodimeric transmembrane glycoproteins that serve as adhesion receptors for ligands in the Extracellular Matrix (ECM) and convert mechanical signals from the ECM into biochemical signals within the cell [15]. Fibrinogen and collagen, the two ECM proteins used in this study, are two of the main glycoproteins present within the ECM that help generate a favorable molecular structure to allow binding of Growth Factors (GF) that interact with cell-surface receptors and initiate gene transcription [16].

Our study aims to understand the effects of various concentrations of oil and nicotine on the cell proliferation, morphology and integrin expression in differentiated Human Foreskin Fibroblast (HFF-1) and undifferentiated Human Mesenchymal Stem Cell (HuMSCs) seeded on fibronectin and collagen coated plates and in 3D fibrin constructs. We observed a decrease in cellular proliferation when exposed to higher oil and nicotine concentrations. Expression of integrins was also affected. In summary, the nicotine carrier, ie. the vapor oils used in e-cigarettes, alter behavior in both undifferentiated (HuMSCs) and differentiated (HFF-1) cells. The nicotine added to the vapor oils slightly enhances the negative effects of the added oil.

Materials and Methods

E-Cigarette oils

Nicotine oils were obtained from a local company Cedar Vape near California State University Channel Islands in Camarillo, CA. Two oils were obtained: one containing no nicotine (oil only) and another that contains both oil + nicotine at a 6mg/ml nicotine concentration.

Cells

Normal Human Foreskin Derived Fibroblast Cells (ATCC, Manassas, VA, United States), passage 4 or 5 were grown in T75 boat flask (Thermo Scientific, Waltham, MA, United States) with HFF-1 prepared media: DMEM High modified (Hyclone, Logan, UT, United States), 1% L-Glutamine (Life Technologies, Carlsbad, CA, United States), 15% Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, Canoga Park, CA, United States), and 1% Penicillin/Streptomycin (Thermo Fisher Scientific, Canoga Park, CA, United States) for 5 to 7 days for confluency. Normal Human Adipose Derived Mesenchymal Stem Cells (ATCC, Manassas, VA, United States), passage 2 or 3 were grown in T75 boat flask (Thermo Scientific, Waltham, MA, United States) with Advance STEM MSC basal medium with the Advance STEM MSC growth supplement kit (Thermo Fisher Scientific, Canoga Park, CA, United States) for 5 to 7 days for confluency. Collagen and fibronectin (Sigma Aldrich, St. Louis, MO, United States) were diluted with sterile PBS (Thermo Fisher Scientific, Canoga Park, CA,

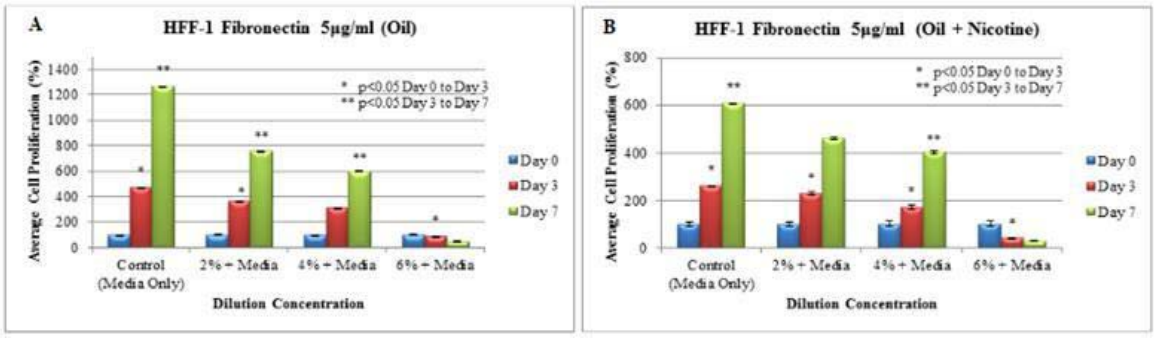

Figure 3: HFF-1 2D cell proliferation on fibronectin 5µg/ml coated wells treated with oil and oil + nicotine at various dilutions on day 0, 3 and 7. (A) HFF-1 cell proliferation effected by 0%, 2%, 4% and 6% oil on day 0, 3 and 7 in fibronectin 5µg/ml conditions. (B) HFF-1 cell proliferation effected by 0%, 2%, 4% and 6% oil + nicotine on day 0, 3 and 7 in fibronectin 5µg/ml coated conditions.

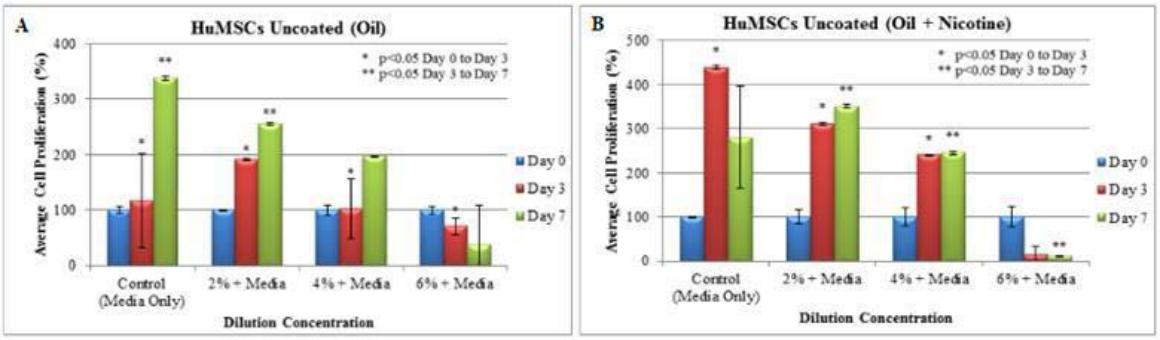

Figure 4: HuMSC 2D cell proliferation on uncoated wells treated with oil and oil + nicotine at various dilutions on day 0, 3 and 7. (A) HuMSC proliferation effected by 0%, 2%, 4% and 6% oil on day 0, 3 and 7 in uncoated conditions. (B) HuMSC proliferation effected by 0%, 2%, 4% and 6% oil + nicotine on day 0, 3 and 7 in uncoated conditions.

