TED ANKARA COLLEGE FOUNDATION PRIVATE HIGH SCHOOL

A study of the effect of corticosteroids on the differentiation and growth of the adipocytes derived from mesenchymal stem cells

BIOLOGY EXTENDED ESSAY

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Word Count: 3925

Abstract

The purpose of my extended essay is to investigate the effect of corticosteroids (CS) on the differentiation and growth of adipocytes from mesenchymal stem cells (MSCs).

My research question was "Do CSs have any impact on the differentiation and growth of adipocytes from MSCs?"

Six wells were used; Well 1-only MSC (control); Well 2-0.2 mg dexamethasone (DXM) with MSC; Well 3- 5 mg DXM with MSC; Well 4- Adipogenic differentiation solution (ADS) with MSC (ADS control); Well 5- 0.2 mg DXM with ADS and MSC; Well 6- 5 mg DXM with ADS and MSC. Adipogenic differentiation and growth were assessed by an inverted phase-contrast microscope by noting the appearance of fat cells and making cell counts at the 5^{th}, 10^{th} and 15^{th} days. At the 5^{th} day, small fat vacuoles that indicated adipogenic differentiation was noted only in Well 6. At the 10^{th} day, such differentiation was noted in Wells 3, 4, 5 as well as in Well 6 with the maximum cell count in Well 6 (Well 6 versus Wells 1, 2 and 3, $p<0.05$). The size of the lipid droplets in Well 6 increased when compared to Day 5 assessment. At the 15^{th} day, the growth and differentiation further increased in Wells 4, 5 and 6; Wells 1, 2 and 3 had significantly less cell counts when compared to Wells 4, 5 and 6 ($p<0.05$). Moreover, at Day 15, the sizes of the lipid droplets in Wells 4, 5, 6 and also Well 3 increased when compared to Day 10 assessment.

I conclude that DXM, in a dose-response manner, affect both the development (hyperplasia) and the size (hypertrophia) of adipocytes in the MSC model. These data contribute to our understanding of development of obesity as a side effect of CS treatment.

Word count: 293

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Abbreviations

ACS: Adipogenic culture solution

ADS: Adipogenic differentiation solution

AL: Acute leukemia

CS: Corticosteroid

DXM: Dexamethasone

MSC: Mesenchymal stem cell

Introduction

Acute leukemia (AL) is the most common cancer that is seen in childhood. Fever, bleeding and poor appetite are the most common complaints for presentation of such patients (1). My mother is a pediatric hematologist and I have visited her clinic since I was 3-year old. During my visits, I have met many children with AL and several became my friends. I noticed that sometimes they had weight gain with full-face appearance. I wondered how they gained such kilos in such short periods of time and learned from my mother that a drug, which is used in AL, might have such side effects that increases appetite and results in weight gain.

I went to the literature and learned that children treated for AL were not obese at the initial diagnosis. However, the first year of treatment is reported to be the period of most marked excess weight gain (2,3).

Two years ago in our biology class we studied the effects and side effects of various hormones. One of the hormones that we studied was adrenal cortex hormone, called cortisol. We learned that overproduction of cortisol might cause Cushing syndrome, featured by rapid weight gain, particularly of the trunk and face with sparing of the limbs (central obesity) (4). When I saw a picture of a patient with Cushing syndrome, it reminded me my mother's patients with AL with very similar facial appearances (Figure 1) (5). Therefore, I did a search and I discovered that the drug form of cortisol is widely used in several diseases including AL.

I learned that such treatment was crucial for the treatment of AL but had several side effects, including obesity (1,2).

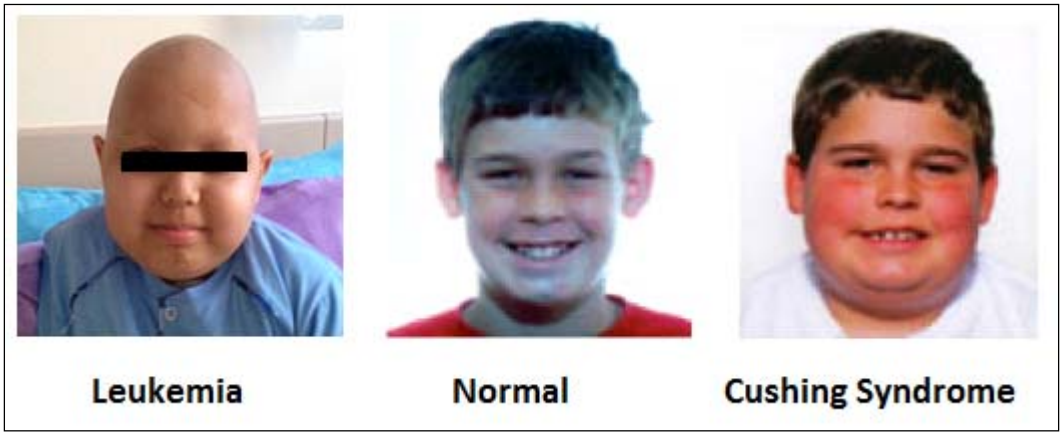

Figure 1. One of my mother's patients with AL who uses steroid therapy (left). Another patient before (middle) and after (right) development of Cushing syndrome (5).

Cortisol is a steroid hormone synthesized by the adrenal cortex and is essential for the body to respond stress. Cortisol levels in the normal range are mandatory to sustain life. However, long-term elevations of circulating cortisol levels are harmful to many organ systems (6).

Steroid medicines, called corticosteroids (CSs), are artificially produced and are similar to those of natural hormone-cortisol, in action. They inhibit inflammation and hence are frequently used in allergic and rheumatologic diseases. They are also commonly used for the treatment of cancer due to their tumor cell lytic effects secondary to DNA blockage (7). However they have several side effects such as disturbed immune system, delayed wound healing, osteoporosis, gastritis, mood abnormalities and weight gain (5,7). In addition to causing weight gain, CSs also tend to redistribute body fat to the face (moon face), back of the neck (buffalo hump), and abdomen (4,5).

Excessive fat deposition results in human obesity. Fat cells also referred to as adipocytes, store excess energy gained from food as fat. Adipocytes originate from fibroblast-like precursor cells under appropriate stimulatory conditions. The precursor cells lack any morphological or enzymatic marker that can be used to determine whether they will differentiate to adipocytes. Adipocytes are identified

by demonstration of intracellular lipid accumulation. The size of the adipose tissue mass is determined by both the number and size of the adipocytes. “Hyperplastic growth” refers to an increase in adipose tissue mass due to an increase in the number of adipocytes, whereas “hypertrophic growth” refers to an increase in adipose tissue mass due to an increase in the size of adipocytes (8). Increase in size occurs primarily by lipid accumulation within the cell. The developmental sequence of adipose tissue in humans has not been concisely defined. The baby is born relatively fat. Two periods of hyperplastic growth are during the third trimester (7-9 months) of pregnancy and just prior to and during adolescence. Once new adipocytes are formed, they remain throughout life and only a reduction in size of the cell is possible (8).

Since CS use is associated with weight gain, I wondered if CSs had hypertrophic and/or hyperplastic growth effect on fat cells. I also wondered if CSs had any effect on hyperplasia of fat cells, one might expect that such treated children prior or during puberty might be more prone to develop obesity in later life.

My research question is “Do CSs, at different dosages, have any impact on the differentiation and proliferation of adipocytes from mesenchymal stem cells (MSCs)?”

Hypothesis

Obesity is a worldwide health problem increasing cardiovascular morbidity and mortality (9); it also affects AL survivors. In patients with AL, the first year of treatment has been associated with the most marked excessive weight gain; at the end of treatment, 9-48% of treated children have been overweight. In addition to increased energy intake and reduced physical activity, CS use induce weight gain in such children (2,9).

The main goal of my project is to investigate the effects of CSs on the differentiation and growth of adipocytes from mesenchymal stem cells (MSC).

Research question: Does CS administration at different dosages have any effect on the differentiation and growth of adipocytes derived from MSCs?

Null Hypothesis: Corticosteroid administration, at different dosages, does not have any effect on the differentiation and growth of adipocytes derived from MSCs.

Method Development and Planning

In order to conduct my study, I started to search where and how I could get adipose tissue samples. Adipose tissue is either subcutaneous, found between the skin and muscles, or visceral around the organs in the main body cavities, primarily in the abdominal cavity (10). Since visceral fat may be provided during abdominal surgery, I wondered if I could have such a sample during surgery. However, my mother told me that visceral fat would not be an ideal tissue as a model, since the vast majority of such cells have already been differentiated and would not permit me to investigate the effect of CSs on the differentiation.

I further discussed this issue with my mother and she told me that fat cells could also differentiate from MSCs found in the yellow bone marrow. She also told me that MSC samples and research set-up were available in the Stem Cell Laboratory of Ankara Children's Hematology and Oncology Hospital. I thereafter decided to visit the facility to see the set-up. My mother introduced me the director of the stem cell laboratory, Meltem Özgüner, MD. Dr. Özgüner is a professor of histology and embryology; she also works in the Department of Histology and Embryology, Ankara Yıldırım Beyazıt University. Following discussion of what I thought to investigate, Dr. Özgüner was so kind to accept conductance of my research project and asked her biologist, Yasin Köksal, to assist me.

Dr. Özgüner detailed the properties of the stem cell as follows (taken from her presentation) (11):

"Stem cells are biological cells found in all organisms. A stem cell has two fundamental properties (Figure 2):

1 Self-renewal: The ability to go through numerous cycles of cell division while maintaining the undifferentiated state.

2 Potency: The capacity to differentiate into specialized cell types (multipotency)

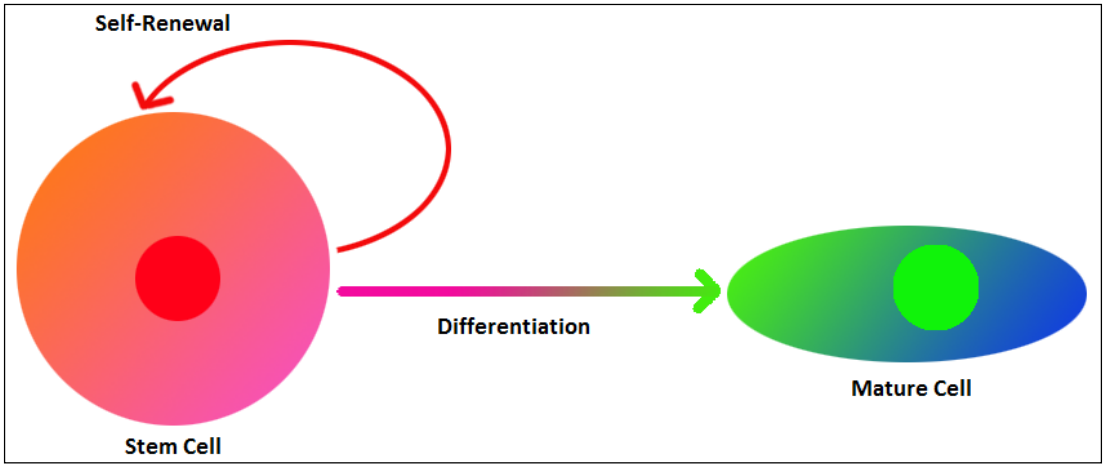

Figure 2: Properties of stem cell (12).

