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Research Article

Part 2: Cinnamon Extract Decreases Size and Lipid Volume In 3T3-L1 Preadipocytes Grown in Three-Dimensional Agarose Culture

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Abstract

In part 1 of the study, the 3D agarose model was presented for its use in 3T3-L1 cells. Growth in 3D agarose allowed 3T3-L1 preadipocytes to differentiate spontaneously without the chemical induction that is used to induce differentiation in monolayer cultures. Part 2 utilizes the previously published 3D agarose culture model to examine the effects of cinnamon extract on the 3T3-L1 cells. Here we report that the morphological changes associated with the 3D agarose cultured 3T3-L1 cells are not altered by the presence of cinnamon extract at the concentration tested. The treated cells remained round with accumulated lipid, as visualized with oil red O staining. However, a significant reduction in median cell size was noted in cultures treated with cinnamon extract. Differentiation was confirmed at all time points by both oil red O staining and immunohistochemistry detection of a known marker of differentiation, PPARγ. Results demonstrate that the decreased size of the cinnamon treated cells is associated with a decreased lipid volume, but only in cells grown in the 3D agarose culture. The same trend was not observed in monolayer treated cells. Modified monolayer experiments, designed to explore the effects of cell rounding on lipase activity, suggest that lipase activity is suppressed when rounded cells are treated with cinnamon extract, an observation that is not replicated in cinnamon treated flat cells. Extrapolation of this data suggests a possible use for cinnamon that could lead to reduced lipid volume storage in adipose cells, although additional studies should be conducted to determine safety and extraneous reactions possible by cinnamon.

Keywords: Three-dimensional culture; 3D culture, Agarose culture; Adipogenesis; Differentiation; 3T3-L1 cells, Cinnamon extract; PPARγ

Introduction

Previous studies have demonstrated the importance of three-dimensional culture in obtaining a more physiologically relevant environment [1-4]. The model presented in part 1 of this 2-part series demonstrated the potential for a 3D agarose system to be used with 3T3-L1 preadipocyte cells that allowed studies on the morphology of the cells as well as utilizing immunohistochemistry techniques to look for protein accumulation [4]. In addition to allowing more conventional dosing regimens and concentrations, the 3D culture method permits an unhindered spatial environment for which cells can expand in a more physiologically relevant arrangement which can also alter cell signaling and gene expression [1,11-16]. In the current study, the second part of a 2-part series is presented using the previously published novel three-dimensional model method for 3T3-L1 cells to examine the effects of treatment of aqueous cinnamon extract on preadipocyte cells [4]. Like the model described in part 1, there is no chemical induction utilized in order to stimulate differentiation into mature adipocytes in this study. Cinnamon was selected for the study because of its documented anti-diabetic and lipid lowering effects [17-22]. Cinnamon has long been used medicinally for gastro-intestinal discomfort but has, in recent decades, caught interest due to its effectiveness at lowering blood glucose [17-22]. Studies in patients with diabetes have also led to the discovery of the lipid lowering effects of cinnamon, particularly the triglycerides [20,22]. Cinnamon has also been demonstrated to stimulate the initiation state of 3T3-L1 adipocyte differentiation [23]. Given that 3T3-L1 cells spontaneously differentiate in our 3D model, it was important to examine the effects of cinnamon on these cells in our model [4]. Many metabolic studies completed in cell culture have selected the 3T3-L1 preadipocytes as a model system because of its expression of the GLUT-4 transporter found in both adipocytes and muscle cells [24,25]. For this reason and due to laboratory studies with cinnamon in the past, we seek to determine the effects of cinnamon on the 3T3-L1 cells grown in the 3D agarose culture method characterized in part 1 of the study [4]. Use of the 3D agarose culture system provides an affordable and physiologically more relevant system than monolayer alone, which is demonstrated in this study when monolayer and 3D methods are compared.

Studies of metabolic dysfunction often utilize the 3T3-L1 preadipocyte cells to examine potential effects on cell signaling and treatments for obesity and diabetes. Based on our desire to examine physiologically relevant and longer-term treatments with cinnamon, we modified the agarose culture system used previously by our lab to be used with the 3T3-L1 cell line with cinnamon treatment [1,4,26,27]. The use of this system and the difference in results between monolayer and 3D treatments demonstrate the importance of completing such studies in systems such as our 3D agarose model. As a result of experiments in our model, we are able to hypothesize about the physiological relevance of our findings.