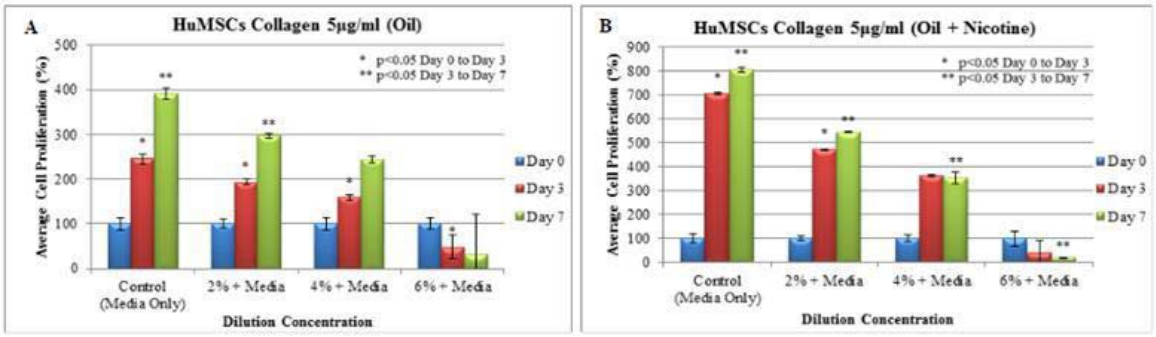

Figure 5: HuMSC 2D cell proliferation on collagen 5µg/ml coated wells treated with oil and oil + nicotine at various dilutions on day 0, 3 and 7. (A) HuMSC average proliferation effected by 0%, 2%, 4% and 6% oil on day 0, 3 and 7 on collagen 5µg/ml coated conditions. (B) HuMSC average cell proliferation effected by 0%, 2%, 4% and 6% oil + nicotine on day 0, 3 and 7 in collagen 5µg/ml coated conditions.

United States) to 5µg/ml for plate coating. Bovine derived Thrombin 10units/ml and bovine derived fibrinogen 10mg/ml (Sigma Aldrich, St. Louis, MO, United States) were used to form a 3D fibrin constructs to examine the 3D morphology of HFF-1 cells. Mouse Anti-Human CD49E Conjugate Monoclonal, α5 antibody (EMD Millipore, Billerica, MA, United States) and FITC Mouse Anti-Human CD29, β1 Antibody (Quantobio, Beverly, MA) were used for FACS analysis for both HuMSCs and HFF-1 cells.

2D Proliferation and fluorescent imaging

HuMSCs and HFF-1 cells were grown in T75 boat flasks for 4 to 5 days to ensure confluency. Old media was removed and the flask was washed with 5ml of sterile PBS. PBS was removed and 5ml of trypsin 0.25% (Thermo Fisher Scientific, Canoga Park, CA, United States) was added and the flask was left at room temperature for 5 to 10 minutes. Trypsin was neutralized with media containing serum. The cell solution was transferred to a 15ml conical tube (Thermo Fisher Scientific, Canoga Park, CA, United States) and spun down in the centrifuge (Eppendorf, Hamburg-Eppendorf, Hamburg, Germany) for 5 minutes at 200g. The supernatant was removed and the cell pellet was resuspended in 1ml of media containing serum. 20µl of the resuspended solution was added to a Cellometer slide

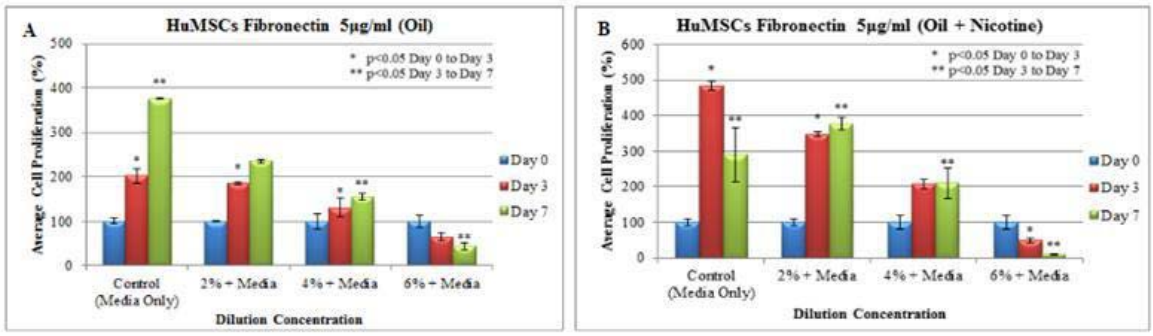

Figure 6: HuMSC 2D cell proliferation on fibronectin 5µg/ml coated wells treated with oil and oil + nicotine at various dilutions on day 0, 3 and 7. (A) HuMSC proliferation effected by 0%, 2%, 4% and 6% oil on day 0, 3 and 7 in fibronectin 5µg/ml coated conditions. (B) HuMSC cell proliferation effected by 0%, 2%, 4% and 6% oil + nicotine on day 0, 3 and 7 in fibronectin 5µg/ml coated conditions.

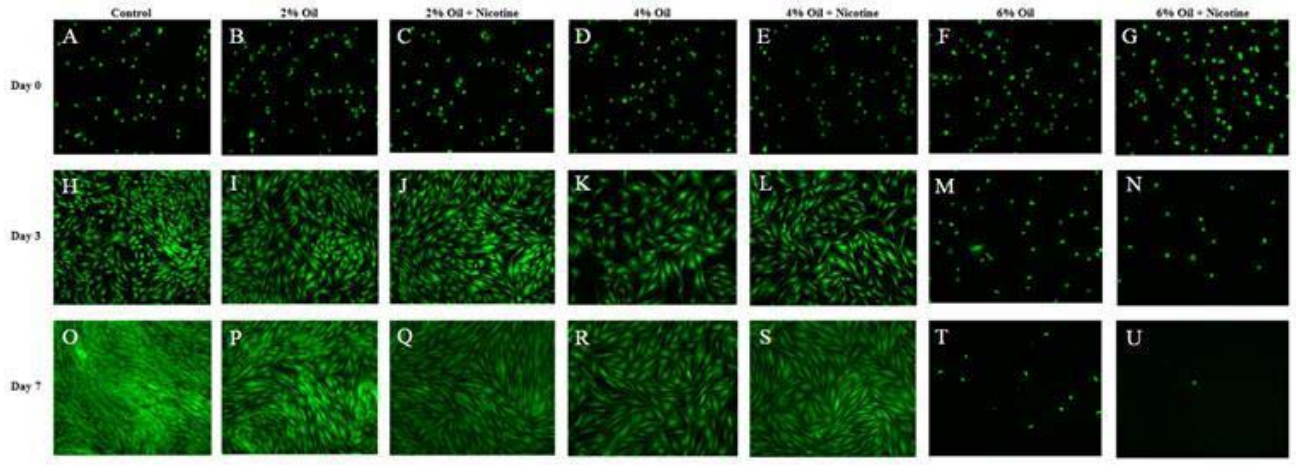

Figure 7: HFF-1 cell proliferation on fibronectin 5µg/ml coated wells treated with oil and oil + nicotine at various dilutions for a 1 week period. (A), (B), (C), (D), (E), (F), (G) HFF-1 cell proliferation effected by 0%, 2%, 4% and 6% oil and oil + nicotine on day 0. (H), (I), (J), (K), (L), (M), (N) HFF-1 cell proliferation effected by 0%, 2%, 4% and 6% oil without nicotine and oil with nicotine on day 3. (O), (P), (Q), (R), (S), (T), (U) HFF-1 cell proliferation effected by 0%, 2%, 4% and 6% oil and oil + nicotine on day 7.