Mesenchymal stem cell is an example of multipotent stem cell; these stem cells may differentiate to a wide variety of mature cell types.

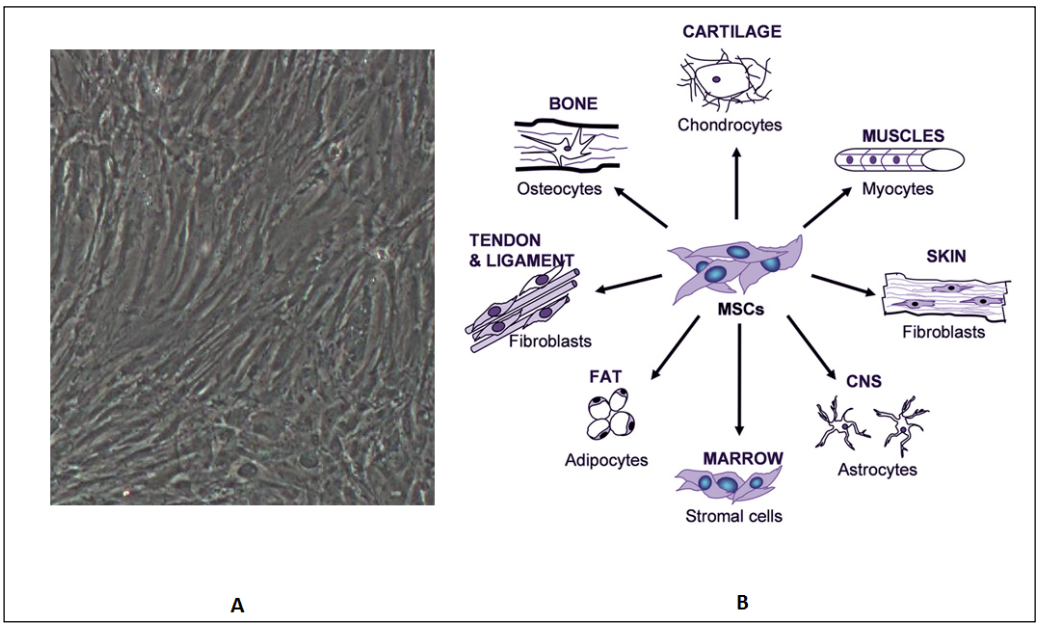

Figure 3. (A) Microscopic appearance of the MSC, **(B)** Differentiation of MSC into various cell lines (13).

Although I have studied bone and bone marrow in this term in my biology class, Dr. Özgüner also told me that:

"There are two types of bone marrow: red marrow, which consists mainly of hematopoietic tissue to produce blood cells, and yellow marrow, which is mainly made-up of fat cells. Hematopoietic stem cells differentiate to red blood cells, white blood cells and thrombocytes in red marrow. Yellow bone marrow, which is not directly involved in blood production, makes up the majority of bone marrow stroma. The bone marrow stroma contains MSCs (Figure 3A). These cells have been shown to differentiate, in vitro or in vivo, into precursors of bone cells called, osteoblasts, cartilage cells called chondrocytes, muscle cells called myocytes and fat cells called adipocytes (Figure 3B)."

I made a quick search about the characteristics of bone marrow adipocytes. The adipocyte is the most common stromal cell in human bone marrow. Adipocytes may have several functions, including occupying excess space in the bone marrow cavity, playing an active role in systemic lipid metabolism and providing a localized energy reservoir for the bone marrow (14). I made the assumption that differentiation of MSCs in bone marrow to adipocytes can be taken as a model to investigate the obesity promoting effects of CSs.

I was informed that in the stem cell laboratory, frozen MSC samples were stored for research. Following thawing and treating such cells with adipogenic culture solution (ACS), they would differentiate to fat cells in a 10 to 20-day period. Adipogenic culture solution has been developed for the adipogenic differentiation of MSCs in tissue culture vessels; it contains various reagents to induce MSCs to differentiate to adipocytes as well as stimulate their growth. By using inverted phase-contrast microscope, one can also observe and follow the differentiation of MSCs to adipocytes during which phases of fat deposition and effect of steroids in a single cell can be noted. Inverted microscopes are useful for observing living cells at the bottom of a dish e.g. tissue culture flask under natural conditions (15). In the laboratory Olympus CK X41 model inverted phase-contrast microscope has been used.

Having all these information, I decided to use MSC for the source of fat cells. I intended to use “dexamethasone” (DXM) which is a type of CS used for the treatment in AL. Dexamethasone has been available in the pharmacy as Dekort ampule (8 mg/2 mL, Deva). I was also informed that, in experimental research, in order to evaluate the impact of one treatment on outcome, it is essential to have a control group. Therefore, I took MSC without DXM as the control group (Well 1). In order to evaluate the dose-response effect, I planned to supplement DXM to the MSC culture in two different concentrations (0.2 mg-Well 2; 5 mg-Well 3). We decided to use 0.2 mg DXM because this dose has been successfully used for bone differentiation from MSC; hence, one may expect this dosing as the standard dose for my set-up and the 5 mg dose as the high dose. I also wondered if DXM had a synergistic effect with ADS on adipogenic differentiation. Hence, in the MSC culture, ADS without DXM formed Well 4; ADS with 0.2 mg DXM formed Well 5 and finally ADS with 5 mg DXM formed Well 6.

Mesenchymal stem cells are long, thin, spindle-shaped cells with large, round nucleus (Figure 3A). The techniques of isolation and culturing MSCs have been discussed in the Appendix 1. Adipocytes contain lipid droplets with flatted nucleus and located on the periphery. Intracellular lipid droplets are typically stained with Oil red O dye and identified by their bright red color (Figure 4).

I intended to make daily observations with inverted phase-contrast microscope. Once I noted the lipid droplets, which were the evidence of adipocytic differentiation, I took the images of all wells. I counted the fat cells, which had 2 or more droplets at the 5^{th}, 10^{th} and 15^{th} days.

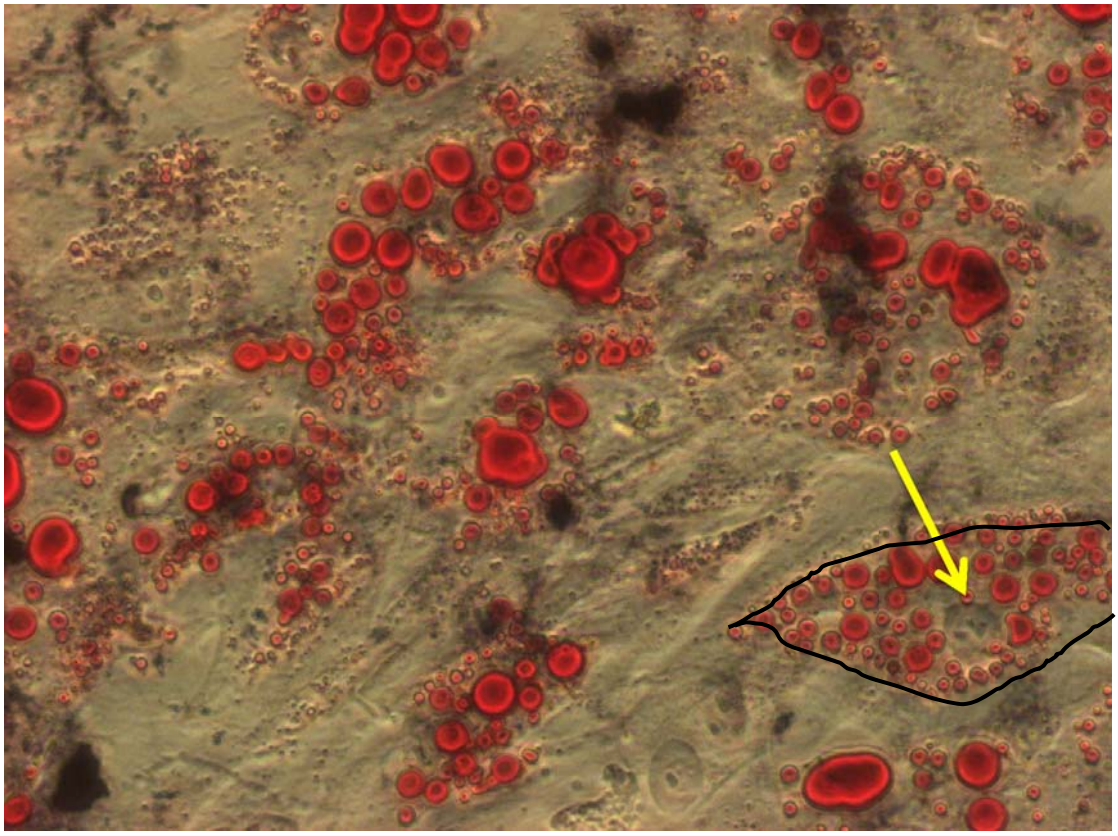

Figure 4: Adipocytes that have lipid droplets are recognized as “reddish-orange spheres” with Oil red O stain. Yellow arrow denotes the nucleus of the cell and black line shows the cytoplasmic border.

Material and methods

My study was held at the Ankara Children's Hematology and Oncology Hospital-Stem Cell Laboratory with the supervision of Professor Meltem Özgüner, MD. All equipment, including disposable material, was kindly provided by Ankara Children's Hematology and Oncology Hospital- Stem Cell Laboratory.