Materials and Methods

Monolayer cell culture

The 3T3-L1 cell line (ATCC® CL-173™) was purchased from American Tissue Culture Collection (ATCC). Cells were grown in monolayer in expansion media, 1X Dulbecco's Modified Eagle's Media (DMEM) with 10% Bovine Calf Serum (BCS) and 0.1% penicillin/streptomycin, as recommended by the manufacturer and incubated at 37°C in a humidified CO_2 incubator until approximately 70% confluence. All media and serum were purchased from ATCC. Culture plates, dishes and supplies were purchased from Corning. Cinnamon extract treated cells were treated at least 24 hours post plating with a final concentration of 0.15 mg/ml of cinnamon extract (0.06 ml of 15 mg/ml stock per 6 ml of media volume). Vehicle control samples were treated with the same 0.06 ml of water rather than cinnamon extract. Normal control samples were treated with standard media with no water or cinnamon extract added.

3D agarose culture

3T3-L1 cells were grown to 70% confluence under standard monolayer conditions as recommended by the manufacturer and collected via a 5-minute incubation at 37°C with 1X Trypsin EDTA (ATCC). Cells were filtered through a single-use sterile 70 µm cell strainer (Fisher Scientific) to remove cell clumps. Single cells were counted with a hemocytometer, pelleted and resuspended at a concentration of 5 x 10^5 cells per ml in 0.5% low temperature agarose in DMEM. The 0.5% low temperature agarose (Bio-Rad) was created by mixing equal volumes of 1% low temperature agarose with 2X DMEM. 10µL of the 0.5% low temperature agarose/cell suspension was plated in the center of a 35 mm cell culture dish previously coated with a 1% high temperature agarose (BioRad). Cultures were allowed to gel at 4°C for 15 minutes prior to feeding with 2 ml of media (DMEM, 10% FBS or 10% BCS, 0.1% pen-strep), then incubated at 37°C in a humidified CO_2 incubator. Cells were fed by complete media change of 2 ml at half-week intervals. A detailed description of the protocol for 3D agarose is described in Kinder and Aulthouse, 2004 (1). Cultures treated with cinnamon extract were treated to a final concentration of 0.15 mg/ml of cinnamon extract by addition to the media. Cells were treated continuously for the full 0.5,1, and 2 week period at a final concentration of 0.15 mg/ml or 0.02 ml. Vehicle control samples were treated with an 0.02 ml of water added to the media. Normal control samples contain standard media with no addition

of water or cinnamon extract.

Cell viability

At all time-points and treatments, at least 3 cultures were analyzed for viability using the trypan blue (Sigma) exclusion assay as described by the manufacturer and others (28, 29). Trypan blue exclusion was visualized using an inverted Nikon M2 microscope prior to oil red O staining. Cells that were blue indicated membrane disruption and would be considered non-viable. As with the other cells cultured using this method by the investigator, the 3T3-L1 cells were highly viable with less than 0.2% taking up the trypan blue dye.

Statistical analysis

Appropriate statistical analysis was completed using Graph Pad Prism. In brief, lipid content data was analyzed using an unpaired t-test with Welch's correction. Lipid volume differences between cultures grown with different serums and treatments were analyzed using an ordinary two-way ANOVA. Cell size differences were analyzed using an ordinary one-way ANOVA with Brown-Forsythe test.

Measurement

Cell diameter was measured using an Olympus IM inverted microscope calibrated with a stage micrometer. The cultures were first centered at 4x and each quadrant of the culture was then analyzed at 20x. Cells measured were selected randomly from the field of vision. Measurements were taken on randomly selected culture dishes blind from treatment conditions. Twenty-five cells were measured per quadrant giving 100 cells per culture. Lipid droplets were measured using the same technique and the average lipid droplet size per quadrant was used to estimate spherical volume.

Oil red O staining

Both monolayer and agarose cultures were stained with oil red O (Sigma) to visualize and detect lipid droplets for photography. Monolayer and agarose cultures were rinsed twice with phosphate buffered saline (PBS) (Sigma), fixed with 10% neutral buffered formalin (NBF) (Fisher Scientific) then rinsed twice in distilled water. Cultures were incubated at room temperature for 15 minutes in 60% isopropanol and stained with oil red O solution for 30-60 minutes then rinsed with tap water until clear. Residual dye was isolated to the high temperature agarose dish coating and did not interfere with microscopy images.

Lipid quantitation using oil red O stain

A. Lipid content quantitation in 3D agarose cultures

Lipid content was estimated in each oil red O (Sigma) stained 3D agarose culture by randomly centering the field on each quadrant of the plated culture to ensure no bias was involved in selection of the field area. The lipid droplet density was evaluated blindly with no knowledge of the culture treatments or growth times. The average diameter of a micro-lipid droplet was calculated per quadrant with an overall average of 0.5 μm. Spherical volume was calculated using the equation $4/3\pi r^3$ where the radius was calculated from the measured micro-lipid droplet diameter. Once the lipid droplet volume was calculated, it was multiplied by the quadrant droplet number in order to obtain a total lipid volume for that quadrant. Residual dye in the high temperature agarose coating was not adjacent to the micro culture droplet (10 μL centered on the coated dish) and did not interfere with oil red O visualization.