(Nexcelom Bioscience, Lawrence, MA, United States) and inserted into the Cellometer Auto T4 (Nexcelom Bioscience, Lawrence, MA, United States) in order to get a cell count. After performing calculations in order to get 5,000 cells/well the correct amount of cell solution was added to media and then 150µl cell solution per well was transferred to 3 plates of 48 well plates (Corning Incorporated, Corning, NY, United States). Cells were seeded on either uncoated or coated with fibronectin 5µg/ml or collagen 5µg/ml. At different time points: day 0, 3 and 7, plates were taken out of the incubator; wells were washed with PBS 3 times and then incubated with Calcein AM (Thermo Fisher Scientific, Canoga Park, CA, United States) and left in the dark for 15 minutes. Cell proliferation was then measured using a Filtermax F5 Microplate Reader (Molecular Devices, Sunnyvale, CA, United States). Fluorescent images were taken on day 0, 3 and 7 of the Calcein stained cells with the IX71 Fluorescent Microscope (Olympus Corporation, Shinjuku, Tokyo, Japan (Figure 11).

3D fibrin constructs

HFF-1 cells were grown in T75 boat flasks for 4-5 days and removed from the flask using the same method described above. After resuspension and proper calculations in order to obtain 5,000cells/well, the cell solution was transferred to a tube containing 5mg/ml fibrinogen. In a 48 well plate 75µl of fibrinogen (5mg/ml) was added and 75µl thrombin (5units/ml) was immediately added afterwards one well at a time. The plates were then tilted back and forth until the construct is solidified. 5mg/ml fibrinogen and 5 units/ml concentration was chosen based on the physiological fibrinogen and thrombin concentration. The day 0 plate was placed into the incubator at 37 C in 5% CO_2 for 1 hour. The plate was stained with Calcein AM (75µl/well), left at room temperature in the dark for 15 minutes then images were taken with the fluorescent microscope as described above. The same steps were conducted for days 3 and 7 (Figure 12).

Fluorescence activating cell sorting (FACS)

The HFF-1 cells were grown in T75 boat flasks for 4-5 days and removed from the flask using the same method described above. FACS analysis was performed on both HuMSCs and HFF-1 cells with antibodies against α5 and β1 integrin subunits. A Guava easyCyte (EMD Millipore, Billerica, MA, United States) was used to perform FACS analysis for day 0 and day 7. A day 7 plate also had 2% and 4% oil and oil + nicotine diluted media in selected wells in order to

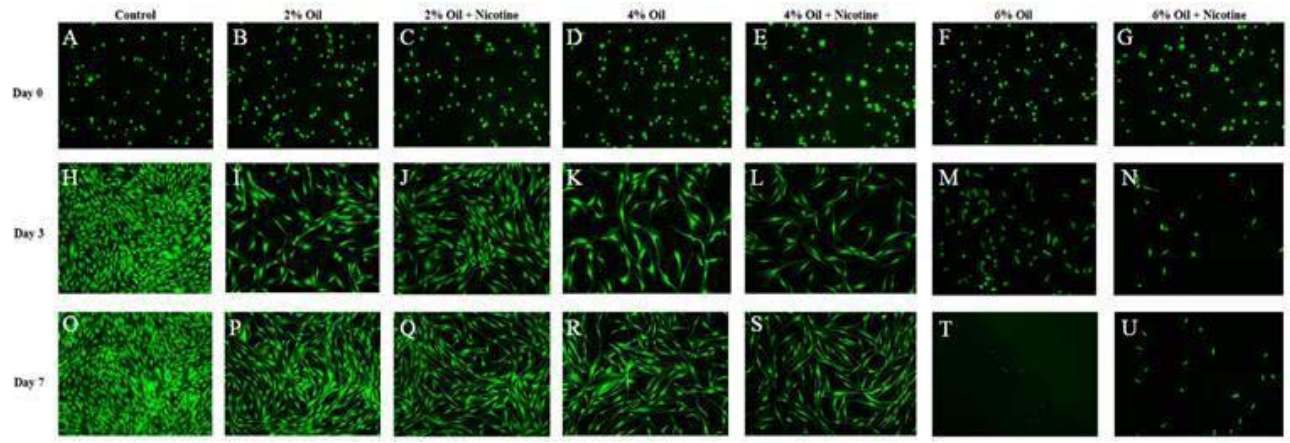

Figure 8: HuMSC proliferation on fibronectin 5µg/ml coated wells treated with oil and oil + nicotine at various dilutions for a 1 week period. (A), (B), (C), (D), (E), (F), (G) HuMSC proliferation effected by 0%, 2%, 4% and 6% oil and oil + nicotine on day 0. (H), (I), (J), (K), (L), (M), (N) HuMSC proliferation effected by 0%, 2%, 4% and 6% oil and oil + nicotine on day 3. (O), (P), (Q), (R), (S), (T), (U) HuMSC proliferation effected by 0%, 2%, 4% and 6% oil and oil + nicotine on day 7.

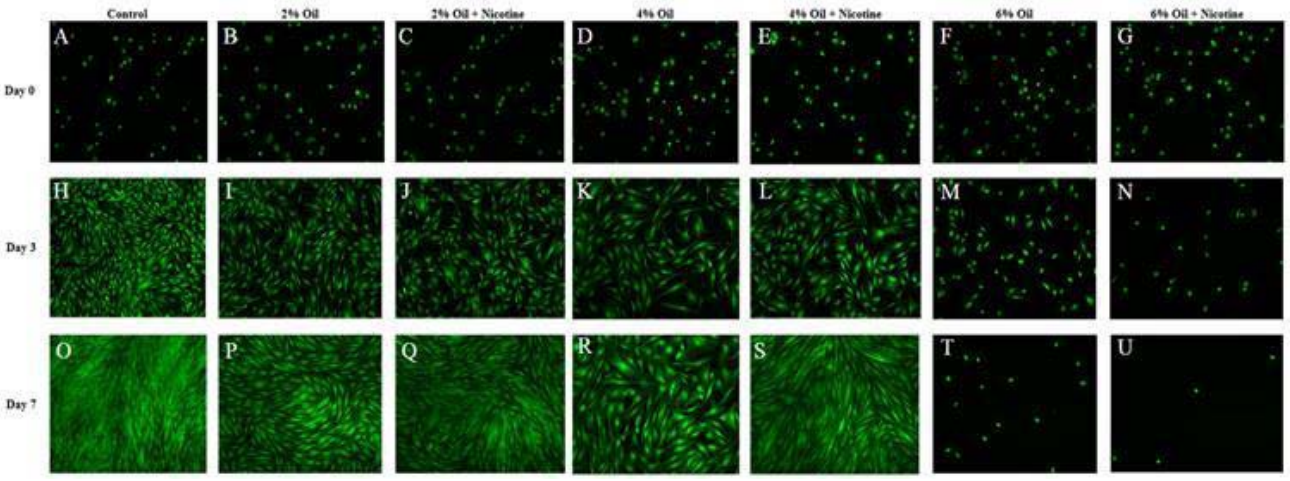

Figure 9: HFF-1 cell proliferation on collagen 5µg/ml coated wells treated with oil and oil + nicotine at various dilutions for a 1 week period. (A), (B), (C), (D), (E), (F), (G) HFF-1 cell proliferation effected by 0%, 2%, 4% and 6% oil and oil + nicotine on day 0. (H), (I), (J), (K), (L), (M), (N) HFF-1 cell proliferation effected by 0%, 2%, 4% and 6% oil and oil + nicotine on day 3. (O), (P), (Q), (R), (S), (T), (U) HFF-1 cell proliferation effected by 0%, 2%, 4% and 6% oil and oil + nicotine on day 7.