Material List:

- Dulbeco's Modified Eagle's Medium-LG
- Penicillin, streptomycin
- L-glutamine
- Fetal bovine serum
- Trypsin -EDTA
- 6 -well plate
- T75 culture flask
- Incubator
- Centrifuge
- Sterile gloves
- Mask
- Biosafety cabinet
- Pasteur pipette
- Mesencult Adipogenic Differentiation Supplement Human
- Inverted phase contrast microscope
- Cryopreserved at second passage mesenchymal stem cell
- 0.2 mg and 5 mg Dexamethasone ampule (8 mg/2 mL)

<table>
<tr>
<td> 
Incubator</td>
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Pasteur pipettes</td>
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<td> 
Solutions: Dulbeco’s Modified Eagle’s Medium-LG; L- glutamine; Penicillin, streptomycin; trypsin</td>
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Adipogenic differentiation supplement</td>
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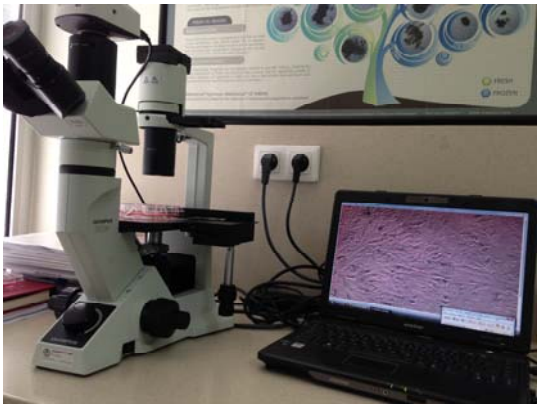
<td> 
Inverted phase-contrast microscope</td>
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Figure 4. Photos of some of the materials that I used in my experiment

Method

In my study, I used MSCs that had been isolated from human mononuclear cells and frozen at -196°C in liquid nitrogen. Isolation of the mononuclear cell, freezing and thawing procedures were described in the Appendix 1 and Appendix 2. Biologist Yasin Köksal performed all these procedures.

Preparation of MSC culture medium:

- 60% DMEM media-Low Glucose
- 30% MCDB medium
- 10% Fetal bovine serum
- 1% Penicillin, streptomycin

  All solutions were mixed and stored at +4 ^{o}C after filtration.

Preparation of cell culture for adipocytes

First, I put 2 mL of MSC culture medium and 50.000 MSCs in each of the wells of the 6-well plate. The preparation (Appendix 1) and counting was done by Biologist Yasin Köksal employing automated blood counting machine. Cell culture medium was changed on a daily basis and 70 % confluency was planned to be attained. In cell culture biology, confluency refers to the proportion of the surface covered by the cells; for example 50% confluency means roughly half of the surface is covered whereas 100% confluency means that the whole surface is covered (16).

After 4 days of culture, 70% confluency was noted and MSCs were ready to be used for my project.

Procedure:

Well 1: MSC with culture medium only (control)

Well 2: 0.2 mg DXM was added to MSC and culture medium

Well 3: 5 mg DXM was added to MSC and culture medium

Well 4: 2 mL ADS was added to MSC and culture medium (adipogenic control)

Well 5: 0.2 mg DXM and 2 mL ADS were added to MSC and culture medium

Well 6: 5 mg DXM and 2 mL ADS were added to MSC and culture medium

Figure 6. The 6-well plate used for culturing.

The 6-well plate was put in the incubator at 37°C with humidified 5% CO_2 in air. Culture medium was changed every other day; DXM and ADS were added to the respective wells while changing the medium. Cell differentiation and growth were noted under inverted phase-contrast microscopic examination every other day for 15 days.

Count of fat cells:

Images were taken by an Olympus X41 inverted phase contrast microscope by capturing 5 random fields in each well at days 5, 10 and 15. The images obtained from the microscope with x10 and x40 magnification were digitally processed. Cells noted to contain vacuoles were accepted to be adipocytes.

Oil Red O staining:

Oil Red O staining (Appendix 3) was used to verify adipogenic differentiation of MSCs at day 15. Cells containing a visibly stained vacuole were considered positive for staining (17).

Data Collection and Results-**Inverted phase-contrast microscopic findings:**

Well 1: In this control well, there was only MSC and culture medium. The structure of the cytoplasm and nucleus of MSCs were noted to be stable on days 5, 10 and 15. The cells had healthy appearance without any fat deposition throughout the study period (Figures 7, 8).

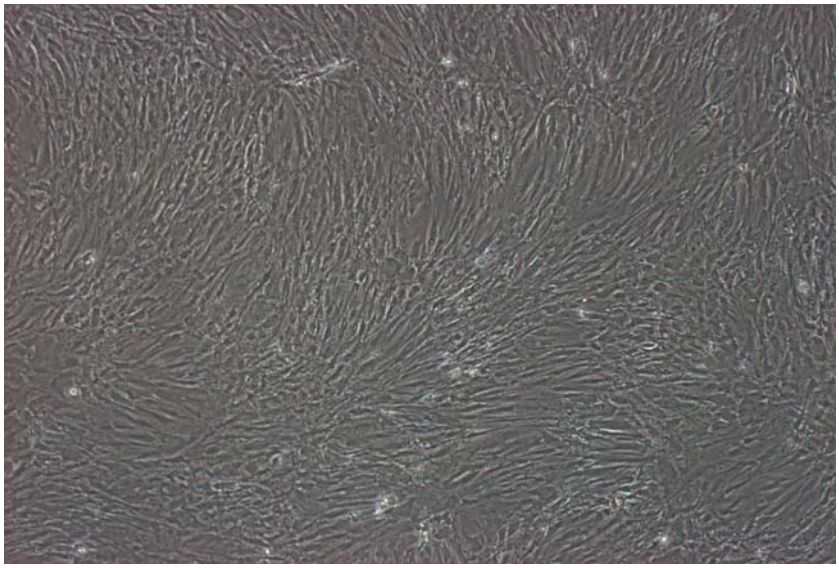


Figure 7: Well 1-Control group, Day 5 assessment: Proliferation capacity of MSCs, with typical fusiform appearance, is normal (without staining, x10 magnification).

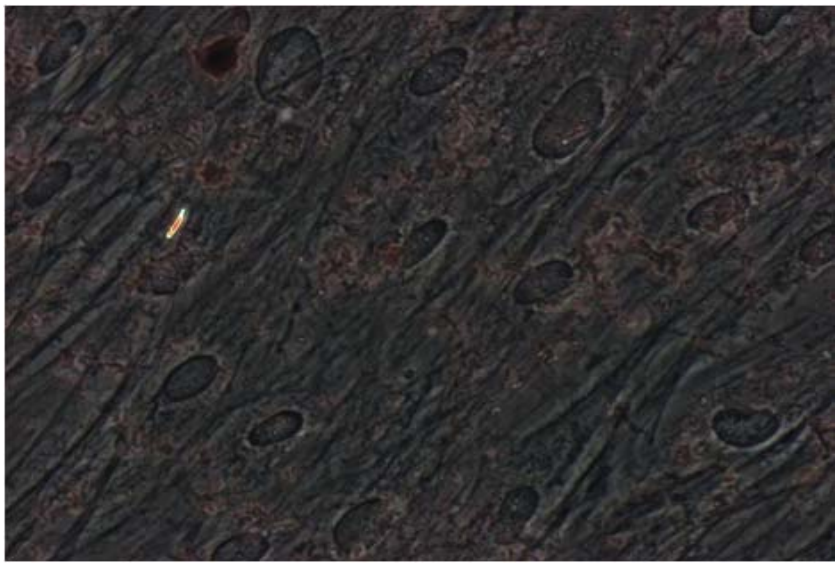


Figure 8: Well 1-Control group, Day 15 assessment: No fat deposition is observed (Oil red O staining, x 40 magnification)

Well 2: In this well, MSCs were cultured with 0.2mg DXM without ADS. At days 5 and 10, no fat cell was noted. At day 15, some lipid droplets were observed in a cell, which was not stained with, oil red O. I was told that this might have been due to the presence of one fat cell in that field (Figures 9, 10).

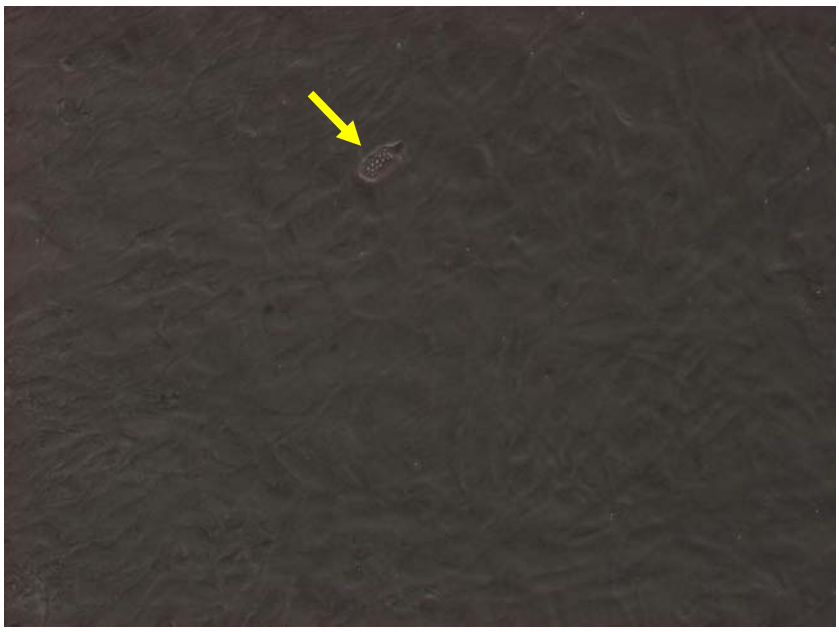


Figure 9: Well 2, Day 15 assessment: One fat cell (yellow arrow) was noted (without staining, x10 magnification)

Figure 10: Well 2, Day 15 assessment: No fat deposition (Oil red O staining, x 40 magnification)

Well 3: In this well, MSCs were cultured with 5 mg DXM without ADS. At day 5, differentiation to adipocytes was not noted. At day 10, some small droplets appeared and at day 15, both the number of cells that contained droplets and the size of the droplets increased (Figure 11, 12, 13).