B. Lipid content quantitation via dye extraction in monolayer cultures.

Cells grown in monolayer were stained with oil red O, were dried overnight, and the dye was extracted with 98% isopropanol for 20-30 minutes at room temperature. Samples were assayed at 520 nm spectrophotometrically using a Bio-Tek plate reader. These lipid volume values were comparable to the calculated method used for the 3D cultures where instead of calculating spherical volume, we calculated the surface area (using the formula $4\pi r^2$) of the lipid droplet in monolayer to estimate lipid content. The dye extraction method is less labor intensive and was used as the primary method of lipid quantitation in all remaining monolayer cultures stained with oil red O. Agarose 3D cultures were not subjected to dye extraction due to interference of the agarose with the spectrophotometer reading.

Photography

Monolayer cells were photographed using a Nikon Digital Sight DS-5M camera mounted on a Meiji Techno inverted microscope. The microscope utilized for oil red O visualization and photography was a Zeiss Primovert microscope equipped with a Zeiss Axiocam Erc5s camera for imaging. The microscope utilized for DAB IHC photography was a Nikon Eclipse 50*i* with a Moticam 10+MP camera for imaging.

IHC

For IHC, agarose cultures (0.5, 1.5 and 2.5weeks) were rinsed in PBS twice and fixed in 10% neutral buffered formalin for a minimum of 48 hours. Cultures were carefully removed from the dish using a spatula and placed in biopsy cassettes for processing. Cultures were processed on a Tissue Tek II processor for 10 minutes in each ethanol concentration (70%, 80%, 95%, 100%, 100%) and followed by two changes of xylene. Infiltration with molten paraffin involved two 30-minute changes and a third change of 15 minutes under vacuum to ensure complete infiltration. Eosin was added to the second 100% ethanol so the cultures/cells could be visualized during embedding and sectioning. Cultures were

embedded in paraffin and sectioned at 5 μm. The sections were mounted on positively charged slides and heated for 30 minutes at 58°C in an oven. Sections were selected for IHC and stained following the manufacturer (Cell Signal) instruction and using their reagents. Briefly, the sections were deparaffinized in excess xylene and rehydrated in a graded series of ethanol (100%, 100%, 95%, 80%, 70%) and brought to distilled water. Antigen retrieval was accomplished using citrate buffer heated in a microwave to boiling. Cooled sections were then rinsed in distilled water and endogenous peroxides were blocked using 3% H_2O_2 for 10 minutes. Sections were then washed with Tris buffered saline with tween 20 (wash buffer) and incubated in "animal free block" solution for 1 hour at room temperature in a humidified chamber to prevent nonspecific binding. The primary antibody PPARγ (C26H12) rabbit mAB #2435 was diluted according the manufacturer recommendations for IHC (1:1000) and applied to sections after the blocking agent was removed. PBS, minus antibody, served as the negative control. Sections were incubated overnight at 4°C in a humidified chamber. Sections were rinsed with wash buffer and incubated in Signal boost reagent-HRP for 30 minutes in a humidified chamber at room temperature. Sections were then rinsed in wash buffer and incubated in the DAB chromogen for 7 minutes, rinsed in distilled water, and counterstained for 30 seconds with modified Harris Hematoxylin. Sections were then rinsed in deionized water, dehydrated in ethanol followed with xylene and cover-slipped using Permount. A PAP pen was used to encircle the sections to reduce the number of reagents used.

Cinnamon Extract Production

Cinnamon extract was produced at a concentration of 15 mg/ml by taking 1500 mg of *Cinnamomum cassia* bark and grinding it into a fine powder in a coffee grinder reserved only for cinnamon grinding. The powder was added to 100 ml of ultra-pure water and heated to 95°C for a period of 1 hour while covered to prevent evaporation. The extract was then filtered through Whatman #1 filter paper and stored at 8°C until use. Extract was filtered through a 0.22 um filter prior to cell treatment.

Results

Cinnamon extract treatment does not affect the morphological shape of the cells grown in 3D agarose but does significantly reduce cell size.