measure integrin expression in the presence of oil and oil + nicotine. Histogram plot measurements were obtained for day 0 and 7 then plotted together with specific colors for different conditions with green (β1) and yellow (α5) fluorescence. For cell proliferation tests, day 0 was used as 100% and day 3 and day 7 were compared to day 0.

Statistical analysis

Data is presented as mean ± Standard Deviation (SD). A Paired Student t-Test is used to determine significance of observed differences between the study groups. A value of $p < 0.05$ is considered statistically significant. The tests were repeated 3 times in triplicate each time with a total of n=9 unless otherwise documented in the figure legends.

Results

HFF-1 cell proliferation in presence of oil and oil + nicotine

We, first, examined the HFF-1 cell proliferation on uncoated and collagen or fibronectin coated conditions in the presence or absence of oil or oil + nicotine.

Uncoated: HFF-1 cells plated on uncoated wells in the presence of various dilutions of both oil and oil + nicotine conditions experienced a lower cellular proliferation in comparison to the control (no oil or nicotine) across all time points (Figure 1A,1B). More specifically, we observed at day 3, a 4 to 3 fold increase in cell proliferation over day 0 in the presence of 2% or 4% oil with or without nicotine respectively. However, the increase was 3 fold less than cells grown in the absence of oil. On the other hand, a decrease in cell proliferation was observed at day 3 in the presence of 6% oil with and without nicotine compared to day 0. By day 7, there was an increase in cell proliferation by 2 to 1.5 fold in the presence of 2% and 4% oil with and without nicotine in comparison to day 3. However, the increase in growth was still much less than cells grown in the absence of oil or oil + nicotine. For the 6% oil, at day 7, with and without nicotine there was a further decrease in cell proliferation from day 3. In conclusion, we observed a decrease in cell proliferation of HFF-1 cells seeded on uncoated wells

over a period of 7 days in the presence of oil. There was a correlation between the decrease of cell proliferation and the increase of oil concentration. Furthermore, the addition of nicotine seems to have a further slight effect on cell proliferation.

Collagen: We have observed similar data on HFF-1 cells seeded on collagen coated wells to the data described above on uncoated wells. Briefly, the increase in cell proliferation in the presence of 2% and 4% oil was 2 folds less than cells growing in the absence of oil (Figure 2A). At 6%, we observed a decrease number of cells at day 3 and day 7 in comparisons to day 0; suggesting cells are either dying or losing their ability to adhere to the wells. The addition of nicotine to the oil has a minimal further effect on cell proliferation (Figure 2B). Furthermore, while HFF-1 proliferated better on collagen in the absence of oil or oil + nicotine when compared to uncoated (18 folds *vs.* 13 folds respectively), growing on collagen did not seem to counter the negative effect of the oil on cell proliferation. In conclusion, HFF-1 cells in the presence of oil and oil + nicotine demonstrate a decrease in cell proliferation. The addition of nicotine leads to a further decrease across all conditions.

Fibronectin: HFF-1 cell proliferation on fibronectin displayed a twofold decrease, with an increase in oil concentration (Figure 3A,3B). Thus, similar to uncoated and collagen – coated, HFF-1 cells in the presence of oil and oil + nicotine showed a decrease in cell proliferation on fibronectin – coated wells.

HFF-1 Morphology in the presence of oil and oil + nicotine

Uncoated: We examined the cell morphology on uncoated plates in the presence of oil and oil + nicotine over a one week period. At day 0, cells, as expected, had small round morphology regardless of the absence or presence of oil or oil + nicotine. By day 3, cells growing in the presence of 2% oil had similar morphology to cells seeded in the absence of oil (Figure not shown). However, in the presence of 4% oil, cells appeared more stretched cell bodies (Figure not shown) while in the presence of the 6% oil dilution showed some slightly stretched cells, but mostly no change in morphology i.e, cells remained rounded (Figure not shown). By day 7, the change in morphology became more prominent, cells in the presence of the 2% oil, showed stretched cell bodies in comparison to day 3 and in the presence of 4% oil, cells stretched more than day 3 (Figure not shown). Interestingly, in the presence of 6%, there was no change in cell morphology from day 0 to day 7 with the cells staying rounded (Figure not shown). In summary, the addition of oil leads to changes in HFF-1 morphology seeded on uncoated coated wells. There was a correlation between changes in morphology and the increase of oil concentration. Adding the nicotine seems to have no further effect on cell morphology.

Collagen: We examined the cell morphology on collagen coated plates in the presence of oil and oil + nicotine over a one week period. At day 0, under all conditions, all cells were rounded (Figure 9A-9G). At day 3, in the presence of 2% oil, cell morphology was very similar to the day 3 control (Figure 9I) and at day 7, the cells showed stretched cell bodies (Figure 9P). Cells in the presence of 4% oil dilution showed stretched cell body morphology on day 3 and at day 7; they were even more stretched in comparison to day 3 (Figure 9K,9R). In the presence of 6% oil dilution, cells bodies were slightly stretched at day 3 (Figure 9F,9M) and they were more rounded at day 7, in comparison to day 0, 3 and the control. In conclusion, HFF-1 cells seem to become more stretched in the presence of 2% and 4% oil over a period of 7 days, while remain rounded in the presence of 6% oil. The addition of nicotine did not further change the cell morphology.

Fibronectin: We examined the HFF-1 cell morphology on fibronectin coated plates in the presence of oil and oil + nicotine over a one week period. Our data demonstrates that HFF-1 on fibronectin coated wells shows changed morphology as the oil dilution increase. In the presence of 2% or 4% oil dilutions cells were stretched in comparison to control (Figure 7I,7J). On the other hand, cells in the presence of 6% oil showed rounded morphology when compared to day 0 and control (Figure 7F). The cells have similar stretched morphology at day 7 in the presence of 2% and 4% oil and rounded morphology in the presence of 6% oil (Figure 7T). The addition of nicotine illustrated minimal change in HFF-1 morphology throughout the week time period (Figure 7G,7N,7U). In conclusion, similar to cells seeded on collagen – coated wells, HFF-1 cells seeded on fibronectin – coated wells showed a stretched morphology in the presence of low oil concentration (2% and 4%) and rounded morphology at a higher oil concentration of 6%. No additional change in morphology was observed when nicotine was added to the oil.