Figure 11: Well 3-Day 10 assessment: Some fat cells (yellow arrows) are recognized (without staining, x 10 magnification)

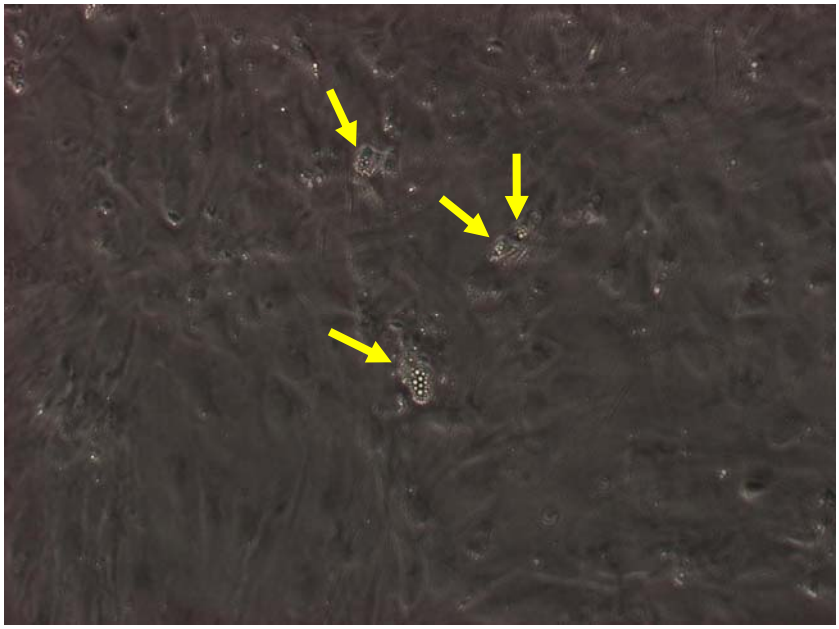


Figure 12: Well 3-Day 15 assessment: Both the number of cells that contain droplets and also the sizes of droplets have increased (without staining, x 10 magnification)

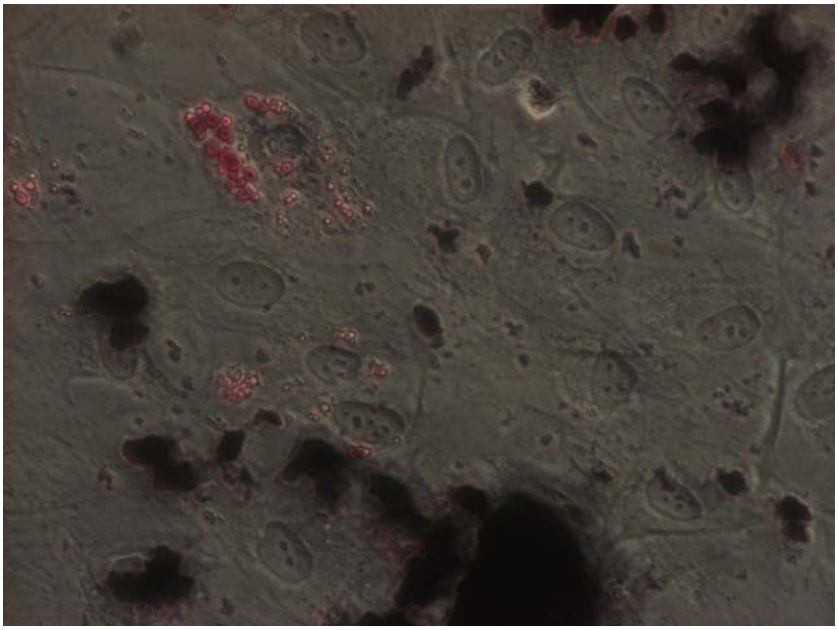


Figure 13: Well 3-Day 15 assessment: Reddish orange spheres indicate lipid droplets (Oil red O staining, x 40 magnification)

Well 4: In this well MSCs were cultured with 2 mL of ADS. No DXM was added. At day 5, several cells with small droplets were noted. At days 10 and 15, both the number and the size of the cells increased. Adipogenic differentiation capacity was detected with Oil Red O staining at day 15 (Figures 14, 15, 16, 17).

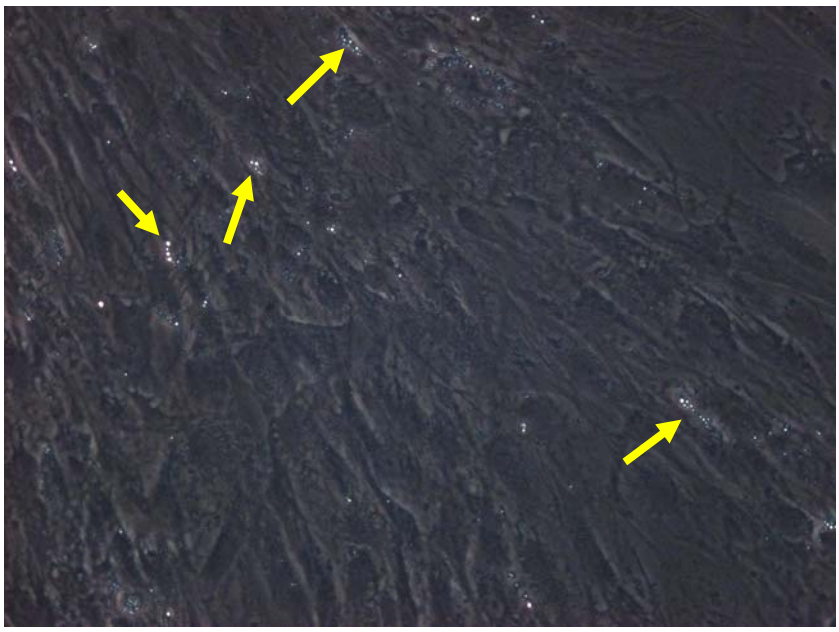


Figure 14: Well 4-Day 5 assessment: Fat cells with small fat droplets (yellow arrows) are noted (without staining, x 10 magnification)

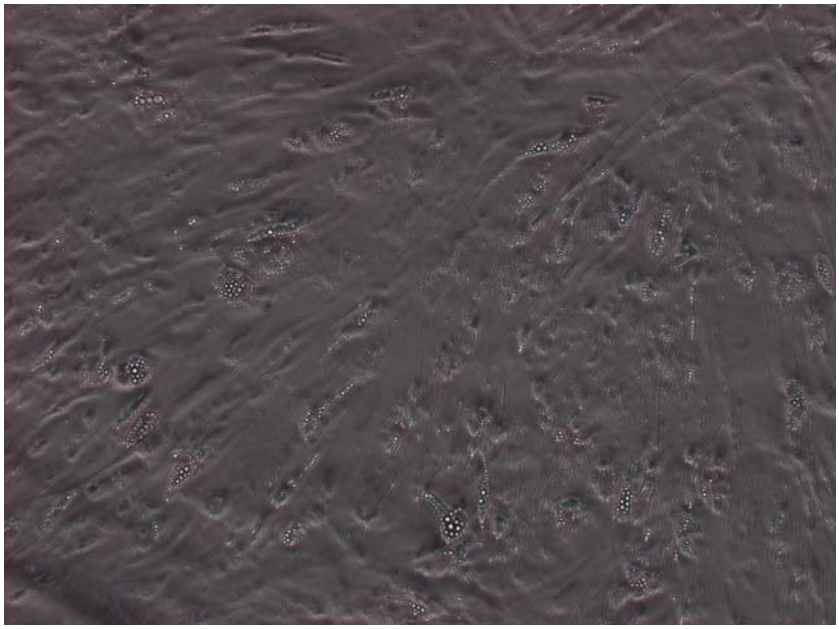


Figure 15: Well 4-Day 10 assessment: Several adipocytes with fat droplets (small white points) are recognized (without staining, x10 magnification)

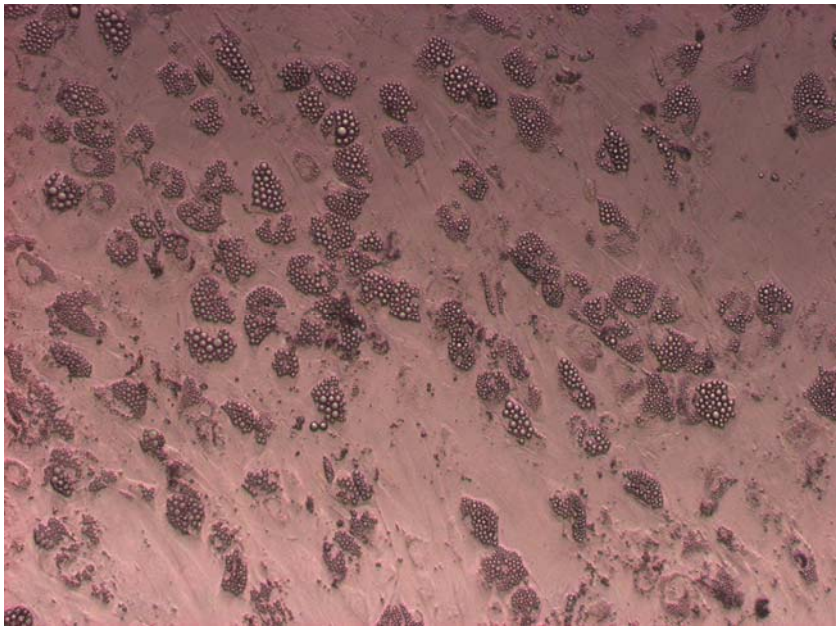


Figure 16: Well 4-Day 15 assessment: Many adipocytes are noted (without staining, x 10 magnification)

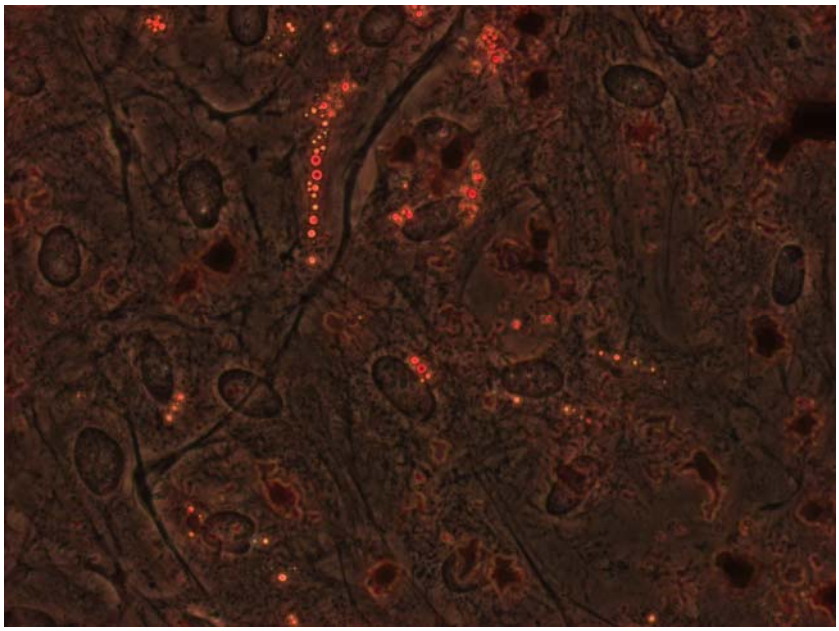


Figure 17: Well 4-Day 15 assessment: Reddish orange droplets indicate fat deposition (Oil red O staining, x40 magnification).