In part 1 of the study, the change in morphology between 3T3-L1 cells grown in monolayer and in the proposed 3D agarose model were presented. It was concluded that growth of the 3T3-L1 cell in 3D agarose culture, allowed them to differentiate from preadipocytes to mature adipocytes without addition of induction cocktail. Results demonstrated the morphological change from flat to round and the accumulation of lipids as evidenced by oil red O staining [4]. In the current study, the 3D agarose model system was used to explore the effects of cinnamon extract on the cells grown. In order to ensure that cinnamon extract treatment did not significantly alter the morphology of the cells and to determine if there was any change in lipid accumulation, the plated 3T3-L1 preadipocytes were initially expanded in monolayer. These cells were filtered to ensure a single cell suspension, counted and suspended in low temperature agarose as described previously [4]. All cultures were plated in triplicate for each cinnamon extract treatment time point at 0.5, 1, and 2 weeks. Normal and vehicle controls were also plated in triplicate. Representative cultures were tested for trypan blue exclusion screening for cell viability as described previously [4]. Neither the controls nor the treatment group exhibited any loss of viability at the concentrations tested with less than 0.2% cell death. Figure 1 shows the oil red O stained cells grown in agarose at 0.5, 1 and 2 weeks in columns 1, 2, and 3, respectively. Row A represents cinnamon treated cultures, while row B and C are the vehicle and normal controls respectively. All cells had similar morphology, differing primarily in size and lipid volume accumulation. This confirms that the treatment of the cells with cinnamon extract under these 3D culture conditions does not prevent lipid accumulation in its entirety nor does it prevent differentiation, which is further confirmed by the detection of PPARγ in the immunohistochemistry results.

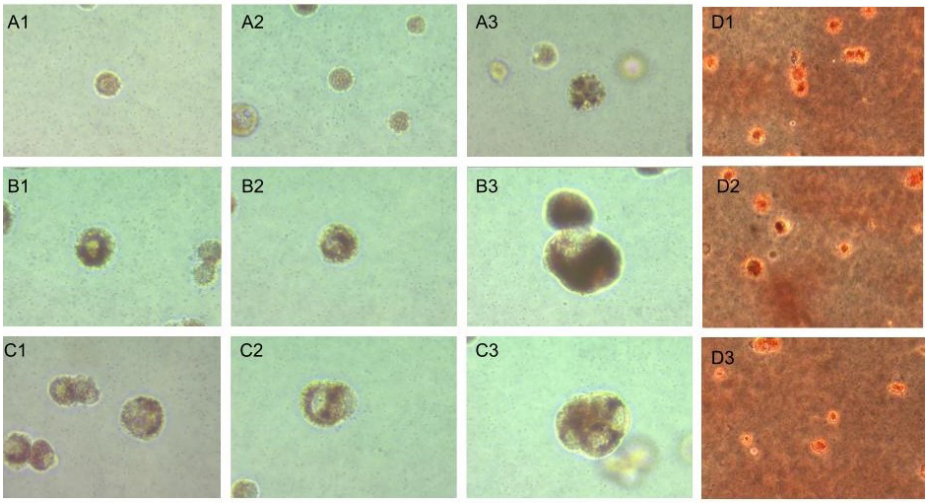

Figure 1: Oil red O staining of 3D agarose cultures at 0.5 weeks (column 1), 1 week (column 2), and 2 weeks (column 3). Row A represents cinnamon treated cultures at 0.5 (A1), 1 (A2) and 2 (A3) weeks. Row B is vehicle control at 0.5 (B1), 1 (B2), and 2 (B3) weeks. Row C is normal control at 0.5 (C1), 1 (C2) and 2 (C3) weeks. All photos were taken at 400X using oil immersion. Column D (1, 2, 3) shows 3 different fields of a representative culture of cinnamon treated cells. These are taken at 20X with an inverted scope.

The diameters of a representative sample from each culture quadrant were measured. The mean cell diameters are shown in figure 2. There is a statistically significant difference between vehicle control and the normal control (data not shown). The vehicle is water and so there is an expectation that cell diameter may decrease slightly based on osmotic volume loss, which could explain the difference in cell size between the normal controls (both FBS and BCS) and vehicle control. The further decrease in cell diameter observed in the cinnamon treated cultures are statistically significant when compared to the vehicle control, indicating that the cell size reduction is not solely the result of osmotic differences. The mean cell diameter of the cinnamon treated cells was nearly half of the vehicle control cells. Similar size reductions were observed in monolayer, albeit to a lesser extent. Monolayer cultures had some rounded cells and some flat cells making the determination of average diameter an unreliable measurement of cell size in monolayer.