HuMSC proliferation in the presence of oil and oil + nicotine

Next, we examined the HuMSC proliferation on uncoated and collagen or fibronectin coated plates in the presence of oil or oil + nicotine.

Uncoated: HuMSCs on uncoated wells, similar to HFF-1 cells, experienced a decrease in cell proliferation in the presence of both oil and oil + nicotine across all dilutions in comparison to the control (no oil or nicotine) across all time points (Figure 4A,4B). At day 3, the 2% or 4% oil with or without nicotine conditions showed a 2 to 3 fold increase in cell proliferation respectively, however, the increase was 1 to 2 folds less than cells in the absence of oil with or without nicotine. By day 7, there was a 2.5 to 0.1 fold increase in average cell proliferation in the presence of 2% and 4% oil with and without nicotine. In the presence of 6% oil with and without nicotine, a decrease in cell proliferation was observed at day 3 compared to day 0; further decrease occurred at day 7. In conclusion and similar to HFF-1, we observed a decrease in HuMSCs seeded on uncoated wells over a period of 7 days in the presence of oil. We observed less proliferation in the 2% and 4% oil + nicotine in comparison to the control but higher proliferation in contrast to the 2% and 4% oil without nicotine over the 7 day period. In conclusion, there was a correlation between the decrease of cell proliferation and increasing oil concentration.

Collagen: We have observed similar data on HuMSCs seeded on collagen coated wells to the data described above on uncoated wells. In summary, the increase in cell proliferation in the presence of 2% and 4% oil was 1-1.5 folds less than cells growing in the absence of oil (Figure 5A). A decrease of cells at day 3 and day 7 in comparisons to day 0 was observed in 6% oil with and without nicotine; indicating cells are either dying or losing their ability to adhere to the wells. Adding nicotine to the oil had a proliferative effect on cells in the presence of 2% and 4% oil with and without nicotine (Figure 5A,5B).

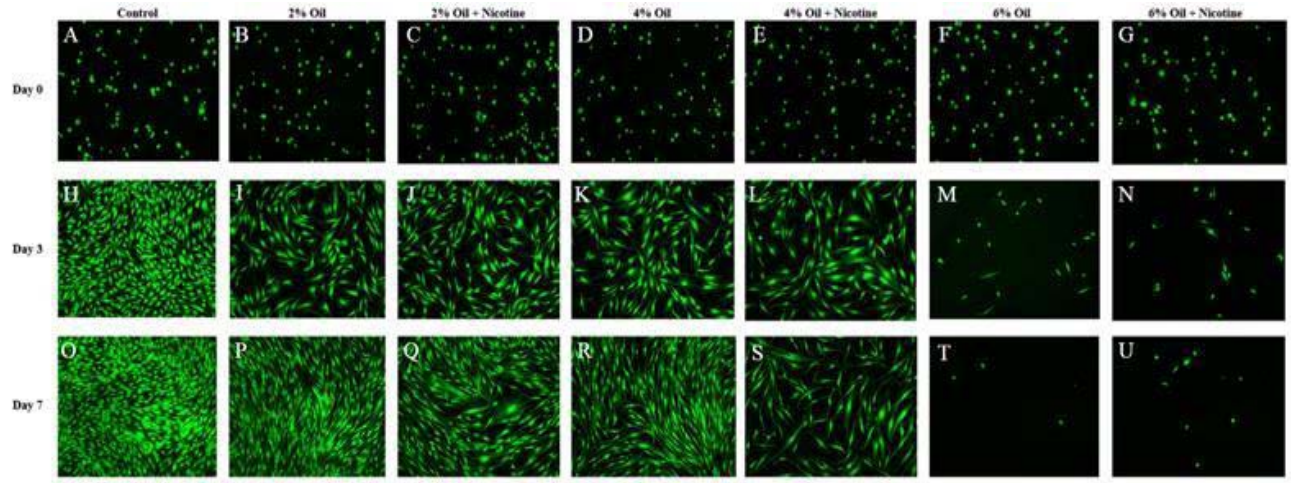

Figure 10: HuMSC proliferation on collagen 5µg/ml coated wells treated with oil and oil + nicotine at various dilutions for a 1 week period. (A), (B), (C), (D), (E), (F), (G) HuMSC proliferation effected by 0%, 2%, 4% and 6% oil and oil + nicotine on day 0. (H), (I), (J), (K), (L), (M), (N) HuMSC proliferation effected by 0%, 2%, 4% and 6% oil and oil + nicotine on day 3. (O), (P), (Q), (R), (S), (T), (U) HuMSC proliferation effected by 0%, 2%, 4% and 6% oil and oil + nicotine on day 7.

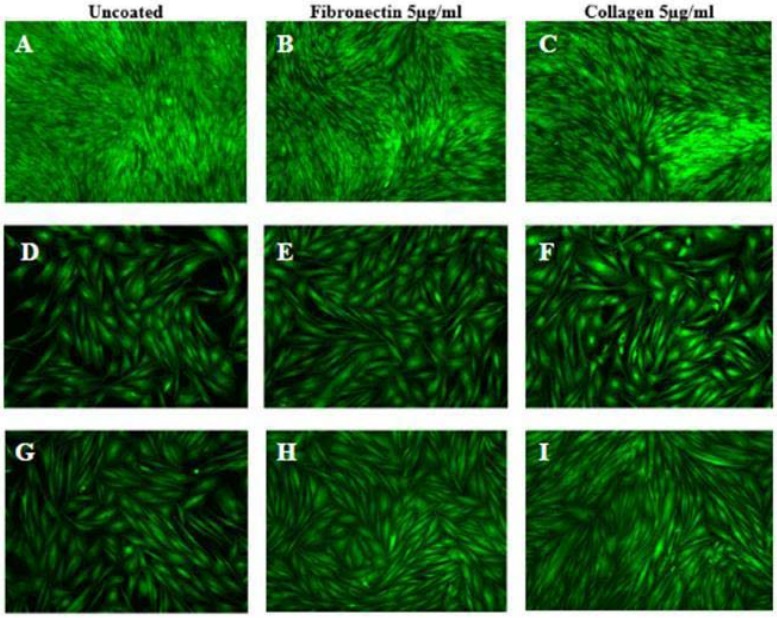

Figure 11: Calcein stained fluorescent images of HFF-1 spreading on uncoated, fibronectin 5µg/ml and collagen 5µg/ml coated wells with oil and oil + nicotine exposure at 4% on day 7. (A), (B), (C) HFF-1 cells in uncoated, fibronectin 5µg/ml and collagen 5µg/ml coated wells with normal HFF-1 media on day 7. (D), (E), (F) HFF-1 cells in uncoated, fibronectin 5µg/ml and collagen 5µg/ml coated wells with HFF-1 media diluted with 4% oil on day 7. (G), (H), (I) HFF-1 cells in uncoated, fibronectin 5µg/ml and collagen 5µg/ml coated wells with normal HFF-1 media diluted with 4% oil + nicotine on day 7.