Well 5: In this well, MSCs were cultured with 2 mL ADS and 0.2 mg DXM. In this well, adipocyte differentiation was noted to commence at day 10. At day 15, the size of the vesicles as well as the number of adipocytes increased (Figures 18, 19, 20).

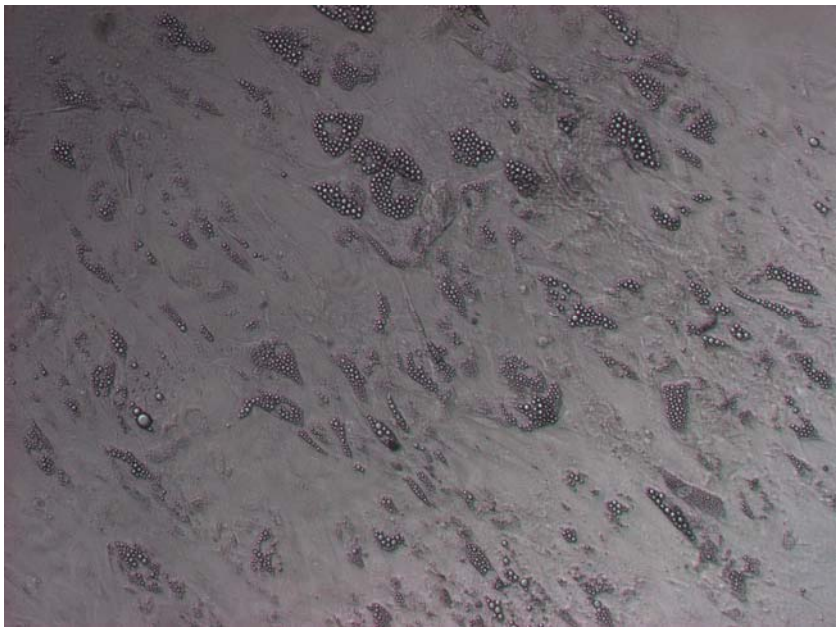


Figure 18: Well 5-Day 10 assessment: Several adipocytes are noted (without staining, x10 magnification).

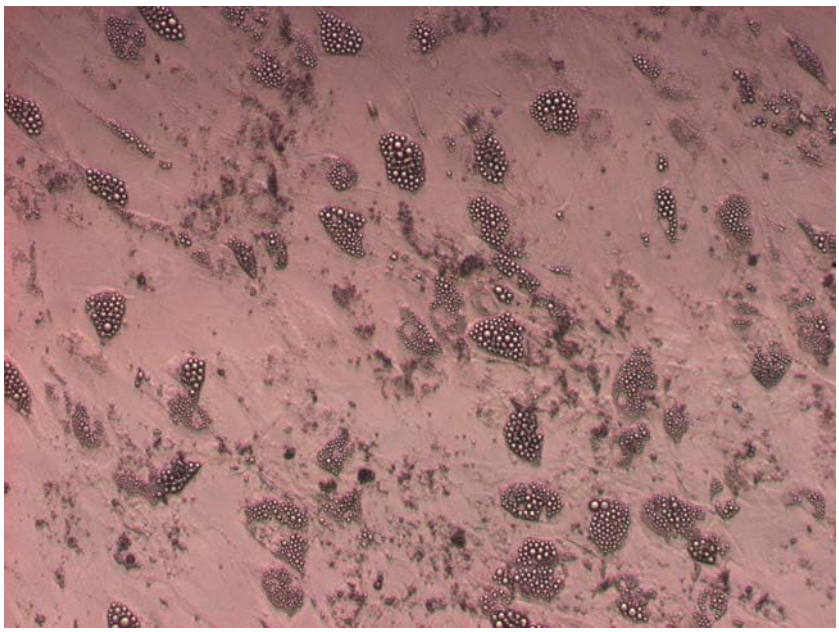


Figure 19: Well 5-Day 15 assessment: The number of adipocytes has further increased with relatively big droplets (without staining, x10 magnification).

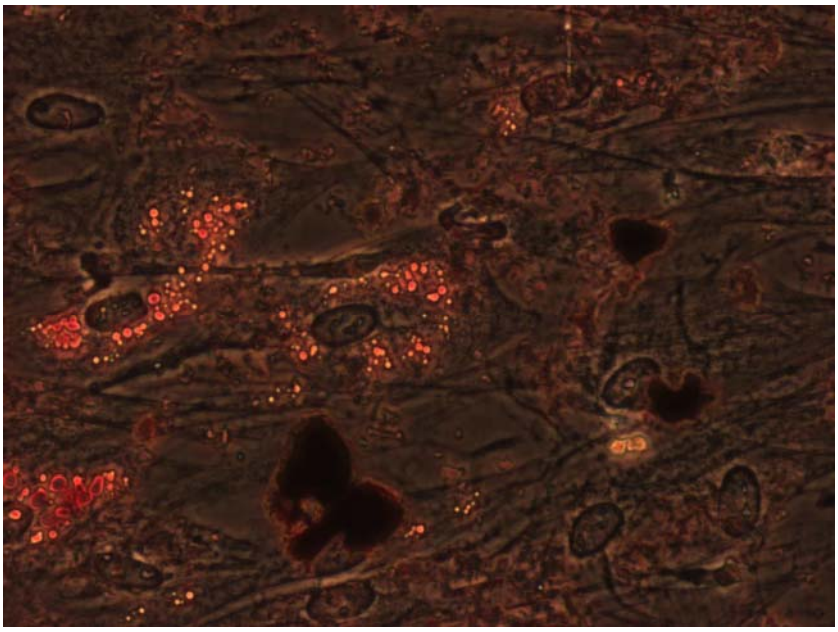


Figure 20: Well 5-Day 15 assessment: Adipocytes stained with Oil red O stain (x40 magnification).

Well 6: In this well, MSCs were cultured with 2 mL ADS and 5 mg DXM. At day 5, several adipocytes were noted and at day 10 both the number of adipocytes and size of the droplets increased. Numerous adipocytes with giant vacuoles were demonstrated at day 15 (Figures 21, 22, 23, 24).

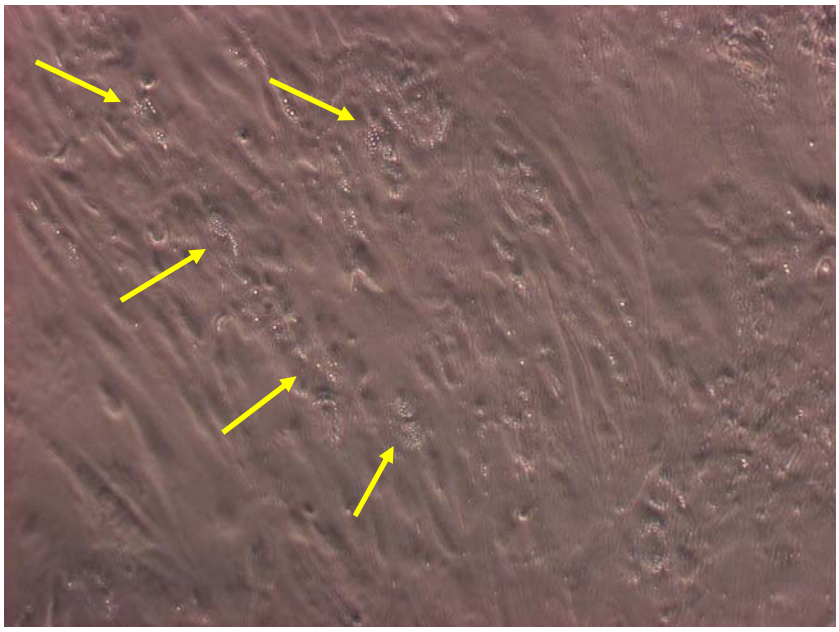


Figure 21: Well 6-Day 5 assessment: Several adipocytes with small fat droplets (yellow arrows) (without staining, x10 magnification).

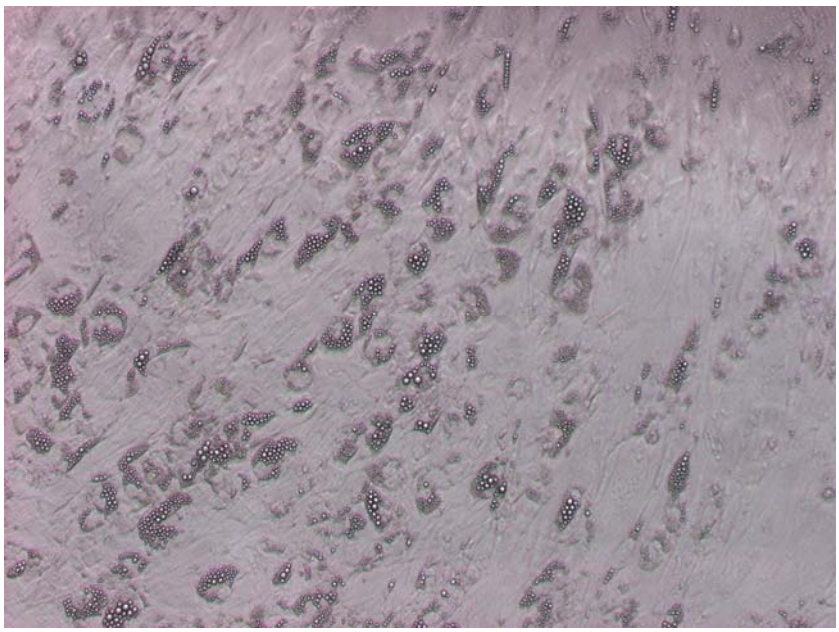


Figure 22: Well 6-Day 10 assessment: Both the number of adipocytes and the vacuole sizes has increased (without staining, x10 magnification).