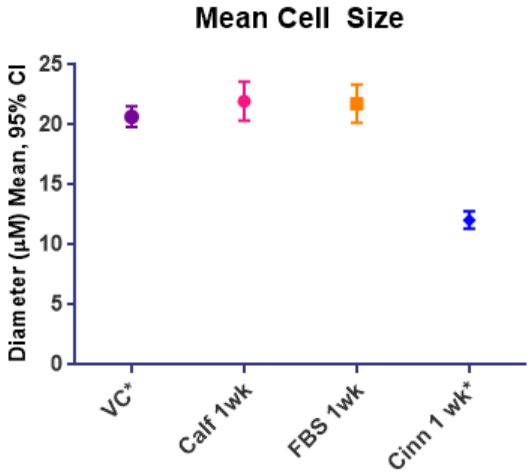

Figure 2: Comparison of the diameter of cells treated with bovine calf serum, fetal bovine serum, or fetal bovine serum with cinnamon extract. Cinnamon treated cell size is significantly different from any of the other samples including vehicle control (VC), which was diluted relative to the normal serum concentrations to the same extent that the cinnamon cultures were diluted. Results were analyzed by an ordinary one way ANOVA with Brown-Forsythe test. P-value <0.0001.

Cinnamon treated cells have significantly decreased lipid volume as determined by droplet size, count and volume.

Following a significant reduction in cell size observed in cinnamon treated cells, determination of the cause of the decreased cell size was warranted. It was important to determine if the cells were smaller as a result of decreased lipid volume accumulation, loss of lipid volume, or decreased adipogenesis. Adipogenesis is confirmed due to the detection of PPARγ in the immunohistochemistry results discussed later in this manuscript and shown in figure 5. Adipogenesis is also detected based on the lipid accumulation stained with oil red O and cell morphology shown in figure 1. Considering these results in conjunction, we conclude that adipogenesis occurred. Another possible test to determine the expression of PPARγ would be RT-qPCR detection of PPARγ, however as determined in the publication of the model, total RNA is not stable during the extraction process from the agarose cultures. For this reason, we completed IHC to demonstrate the presence of PPARγ. In order to estimate the lipid volume for a spatial determination, lipid droplet measurements were taken to determine the average lipid droplet size at each time point under each condition. Each culture was again examined in each of four quadrants by measuring cell size and calculating the average number of droplets per view. Data presented in figure 3 show that at the 1-week time point, the estimated lipid volume was decreased in the cinnamon treated cells as compared to vehicle control. The difference is significant with a p-value less than 0.0001.

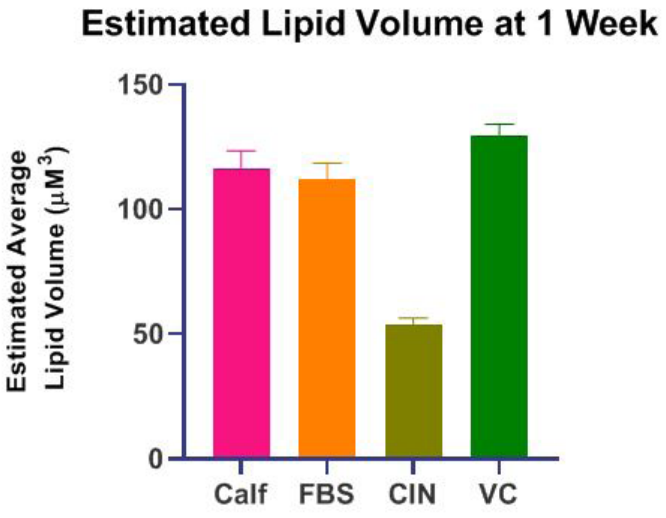

Figure 3: Lipid Volumes were estimated for each cell in 3D culture. Cells treated with Cinnamon showed lower lipid volume. The difference is significant as analyzed using an ordinary one-way ANOVA, $p<0.0001$. Lipid in monolayer cultures was quantitated via dye extraction. In general, 3D cultures accumulated more lipid than monolayer. Cinnamon treated monolayer cultures differed in lipid trends, but more samples are necessary to confirm any changes in lipid accumulation.

After viewing reductions in estimated lipid volume, it was necessary to determine if cinnamon treatment was associated with lipid volume loss, a decrease in lipid accumulation, or reduction of adipogenesis. Again, we can confirm that adipogenesis occurs based on our results shown in figures 1 and 5. Although oil red O dye extraction was not possible in our agarose suspension, lipid volume determination by space fill was not feasible in monolayer due to the mixture of round and flat cells. Lipid volume determination by dye extraction was more reliable for monolayer. Figure 4 shows the estimated micrograms of lipid in both 3D and monolayer cultures. Although two different methods were used to estimate lipid content in the cultures, each data set was normalized in order to compare for trends. It is also important to note that within each culture method, lipid determination was completed identically. Looking at the data from figure 4 for the 3D cultures, it is obvious that cinnamon treated cells had reduced lipid content when compared to vehicle control. This trend was not observed in the monolayer cultures, where very little difference was observed between the cinnamon and vehicle control treatment groups. This data does not conclude that cinnamon reduces lipid volume but it does suggest that the 3D culture condition is important to obtain these results. We do not view these as negative results because the effect was absent in monolayer, but rather believe it demonstrates that the cell environment is important in expression and activity of lipid regulating mechanisms.