Additionally, the HuMSCs proliferated better on collagen in the absence of oil or oil + nicotine when compared to uncoated (4 fold vs. 3.5 folds respectively), growing on collagen did not seem to counter the negative effect of oil on cell proliferation. In conclusion, we observed a decrease in cell proliferation of HuMSCs seeded on collagen coated wells over a period of 7 days in the presence of oil. There was a correlation between the decrease of cell proliferation and the increase of oil concentration. Additionally, less proliferation was observed in the 2% and 4% oil + nicotine in comparison to the control but higher proliferation in contrast to the 2% and 4% oil without nicotine over the 7 day period. Even with collagen present, we observed a similar decreasing trend in average cell proliferation in both the oil and oil + nicotine in comparison to the control (Figure 6).

Fibronectin: We examined similar data on HuMSCs seeded on fibronectin coated plates to the data described above on uncoated wells and collagen coated wells (Figure 8).

HuMSC Morphology and Spreading in Presence of Oil and Oil + Nicotine

We examined the cell morphology on uncoated and collagen or fibronectin coated plates in the presence of oil and oil + nicotine over a one week period.

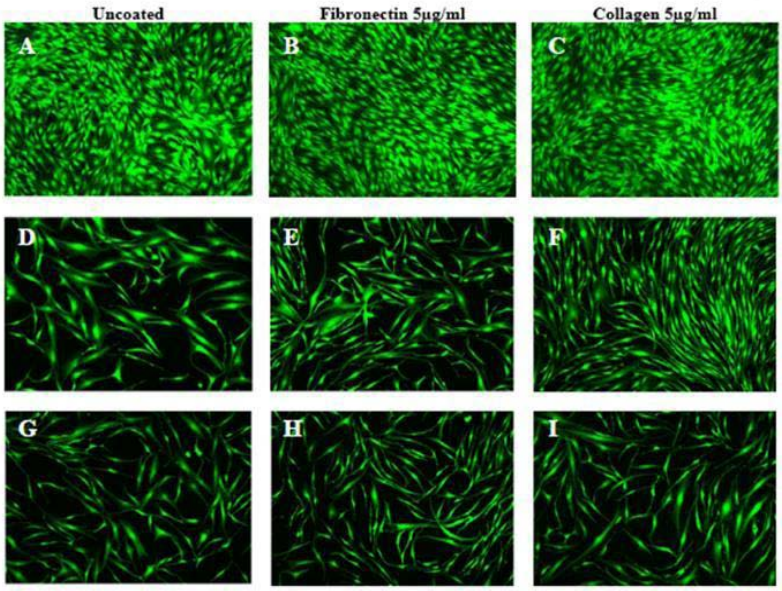

Figure 12: Calcein stained fluorescent images of HuMSC spreading on uncoated, fibronectin 5µg/ml and collagen 5µg/ml coated wells with oil and oil + nicotine exposure at 4% on day 7. (A), (B), (C) HuMSCs in uncoated, fibronectin 5µg/ml and collagen 5µg/ml coated wells with normal HuMSC media on day 7. (D), (E), (F) HuMSCs in uncoated, fibronectin 5µg/ml and collagen 5µg/ml coated wells with HuMSC media diluted with 4% oil on day 7. (G), (H), (I) HuMSCs in uncoated, fibronectin 5µg/ml and collagen 5µg/ml coated wells with normal HuMSC media diluted with 4% oil + nicotine on day 7.

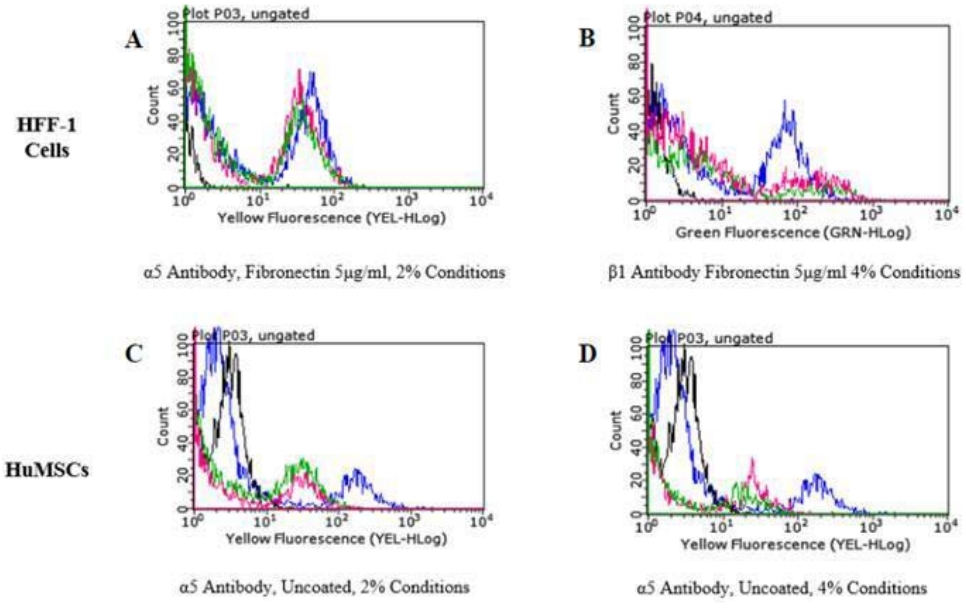

Figure 13: Fluorescent activated cell sorting (FACS) results from the Guava Incyte software of HuMSCs and HFF-1 cells on day 0 and 7. (A) HFF-1 cells in fibronectin 5µg/ml coated wells incubated with α5 antibody in 2% oil and oil + nicotine conditions for one week. (B) HFF-1 cells in fibronectin 5µg/ml coated wells incubated with β1 antibody in 2% oil and oil + nicotine conditions for one week. (C) HuMSCs in uncoated wells incubated with α5 antibody in 2% oil and oil + nicotine conditions for one week. (D) HuMSCs in uncoated wells incubated with α5 antibody in 4% oil and oil + nicotine conditions for one week.

Uncoated: Change in cell morphology was very similar to that seen with HFF-1 i.e., cells became more stretched with branching at 2% and 4% oil with and without nicotine and cells remained rounded with no branching in the 6% oil with and without nicotine. In summary, the addition of oil leads to changes in HuMSC morphology seeded on uncoated wells. There was a correlation between changes in morphology and the increase of oil concentration. Adding the nicotine seems to have no further effect on cell morphology.