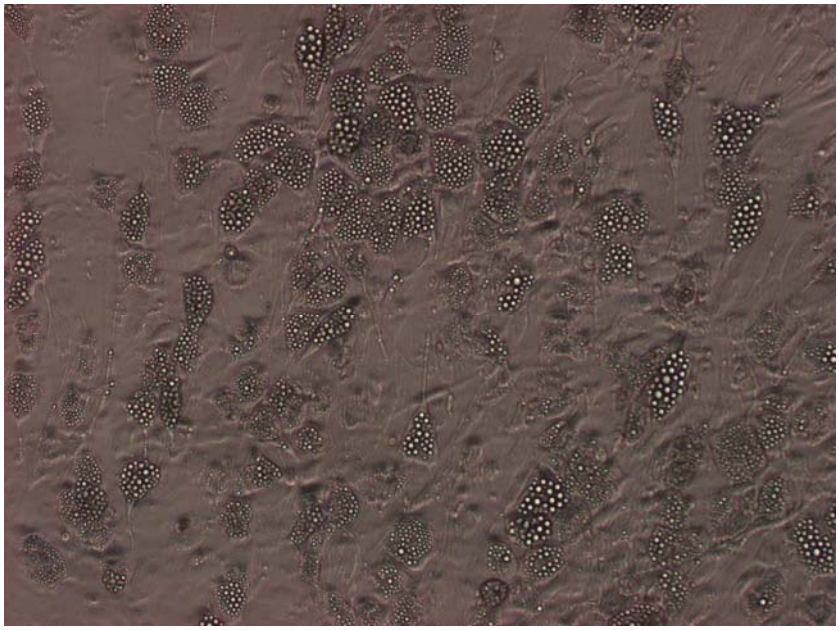


Figure 23: Well 6-Day 15 assessment: The number of adipocytes and the vacuole sizes has further increased (without staining, x10 magnification).

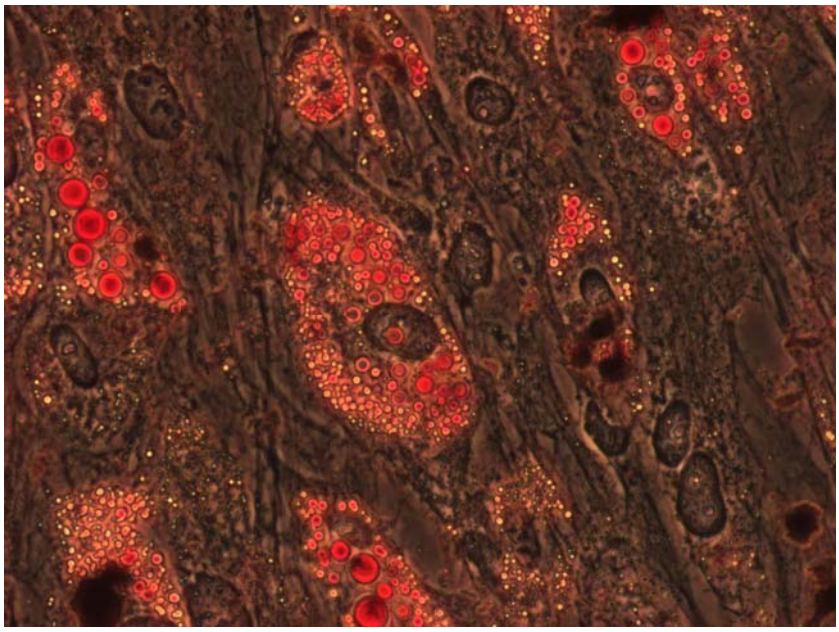


Figure 24: Well 6-Day 15 assessment: Adipocytes stained with Oil red O staining. (x40 magnification).

Fat cell count:

The cell counts of 5 random fields of the 6 wells at the 5^{th}, 10^{th} and 15^{th} days of observation were given in Table 1. The mean adipocyte counts of the 6 wells stratified by well number as well as day of observation were given in Graphs 1 and 2, respectively.

Wells	Trial (Field)	Cell Count		
		Day 5	Day 10	Day 15
1 (Control)	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
	5	0	0	0
2 (MSC+0.2 mg DXM)	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	1
	5	0	0	0
3 (MSC+5 mg DXM)	1	0	12	10
	2	0	1	18
	3	0	4	2
	4	0	8	8
	5	0	3	15
4 (MSC+ADS)	1	0	26	86
	2	1	16	90
	3	0	26	62
	4	0	8	78
	5	0	13	41
5 (MSC+ADS+0.2 mg DXM)	1	0	24	52
	2	0	11	48
	3	0	3	83
	4	0	50	66
	5	0	42	75
6 (MSC+ADS+5 mg DXM)	1	2	30	110
	2	4	42	72
	3	7	60	84
	4	15	34	97
	5	12	48	102

Table 1. The adipose cell counts of 5 random trials (fields) in 6 wells at Day 5, 10, and 15 assessments.

Statistical Analysis:

1. Data were expressed as mean and standard deviation (SD).

   The mean of a group $x_1, x_2, \cdots, x_n$, denoted by $\bar{x}$, was calculated by dividing the sum of the sampled values by the number of items in the group (18):

   $$\bar{x} = \frac{x_1 + x_2 + \cdots + x_n}{n}$$

   Standard Deviation was calculated using the formula given below (19):

   $$\sigma = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(x_i - \mu)^2}$$

2. Analysis of variance (ANOVA) was used for comparison of the mean cell counts of the 6 wells. When statistically significant difference was noted, Tamhane's T2 test was used for post-hoc analysis to delineate, which 2 groups differed significantly (20).

3. Student t-test was used to compare the mean cell counts within each well at days 10 and 15 (within well comparison).

4. Statistical significance (p) was set at 0.05.

5. All statistical analyses were done using Statistical Package for the Social Sciences (SPSS) 22.0 under the supervision of Prof. Dr. Özgüner.

Descriptives[a]

value

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	5	,0000	,00000	,00000	,0000	,0000	,00	,00
MSC+0,2 DXM	5	,0000	,00000	,00000	,0000	,0000	,00	,00
MSC+5 DXM	5	,0000	,00000	,00000	,0000	,0000	,00	,00
MSC+ADS	5	,2000	,44721	,20000	-,3553	,7553	,00	1,00
MSC+ADS+0.2 mg DXM	5	,0000	,00000	,00000	,0000	,0000	,00	,00
MSC+ADS+5 mg DXM	5	8,0000	5,43139	2,42899	1,2560	14,7440	2,00	15,00
Total	30	1,3667	3,63397	,66347	,0097	2,7236	,00	15,00

a. day = Day 5

Table 2. The descriptive of the cell counts of each well at the 5th day of observation assessed by SPSS.

Multiple Comparisons[a]

Dependent Variable: value

Tamhane

(I) grup	(J) grup	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	MSC+0,2 DXM	,00000	,00000	.	,0000	,0000
	MSC+5 DXM	,00000	,00000	.	,0000	,0000
	MSC+ADS	-,20000	,20000	,999	-1,4428	1,0428
	MSC+ADS+0.2 mg DXM	,00000	,00000	.	,0000	,0000
	MSC+ADS+5 mg DXM	-8,00000	2,42899	,368	-23,0932	7,0932
MSC+0,2 DXM	Control	,00000	,00000	.	,0000	,0000
	MSC+5 DXM	,00000	,00000	.	,0000	,0000
	MSC+ADS	-,20000	,20000	,999	-1,4428	1,0428
	MSC+ADS+0.2 mg DXM	,00000	,00000	.	,0000	,0000
	MSC+ADS+5 mg DXM	-8,00000	2,42899	,368	-23,0932	7,0932
MSC+5 DXM	Control	,00000	,00000	.	,0000	,0000
	MSC+0,2 DXM	,00000	,00000	.	,0000	,0000
	MSC+ADS	-,20000	,20000	,999	-1,4428	1,0428
	MSC+ADS+0.2 mg DXM	,00000	,00000	.	,0000	,0000
	MSC+ADS+5 mg DXM	-8,00000	2,42899	,368	-23,0932	7,0932
MSC+ADS	Control	,20000	,20000	,999	-1,0428	1,4428
	MSC+0,2 DXM	,20000	,20000	,999	-1,0428	1,4428
	MSC+5 DXM	,20000	,20000	,999	-1,0428	1,4428
	MSC+ADS+0.2 mg DXM	,20000	,20000	,999	-1,0428	1,4428
	MSC+ADS+5 mg DXM	-7,80000	2,43721	,389	-22,7634	7,1634
MSC+ADS+0.2 mg DXM	Control	,00000	,00000	.	,0000	,0000
	MSC+0,2 DXM	,00000	,00000	.	,0000	,0000
	MSC+5 DXM	,00000	,00000	.	,0000	,0000
	MSC+ADS	-,20000	,20000	,999	-1,4428	1,0428
	MSC+ADS+5 mg DXM	-8,00000	2,42899	,368	-23,0932	7,0932
MSC+ADS+5 mg DXM	Control	8,00000	2,42899	,368	-7,0932	23,0932
	MSC+0,2 DXM	8,00000	2,42899	,368	-7,0932	23,0932
	MSC+5 DXM	8,00000	2,42899	,368	-7,0932	23,0932
	MSC+ADS	7,80000	2,43721	,389	-7,1634	22,7634
	MSC+ADS+0.2 mg DXM	8,00000	2,42899	,368	-7,0932	23,0932

a. day = Day 5

Table 3. Multiple comparisons using ANOVA (SPSS) of the mean cell counts of 6 wells at the 5th day of observation.

Descriptives[a]

value

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	5	,0000	,00000	,00000	,0000	,0000	,00	,00
MSC+0,2 DXM	5	,0000	,00000	,00000	,0000	,0000	,00	,00
MSC+5 DXM	5	5,6000	4,39318	1,96469	,1452	11,0548	1,00	12,00
MSC+ADS	5	17,8000	8,01249	3,58329	7,8512	27,7488	8,00	26,00
MSC+ADS+0.2 mg DXM	5	26,0000	19,93740	8,91628	1,2444	50,7556	3,00	50,00
MSC+ADS+5 mg DXM	5	42,8000	11,88276	5,31413	28,0456	57,5544	30,00	60,00
Total	30	15,3667	18,27469	3,33649	8,5428	22,1905	,00	60,00

a. day = Day 10

Table 4. The descriptive of the cell counts of each well at the 10th day of observation assessed by SPSS.