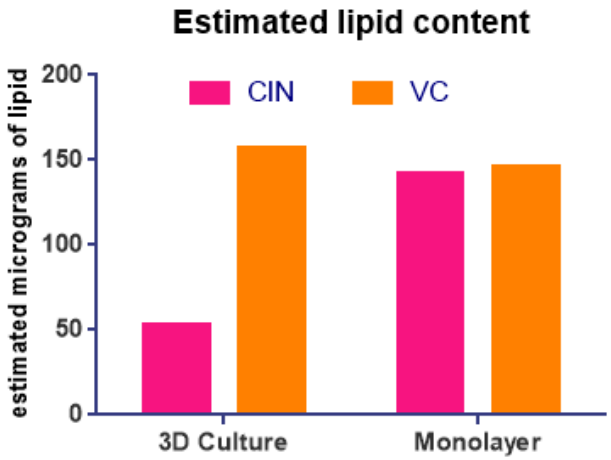

Figure 4: Estimated lipid content (quantitated by dye extraction in monolayer and volume fill in 3D culture) was normalized and graphed for comparison. Data were analyzed using unpaired t test with Welch's correction. Results showed a p value of 0.0023 indicating a significant difference.

Cinnamon treatment does not affect accumulation of PPARgamma observed in 3D culturing.

Additionally, careful examination of the oil red O stained cells at every time point demonstrates visually that less lipid is present in the cinnamon treated cells. For this reason, it is important to confirm that the reduced lipid volume is not due to failure to differentiate. As outlined in part 1 of this series, growth in 3D agarose culture allowed spontaneous differentiation of the preadipocytes into adipocytes. In support of our model, this was confirmed by immunohistochemistry detection of PPARγ. This method was again used to confirm that despite the reduced lipid volume, adipogenesis does occur. Figure 5 shows a comparison of vehicle control and cinnamon treated culture sections at each time point, each subjected to immunodetection and chromogen labeled PPARγ. Panels B, C, E, F, the cinnamon treated and vehicle control panels, exhibit the brown chromogen selected as the label, indicating the presence of PPARγ in all cultures with or without cinnamon treatment. For comparison, similar culture sections were stained only with hematoxylin to differentiate between an unlabeled and labeled PPARγ. These data demonstrate that cinnamon treatment does not prevent the accumulation of PPARγ in the cell and therefore is not altering adipogenesis. These results confirm that adipogenesis occurs in both the untreated and cinnamon treated cells. However, quantification of PPARγ was not possible via this method and therefore it cannot be determined if the amount of PPARγ changes with cinnamon treatment.

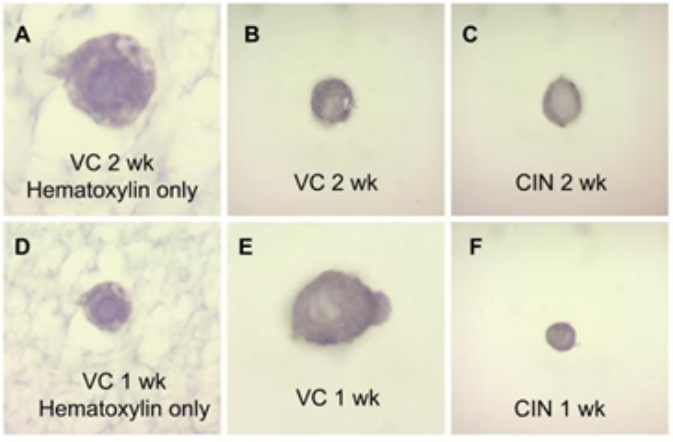

Figure 5: Immunohistochemistry detection of PPARγ. Panel A and D show the hematoxylin stained vehicle control at 2 weeks and 1 week, respectively. Vehicle control at 2 weeks and 1 week in panel B and E, respectively, show the cultures dyed with hematoxylin overlapped with the brown chromagen. Panel C and F represent the cinnamon treated cultures with both hematoxylin and brown chromagen, respectively. Comparison between the hematoxylin only and the chromogen cultures demonstrate the presence of PPARγ. All photos were taken at 1000X with oil immersion.