Collagen: Change in cell morphology was very similar to that seen with HFF-1 seeded on collagen ie., cells became more stretched with increased branching at 2% and 4% oil with and without nicotine and cells remained rounded with little to no branching in the 6% oil with and without nicotine. The addition of nicotine did not further change the cell morphology. In summary, HuMSCs seem to become

more stretched in the presence of 2% and 4% oil over a period of 7 days, while remain rounded in the presence of 6% oil. The addition of nicotine did not further change the cell morphology (Figure 10).

Fibronectin: Change in cell morphology was very similar to that seen with HFF-1 seeded on fibronectin ie., cells became more stretched with increased branching at 2% and 4% oil with and without nicotine and cells remained rounded with little to no branching in the 6% oil with and without nicotine. In summary, the addition of nicotine lead to no change in the morphology; less cells were present in the oil + nicotine conditions making the changes in morphology more apparent.

HFF-1 integrin expression in presence of oil and oil + nicotine

Uncoated: We investigated the integrin expression of HFF-1 cells on uncoated wells in the presence of both oil and oil + nicotine using α5 and β1 antibodies. Day 0 was used as a baseline reading and control for day 7 readings.

Our data showed that HFF-1 cells in the presence of oil (green line) had little to no effect on integrin expression compared to the day 0 results. The cells treated with β1 antibody showed no difference than the control.

HFF-1 cells treated with β1 antibody on day 7 in both the 2% and 4% conditions showed an increase in expression compared to the control. On day 7, the α5 antibody in 2% oil + nicotine condition showed a decrease in expression compared to the control.

In conclusion, HFF-1 cells exhibited a decrease in α5 integrin expression for both the 2% oil. β1 integrin expression increased in the presence of 4% oil. Adding the nicotine did not further change the integrin expression for either conditions.

Fibronectin: The integrin expression of HFF-1 cells were examined on fibronectin coated wells in the presence of both oil and oil + nicotine using α5 and β1 antibodies (Figure 13A,13B).

Our data showed that HFF-1 cells in the presence of 2% oil showed a decrease in α5 expression compared to the control. There was a decrease in β1 expression in the 2% oil treated cells as well. A similar decrease was seen in the 4% oil treated cells incubated with β1 antibody.

Our data showed that HFF-1 cells in the presence of 2% oil + nicotine cell incubated with α5 antibody showed a slight decrease in expression compared to the control indicating that the cells did not adhere well (Figure 13A). A slight increase in β1 integrin expression was seen in the 4% oil + nicotine condition as seen in Figure 13B.

In conclusion, HFF-1 cells in the presence of oil and oil + nicotine on fibronectin coated wells showed a slight decrease in α5 integrin expression and a slight increase in β1 integrin expression. Adding the nicotine did not change how the oil affected integrin expression.

HuMSC integrin expression in the presence of oil and oil + nicotine

Uncoated: The integrin expression of HuMSCs were evaluated on uncoated wells in the presence of both oil treated cells and oil + nicotine treated cells using α5 and β1 antibodies. Day 0 was used as baseline reading and control for day 7.

Our data showed that HuMSCs in the presence of 2% oil had lower α5 integrin expression (Figure 13C). The 4% oil treated cells showed a decrease in α5 integrin expression as well. β1 integrin expression in both the 2% and 4% oil conditions showed no change on day 7 (Figure not shown).

HuMSCs in the presence of oil + nicotine had very similar α5 expression in comparison to the 2% oil conditions (Figure 13C,13D). On day 7, α5 antibody experienced a decrease in integrin expression for the 2% oil + nicotine treated cells. A similar decrease was seen in 4% oil + nicotine condition. β1 integrin expression in both the 2% and 4% conditions showed no change on day 7 in comparison to the control (Figure not shown).

In conclusion, HuMSCs in the presence of oil and oil + nicotine on uncoated wells experienced a decrease in integrin expression for α5 but no change in β1. Adding the nicotine showed a similar decrease in integrin expression for both antibodies compared to the oil without nicotine.

Fibronectin: Our data showed that HuMSCs in the presence of oil had lower α5 integrin expression and little to no effect in β1 integrin expression. The 2% oil + nicotine treated cells incubated with α5 antibody on day 7 showed an increase in integrin expression. 4% oil treated cells incubated with α5 antibody had a decrease in expression compared to the control.

Our data showed that HuMSCs in the presence of oil + nicotine had lower α5 integrin expression and little change in β1 integrin expression. The 2% oil + nicotine treated cells had a decrease in α5 integrin expression similar to the uncoated condition. HuMSCs treated with 4% oil + nicotine incubated with β1 antibody had little to no change in expression on day 7.

In conclusion, HuMSCs in the presence of oil and oil + nicotine on fibronectin coated wells experienced slight decreases in integrin expression. Adding the nicotine did not influence how the oils without nicotine affected integrin expression.

3D fibrin construct HFF-1 cell growth in the presence of 4% oil and oil + nicotine

Since cells grow normally in 3D and not on 2D substrates, we examined cell proliferation and morphology in 3D fibrin constructs in the absence and presence of oil and oil + nicotine.

In the presence of oil, our data showed that cell proliferation on day 3 increased in all conditions (Figure 14 E,14F,14G). The cells in the presence of 4% oil dilution showed increased cell growth compared to the control but the cells also had thinner morphology as seen in the 4% oil 2D conditions (Figure not shown). Cells in the presence of 4% oil dilution showed a decrease in cell proliferation on day 7 when compared to the control. The HFF-1 cell morphology changed when given the addition of nicotine. The proliferation increased for the 4% oil + nicotine treated cells but also exhibited a slimmer morphology. Minimal growth was examined as shown in Figure 14J. Additionally, the 4% oil + nicotine treated cells experienced a decrease in cell growth from day 3 to day 7.

The 3D HFF-1 proliferation results showed similar morphology and growth in both 4% oil and oil + nicotine treated cells. In the presence of oil, HFF-1 experienced a decrease in cell proliferation.