Multiple Comparisons[a]

Dependent Variable: value

Tamhane

(I) grup	(J) grup	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	MSC+0,2 DXM	,00000	,00000	.	,0000	,0000
	MSC+5 DXM	-5,60000	1,96469	,510	-17,8082	6,6082
	MSC+ADS	-17,80000	3,58329	,109	-40,0658	4,4658
	MSC+ADS+0.2 mg DXM	-26,00000	8,91628	,486	-81,4039	29,4039
	MSC+ADS+5 mg DXM	-42,80000*	5,31413	,019	-75,8209	-9,7791
MSC+0,2 DXM	Control	,00000	,00000	.	,0000	,0000
	MSC+5 DXM	-5,60000	1,96469	,510	-17,8082	6,6082
	MSC+ADS	-17,80000	3,58329	,109	-40,0658	4,4658
	MSC+ADS+0.2 mg DXM	-26,00000	8,91628	,486	-81,4039	29,4039
	MSC+ADS+5 mg DXM	-42,80000*	5,31413	,019	-75,8209	-9,7791
MSC+5 DXM	Control	5,60000	1,96469	,510	-6,6082	17,8082
	MSC+0,2 DXM	5,60000	1,96469	,510	-6,6082	17,8082
	MSC+ADS	-12,20000	4,08656	,300	-30,9691	6,5691
	MSC+ADS+0.2 mg DXM	-20,40000	9,13017	,728	-72,8530	32,0530
	MSC+ADS+5 mg DXM	-37,20000*	5,66569	,017	-66,4801	-7,9199
MSC+ADS	Control	17,80000	3,58329	,109	-4,4658	40,0658
	MSC+0,2 DXM	17,80000	3,58329	,109	-4,4658	40,0658
	MSC+5 DXM	12,20000	4,08656	,300	-6,5691	30,9691
	MSC+ADS+0.2 mg DXM	-8,20000	9,60937	1,000	-56,7162	40,3162
	MSC+ADS+5 mg DXM	-25,00000	6,40937	,085	-52,7644	2,7644
MSC+ADS+0.2 mg DXM	Control	26,00000	8,91628	,486	-29,4039	81,4039
	MSC+0,2 DXM	26,00000	8,91628	,486	-29,4039	81,4039
	MSC+5 DXM	20,40000	9,13017	,728	-32,0530	72,8530
	MSC+ADS	8,20000	9,60937	1,000	-40,3162	56,7162
	MSC+ADS+5 mg DXM	-16,80000	10,37979	,917	-63,2995	29,6995
MSC+ADS+5 mg DXM	Control	42,80000*	5,31413	,019	9,7791	75,8209
	MSC+0,2 DXM	42,80000*	5,31413	,019	9,7791	75,8209
	MSC+5 DXM	37,20000*	5,66569	,017	7,9199	66,4801
	MSC+ADS	25,00000	6,40937	,085	-2,7644	52,7644
	MSC+ADS+0.2 mg DXM	16,80000	10,37979	,917	-29,6995	63,2995

*. The mean difference is significant at the .05 level.

a. day = Day 10

Table 5. Multiple comparisons using ANOVA (SPSS) of the mean cell counts of 6 wells at the 10th day of observation.

Descriptives[a]

value

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	5	,0000	,00000	,00000	,0000	,0000	,00	,00
MSC+0,2 DXM	5	,2000	,44721	,20000	-,3553	,7553	,00	1,00
MSC+5 DXM	5	10,6000	6,22896	2,78568	2,8657	18,3343	2,00	18,00
MSC+ADS	5	71,4000	20,09478	8,98666	46,4490	96,3510	41,00	90,00
MSC+ADS+0.2 mg DXM	5	64,8000	14,85598	6,64379	46,3539	83,2461	48,00	83,00
MSC+ADS+5 mg DXM	5	93,0000	15,06652	6,73795	74,2924	111,7076	72,00	110,00
Total	30	40,0000	39,76396	7,25987	25,1519	54,8481	,00	110,00

a. day = Day 15

Table 6. The descriptive of the cell counts of each well at the 15th day of observation assessed by SPSS.

Multiple Comparisons[a]

Dependent Variable: value

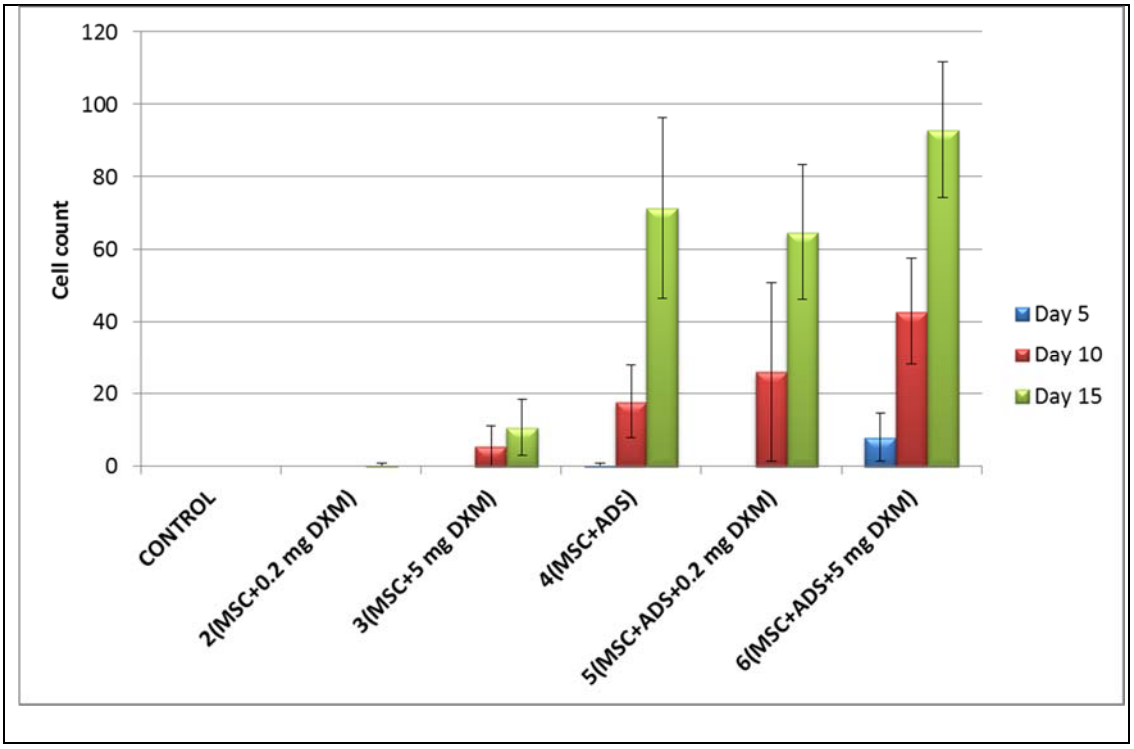
Tamhane

(I) grup	(J) grup	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	MSC+0,2 DXM	-,20000	,20000	,999	-1,4428	1,0428
	MSC+5 DXM	-10,60000	2,78568	,250	-27,9096	6,7096
	MSC+ADS	-71,40000*	8,98666	,020	-127,2412	-15,5588
	MSC+ADS+0.2 mg DXM	-64,80000*	6,64379	,009	-106,0831	-23,5169
	MSC+ADS+5 mg DXM	-93,00000*	6,73795	,002	-134,8682	-51,1318
MSC+0,2 DXM	Control	,20000	,20000	,999	-1,0428	1,4428
	MSC+5 DXM	-10,40000	2,79285	,262	-27,5958	6,7958
	MSC+ADS	-71,20000*	8,98888	,020	-127,0053	-15,3947
	MSC+ADS+0.2 mg DXM	-64,60000*	6,64680	,009	-105,8347	-23,3653
	MSC+ADS+5 mg DXM	-92,80000*	6,74092	,002	-134,6204	-50,9796
MSC+5 DXM	Control	10,60000	2,78568	,250	-6,7096	27,9096
	MSC+0,2 DXM	10,40000	2,79285	,262	-6,7958	27,5958
	MSC+ADS	-60,80000*	9,40851	,024	-111,6003	-9,9997
	MSC+ADS+0.2 mg DXM	-54,20000*	7,20417	,007	-90,1253	-18,2747
	MSC+ADS+5 mg DXM	-82,40000*	7,29109	,001	-118,9097	-45,8903
MSC+ADS	Control	71,40000*	8,98666	,020	15,5588	127,2412
	MSC+0,2 DXM	71,20000*	8,98888	,020	15,3947	127,0053
	MSC+5 DXM	60,80000*	9,40851	,024	9,9997	111,6003
	MSC+ADS+0.2 mg DXM	6,60000	11,17587	1,000	-40,8033	54,0033
	MSC+ADS+5 mg DXM	-21,60000	11,23210	,771	-69,1067	25,9067
MSC+ADS+0.2 mg DXM	Control	64,80000*	6,64379	,009	23,5169	106,0831
	MSC+0,2 DXM	64,60000*	6,64680	,009	23,3653	105,8347
	MSC+5 DXM	54,20000*	7,20417	,007	18,2747	90,1253
	MSC+ADS	-6,60000	11,17587	1,000	-54,0033	40,8033
	MSC+ADS+5 mg DXM	-28,20000	9,46256	,234	-67,0475	10,6475
MSC+ADS+5 mg DXM	Control	93,00000*	6,73795	,002	51,1318	134,8682
	MSC+0,2 DXM	92,80000*	6,74092	,002	50,9796	134,6204
	MSC+5 DXM	82,40000*	7,29109	,001	45,8903	118,9097
	MSC+ADS	21,60000	11,23210	,771	-25,9067	69,1067
	MSC+ADS+0.2 mg DXM	28,20000	9,46256	,234	-10,6475	67,0475