Lipase activity is significantly higher when cells are allowed to attach following trypsin release before treatment occurred.

Due to the nature of the agarose culture system, extraction of native and functional enzymes is possible only with enzymes that are extremely stable. Detection of active lipase was not possible when extracted from agarose culture. In order to determine if cell shape played a role in lipase activity, cells grown in monolayer were treated with cinnamon immediately at plating or following a 24-hour growth period. Furthermore, if treated following 24 hours of growth, one group was released using trypsin and re-plated with cinnamon treatment and one was treated without release. Treatment at plating and treatment following 24-hour growth with subsequent trypsin release samples were exposed to cinnamon prior to attachment. Cells treated 24 hours after plating but without trypsin release, were exposed to cinnamon only after attachment. These preadipocyte cells were not induced chemically for induction prior to experimentation in order to ensure that observed effects were from cell shape rather than adipogenesis. Following an additional 24 hours post treatment cells were harvested and assayed for lipase activity. Results are graphed in figure 6. Although not significant, lipase activity is most notably decreased in cells treated following trypsin release. Minimal differences exist between untreated, treated at plating and treated without release. This suggests that cell shape is an important determinant of how cinnamon will affect the cell and on a relatively short time scale.

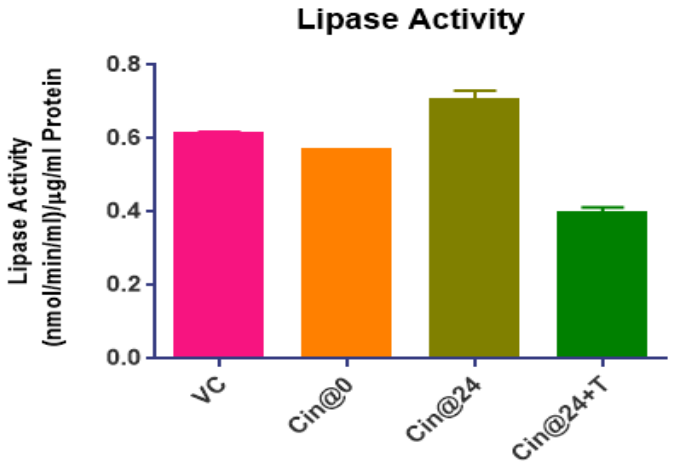

Figure 6: At plating cells were treated with 25 microliters extract/milliliter of media (Cin@0). Cells were allowed to grow for 24 hours, then treated with cinnamon extract. In one sample cells remained attached upon addition of extract (Cin@24) and in another sample cells were released with trypsin and re-plated with treatment (Cin@24+T). All samples were harvested at 24 hours. Lipase activity increased where cells remained flat and attached. Results were analyzed with ordinary one-way ANOVA with the

Brown-Forsythe test. P-value = 0.0002 indicating significant difference.

Discussion

The presented study represents a novel approach to the study of the effects of cinnamon on 3T3-L1. Our 3D model provides a non-chemical induction method of differentiation that can provide direct effects of the cinnamon treatment rather than potential combined effects from the differentiation cocktail along with cinnamon treatment. It also demonstrates clearly a difference in biological behavior between the two culture types, suggesting that the more physiologically relevant 3D model system may be an essential addition to metabolic studies. Numerous studies have demonstrated the importance of such a novel system [4,5,11-16].

Cinnamon treatment of the 3D grown 3T3-L1 cells did not disrupt the morphology of the cells nor did it inhibit adipogenesis. Although the agarose 3D model was previously shown to spontaneously induce adipogenesis in the 3T3-L1 cells, it was predicted that cinnamon may alter that differentiation. Cinnamon has been referred to as an insulin mimic due to its documented, yet still debated, effects on blood glucose levels. Some studies have demonstrated reduction in blood glucose levels both postprandial and fasting, suggesting cinnamon has a multi-target action. The basis of the multi-target activity stems from the fact that fasting blood glucose reductions suggest action at the liver while postprandial glucose reduction indicates a muscle or adipose effect. Although blood glucose control is essential to health, patients with diagnosed type 2 diabetes also commonly have comorbidities such as obesity and hyperlipidemia [30-32]. This blood glucose control alone is not sufficient to improve the overall health of the patient [33,34]. Of particular concern would be any treatment that leads to additional weight gain, as is commonly seen with insulin treatment but not as often with oral anti-hyperglycemia treatments such as metformin [34-37]. The hypothesis was that the blood glucose lowering effects observed with cinnamon may be related to decreased adipogenesis and we were interested in exploring cinnamon use as a potential weight-loss aid. The 3D agarose model allowed differentiation independent of chemical induction; our research question was, would cinnamon treatment stop that adipogenesis. The first experiment demonstrated that the morphological change observed with adipogenesis was indeed still occurring, this was further confirmed by the IHC detection of PPARγ, which is highly expressed during differentiation and is commonly used as a marker of differentiation. Microscopy examination of the culture demonstrated vacuole formation, which until dyed with oil red O, were assumed to be full lipid vacuoles. After completion of the oil red O stain, it became evident that although some lipid accumulation was occurring, it was to a lesser extent in the cinnamon treated cultures. Additionally, consistently smaller cells were observed when the cultures were treated with cinnamon. Cell diameter measurements summarized in figure 2, confirmed a significant difference in cell size, despite the similarities in morphology. Importantly the cinnamon treated cells were significantly smaller than the vehicle control cells, which would be expected to be slightly contracted due to osmotic shifts occurring from the aqueous vehicle. The significant (P-value <0.0001) difference in cell size between the VC and the cinnamon treated cell size fully confirm it is not due to osmotic differences alone.