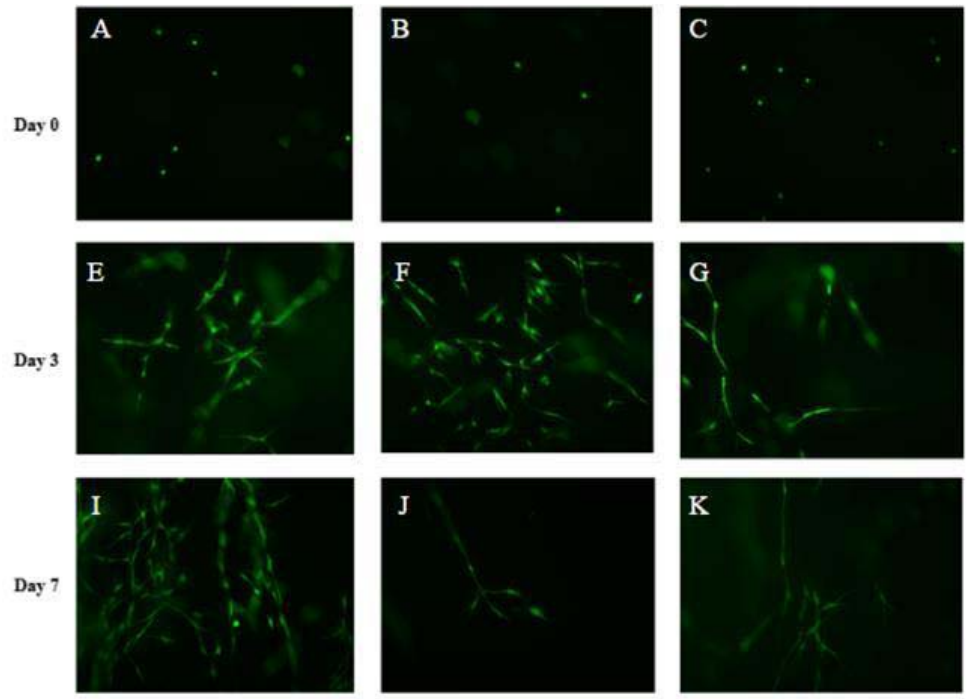

Figure 14: HFF-1 cells in 3D fibrin constructs with fibrinogen 5mg/ml and thrombin 5 units/ml treated with 4% oil and oil + nicotine fluorescent images on day 0, 3 and 7. (A), (E), (I) HFF-1 cells in 3D fibrin clot with regular HFF-1 media (control) on day 0, 3 and 7. (B), (F) and (J) HFF-1 cells in 3D fibrin clot treated with 4% oil diluted HFF-1 media on day 0, 3 and 7. (C), (G), (K) HFF-1 cells in 3D fibrin clot treated with 4% oil + nicotine diluted with HFF-1 media on day 0, 3 and 7.

However, it is further decreased with the addition of nicotine.

Discussion

This study demonstrated that both oil and oil + nicotine alter cell behavior on both collagen and fibronectin substrates. The increase in oil and oil + nicotine dilution percentage resulted in a decrease in cell proliferation and altered morphology. The 6% oil and oil + nicotine dilutions had the greatest impact on cell proliferation (Figure 1 and 2). The 6% dilutions also had the greatest impact on cell morphology on both collagen and fibronectin. The results are consistent with previous studies that have shown that nicotine induces apoptosis (cell death) *in vivo* [17].

Most mammalian cells are anchorage-dependent and attach firmly to the substrate *in vitro* [21]. Our results reveal that both HFF-1 and HuMSCs proliferate best on collagen. In order for the cells to function properly regardless of the presence of oil and oil + nicotine, they must be sufficiently supported and maintain contact with the extracellular matrix [18]. In a study evaluating the effect of Extracellular Matrix (ECM) proteins in controlling cell survival and proliferation, it was found that collagen promotes cell proliferation, cell survival under stress [19]. Cell adhesion is involved in stimulating signals that regulate cell differentiation, cell cycle, cell migration, and cell survival [20]. Integrins mediate cell adhesion by binding to extracellular ligands and encourage force sensing transmission signals to and from the cell through a connection to the actin cytoskeleton [22]. Moreover, it has been observed that cells will spread on stiff substrates as integrins ligands adopt a larger surface area through the development of mature Focal Adhesions (FA) in comparison to softer substrates [23]. FAs respond to mechanical forces generated by either internal contractions of actin cytoskeleton or by external ECM stretching [24]. It is through the convergence of these integrin-mediated adhesions and mechanotransduction pathways that cells are able to carry out critical functions such as migration, proliferation and differentiation [25]. Therefore, we hypothesize the presence of oil and oil + nicotine may be altering the dynamic relationship between focal adhesions and the HFF-1 and HuMSC cells and their cellular proliferation on the synthetic scaffolds used in this experiment.

The generated FACS data focuses on the fibronectin α5β1 integrin binding domain. This is relevant when cells are in contact with surfaces that contain physiological ligands for high-affinity attachment of cells, such as RGD peptides [28]. The RGD motif in fibronectin represents the major binding site for α5β1 integrins [27]. The results ultimately demonstrate that integrin expression is dependent on the condition and type of antibody the cells are treated with. We observe that for both HFF-1 and HuMSCs, oil and oil + nicotine caused a decrease in α5 and β1 expression on both fibronectin and uncoated conditions. This is the first study to our knowledge that examined integrin expression in the presence of oil and oil + nicotine.

The fluorescent images presented from the seeding of HFF-1 cells within 3D fibrin constructs further illustrate the effects of oil and oil + nicotine on cellular proliferation. Fibrin clot formation is associated with most wound healing processes and is thought to guide the recruitment of fibroblast cells [32]. 3D fibrin constructs can be modulated by the addition of fibrinogen complex with thrombin *in vitro* which polymerize into a synthetic fibrin clot [33]. The resulting 3D construct consists of a dense network of thin fibers that allow cells to not only migrate but proliferate [34]. Our fluorescent images reveal a negative correlation between the presence of 4% oil and

cellular proliferation. However, we observe a further decrease HFF-1 proliferation in the presence of nicotine suggesting that the 3D fibrin clot scaffolds reveal an additional deleterious effect captured further than the 2D cellular proliferation assay alone. Cell culture using the 3D technique more closely mimics the environment of cellular tissues and organelles and allows for natural gap junctions and cell-to-cell attachments, as they do *in vivo* [36].

In summary, our study aimed to understand the effects of oil and oil + nicotine on both HFF-1 and HuMSCs cell proliferation on various two dimensional substrates, integrin expression and three dimensional cell seeding in fibrin constructs. The HFF-1 and HuMSCs experienced dramatic decreases in proliferation in the presence of oil or oil + nicotine. Regardless of whether nicotine was present within the oil, the increasing concentrations of oils in media affected both HFF-1 and HuMSC growth. Oil and oil + nicotine altered cellular morphology with increasing amounts of oil and oil + nicotine.

To our knowledge, this is the first study demonstrating the effects of vaporizable oil and oil + nicotine on HFF-1 and HuMSC proliferation, morphology and integrin expression. It is possible that there are unknown compounds, besides the presence of nicotine, in the oils that are affecting initial adhesion, cell proliferation and morphology. While the vaporizable liquid consists primarily of propylene glycol, flavorings and nicotine, little is known about the exact chemical composition of vapor ultimately inhaled by the user [2]. A novel study utilizing GC-MS analysis identified over 64 other ingredients were present in the vapor besides those on the vendor label [38]. These findings present a plausible explanation for the observed changes in cellular behavior in both cell lines. As seen in Figures 13 and 14, integrin expression is altered in the presence of oil and oil + nicotine and shown in Figures 7 through 12 that cell morphology and spreading has changed considerably.

Further *in vivo* research is needed to truly comprehend the effects of oil and oil + nicotine on human health. Additionally, the composition of the oil and oil + nicotine must be evaluated in order to identify the exact mechanism of action affecting the cellular proliferation, integrin expression and morphology.

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