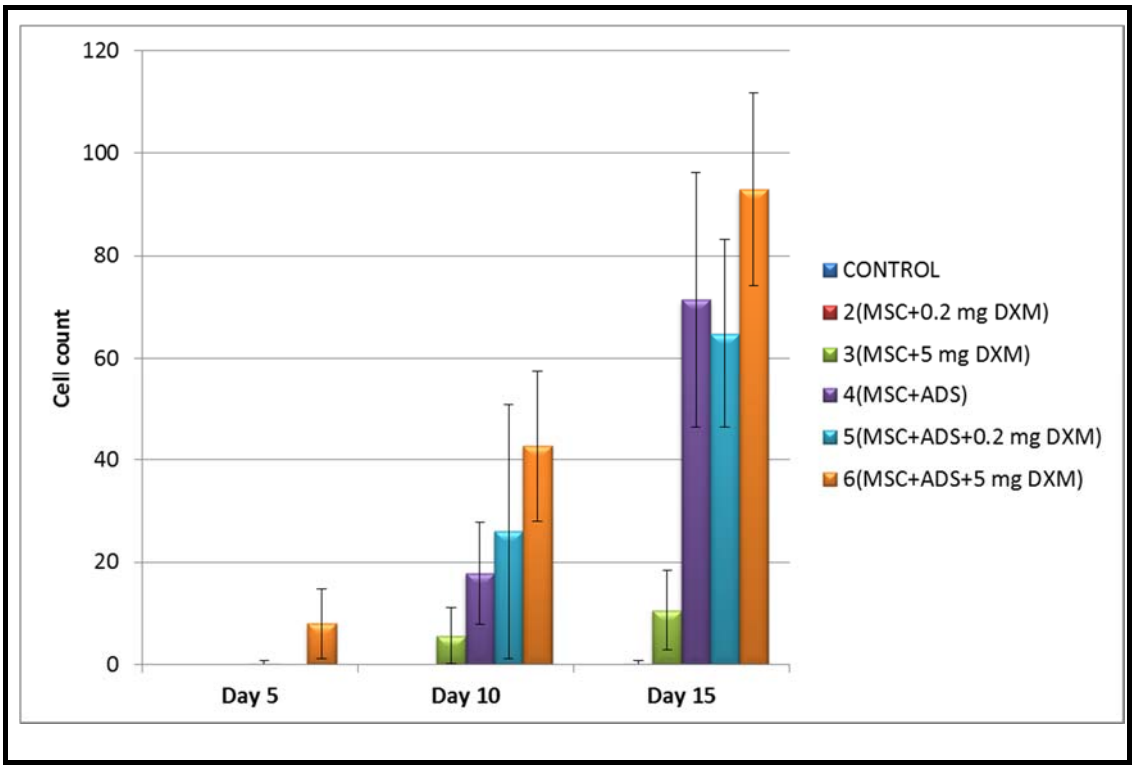
*. The mean difference is significant at the .05 level.

a. day = Day 15

Table 7. Multiple comparisons using ANOVA (SPSS) of the mean cell counts of 6 wells at the 15th day of observation.



Graph 1. The mean and standard error of adipocyte counts of the 6 wells at days 5, 10, and 15 of in-vitro culturing stratified by wells.



Graph 2. The mean and standard error of adipocyte counts of 6 wells stratified by day (Days 5, 10, and 15) of in-vitro culturing.

Conclusion and Evaluation

Obesity is currently a common worldwide problem. Unhealthy dietary habits and sedentary life style are the main contributing factors. Imbalance of various hormones including leptin, thyroid hormone, insulin, sex hormones, and growth hormone may also contribute to obesity by influencing appetite, metabolism, and body fat distribution. Moreover, excessive cortisol production in Cushing syndrome may result in obesity (21).

Obesity is commonly encountered in children who survive treatment of AL. Side effects of chemotherapeutic agents/radiotherapy employed for the treatment of AL along with disturbances in lifestyle and genetic factors contribute to obesity in these patients. Nevertheless, the precise cause of obesity in patients with AL is yet to be established (2,3,9).

Of the drugs used to treat AL, CSs are the only one with their known effect on weight gain. Corticosteroids can promote increased adiposity via a range of possible mechanisms including *i)* effects on appetite/regulation of energy intake and *ii)* alterations in substrate oxidation and energy expenditure (9).

In this experiment, my goal was to investigate the direct adipogenic effect of CSs on precursor cells. To test my hypothesis, I used MSC as the source of adipocyte and DXM as the drug form of cortisol. I assessed the differentiation and growth of adipocytes by using an inverted phase contrast microscope.

Mesenchymal stem cells were treated with 0.2 mg DXM (Well 2); 5 mg DXM (Well 3); 0.2 mg DXM and ADS (Well 5); 5mg DXM and ADS (Well 6). Mesenchymal stem cells cultured with medium only served as the control group (Well 1). Mesenchymal stem cells cultured with ADS but without DXM formed Well 4 and served as the adipogenic control.

Fat cells were assessed morphologically on a daily basis and images of cells were acquired at the 5^{th}, 10^{th} and 15^{th} days and counted. At day 15, all wells were stained with Oil red O stain for confirmation of adipogenic differentiation.

Figures 7-24 show serial images of adipocyte differentiation of MSCs and expansion of lipid vesicles. Adipocyte counts at the 5^{th}, 10^{th} and 15^{th} days of cultures are given in Table 1 and Graphs 1-2. Adipocytes containing lipids were noted at Day 5 in only Well 6, suggesting that only high dose DXM together with ADS can differentiate MSCs to fat cells in the early phase. Performing ANOVA, no statistical difference in cell counts was noted between the 6 wells at the 5^{th} day of observation.

At day 10, differentiation to adipocytes was noted in Wells 3, 4, 5 in addition to Well 6. The size of the lipid droplets (vacuoles) in Well 6 increased when compared to Day 5 assessment (Figure 22). When statistical analysis was performed using ANOVA, only the mean count of Well 6 was statistically higher than Wells 1, 2 and 3 ($p<0.05$). Although there appeared to be a trend in increase in cell counts among Wells 4, 5 and 6 (Graph 2), it did not reach statistical significance probably due to limited sample size.

At day 15, Wells 1, 2 and 3, all three wells without ADS, had significantly less cell counts when compared to Wells 4, 5 and 6, all three groups with ADS ($p<0.05$). There appeared to be no statistically significant difference in cell counts among Wells 4, 5 and 6. Moreover, the sizes of the lipid droplets (vacuoles) in Wells 4, 5, 6 as well as in Well 3 increased when compared to Day 10 assessment.

Based on my data, I conclude that the control well (Well 1) is adequate, since no adipocyte differentiation at all has been observed during the whole observation period. Similar to the control well, the low-dose DXM (0.2 mg) alone (Well 2) is not sufficient to induce adipocyte differentiation, excluding the only differentiated cell observed at Day 15 (Figure 9). The low-dose DXM with ADS (Well 5) induced adipocyte differentiation at Days 10 and 15; however, the lack of significant difference in cell count among the ADS with 0.2 mg DXM well (Well 5) and ADS-only well (Well 4) at days 10 and 15, concludes that this low-dose of DXM, in my model, does not further stimulate the cell count on top of ADS. Although subjectively assessed, the sizes of the lipid droplets (vacuoles) in Well 5 were larger than those of Well 4 both on days 10 and 15. Since the size of the lipid droplet is a marker of

cell growth (hypertrophy), these data may indicate that 0.2 mg DXM lacks hyperplastic but may have limited hypertrophic effect.

Five mg DXM with-ADS (Well 6) was associated with the highest mean cell count at days 10 and 15. Furthermore, there was an accelerated differentiation at day 5, only noted in Well 6 since one normally would not expect differentiation earlier than day 10. Although there was a trend for higher cell counts in Well 6 compared to Well 4, both on days 10 and 15, it did not reach statistical significance possibly due to limited sample size. These data conclude that 5 mg DXM further promotes the stimulatory effect of ADS on the differentiation and growth of adipocytes Furthermore, 5 mg DXM *alone* stimulated adipocyte differentiation, albeit to a lesser extent compared to with ADS, on days 10 and 15 (Figures 11-13).

I conclude that CS exposure alone, at high doses (5 mg in my study), induces adipocyte differentiation and growth in the MSC model. This stimulatory effect is dose- and duration-dependent. These findings may explain the occurrence of obesity as a side effect of CS treatment in survivors of AL.

One of the drawbacks of my study is limited sample size. Only one 6-well plate has been used in the current study. Increasing the number of 6-well plates will improve the power. With increased power, statistical comparisons would be less prone to error. Since staining with Oil red O inevitably results in termination of the culturing, it was only used at Day 15 in the current study. It would have been more fruitful to make staining with Oil red O also at Days 5 and 10 necessitating more 6-well plates to be used. Another drawback is subjective assessment of adipocyte cell count. There might be intra- and inter-personal variation for counting. Automated approach for counting, referred to as automated digital microscopic cell counter, would avoid such errors. Subjective assessment of the hypertrophic growth of cells by accumulation of intracellular lipid droplets (vacuoles) is another limitation of my study. Further studies with different types of CSs with different doses are warranted to delineate the impact of CSs on the differentiation and growth of adipocytes.

Word Count: 3922

Appendices

Appendix 1: Mononuclear cell isolation from bone marrow and culture of human bone marrow derived MSCs

Mononuclear cells were isolated from bone marrow employing density gradient centrifugation method by using Ficoll (Density: 1.077 g/mL) (Biochrom, Germany) at Ankara Children's Hematology and Oncology Hospital, Stem Cell Laboratory. Mononuclear cells were plated on 75cm^2 flasks at least 20x10^6 \ cell with 10 mL growth medium [DMEM-LG (Biochrom, Germany), %10 fetal bovine serum (FBS) (Biochrom, Germany), %1 antibiotics (Penicillin-Streptomycin, Biochrom, Germany)] and were incubated at 37^{o}C and %5 CO_2 (Galaxy 170R, Eppendorf Company, Germany).

After 72 hours, non-adherent cells were removed. The culture medium was changed every three days. When 70%-80% adherent cells were confluent, they were trypsinized (0.05% tyripsin at 37^0 C for 5 minutes, Biochrom, Germany) to detach the unbinded MSCs from the flask.

Appendix 2. Freezing and Thawing Culture Cells

Freezing: The cell suspension was diluted with freezing media (70% DMEM-LG, 20% Fatal bovine serum, 20% Dimethylsulphoxydite) and the suspension was put into sterile freezing vials. The cells were frozen overnight at -80^{o}C and thereafter transferred to the liquid nitrogen tank (-196^{o}C).

Thawing: The frozen cells were removed from the nitrogen tank and transferred to a 37C° incubator or 37C° water tank to thaw. The thawing process takes 1-2 minutes.

Appendix 3: Oil red O staining

The MSCs that were differentiated to adipocytes in the wells were washed twice with phosphate buffered saline at pH 7.4 then were fixed with 10% formaldehyde for 1 h at room temperature. After the cells were washed with 60% isopropanol, they were stained with oil red O (6 parts of 0.5% oil red O dye in 100% isopropanol

to 4 parts of water) for 10 min. After washing with distilled water, three times, the dye was fixed in the cells.

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