It is important to confirm that the decrease in cell size observed in cinnamon treated cells is related to a true change in lipid volume, not an osmotic contraction. Estimation of the lipid volume per lipid droplet and evaluating the percent of cells containing lipid droplets, allowed confirmation that the smaller cells contained smaller lipid volumes rather than more tightly packed lipid droplets. The presence of lipid volume stored in all cells implies that all cells were fully differentiated adipocytes, as preadipocytes would not have the lipid storage required for these volumes of lipid within the cells. The cinnamon treated cells are also estimated to have a lipid volume close to half of that of the untreated cells. This decrease in lipid volume implies that less lipid is being stored within the cells, even when lipid storage is a viable option for the cells. However, it is unclear if this decrease in stored lipid volume can be attributed to a decrease in initial storage of lipid, or an increase in breakdown and secretion of stored lipids from the adipocytes. More studies are required before one mechanism can be determined, however the estimated lipid store volumes in cinnamon-treated cells are lower regardless of the mechanism behind it. Unpublished research in our lab on monolayer 3T3-L1 cells suggest that lipase activity increased with cinnamon treatment, which would explain our observations. Furthermore, our preliminary data suggests an increase in expression of PPARγ in cinnamon treated monolayer cells, a transcription factor which is commonly used as a marker of adipogenesis, but that also is partially responsible for controlling the expression of adipose triglyceride lipase (ATGL) (Stockert lab, unpublished).

The fact that the lipid volume decreased with cinnamon treatment in the 3D cultured cells but not the monolayer cultured cells suggests that the spatial environment of the cells is important in their lipid storage. It appears that having the permissive 3D environment allows the cells to moderate the lipid volume, either through decreased storage or through increased lipase activity. Given the nature of the 3D agarose culture, it was not possible to extract lipase enzyme and effectively measure its activity. As a result of this limitation, a modified experiment was designed that explored the effect of cell release (temporarily allowing cell rounding) on lipase activity. Cells that were released with trypsin and then treated with cinnamon, compared to those that were not released prior to cinnamon treatment had lower lipase activity,

although it was not significant. This result is opposite of what was expected given the fact that the trypsin released cells were allowed to temporarily adopt a round morphology. It is unclear, especially due to lack of significance, if this trend was due to cell shape or if the temporary rounding was not enough to mimic the 3D environment. An alternative to the 3D environment that provides longer rounding time while still allowing for extraction of lipase enzyme would address this uncertainty.

It is clear from the immunohistochemistry that cinnamon treated cells do still express PPARγ and along with the evidence of lipid accumulation, demonstrates that adipogenesis is still occurring in the cinnamon treated cells. Research has suggested that some of the components in cinnamon function as partial agonists of PPARγ by binding the ligand binding domain and stimulating ligand dependent activation of PPARγ. PPARγ is responsible in part for regulation of lipid metabolism, in particular expression of adiponectin and resistin, which are responsible for regulating lipid storage and lipolysis in adipose tissue. Further research in the lab is exploring the ligand activation of PPARγ and may relate back to this research question but is currently outside the scope of this study.

We conclude from this study that the 3D model system is an essential tool for metabolic studies given that results differ based on cell shape. We demonstrate that cinnamon very significantly reduces the cell size when grown under these conditions, which results from an apparent reduction in lipid volume, but not from reduced adipogenesis. Furthermore, we conclude that the reduction in lipid volume is not observed to the same extent in monolayer, which we view as a demonstration of the importance of the studies in the 3D culture method, such as the one presented.

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