PHENOTYPIC DIFFERENCES OF MALE AND FEMALE MICE ADIPOSE-DERIVED STEM CELLS (MASCS) UNDER AN ACUTE STRESS CONDITION

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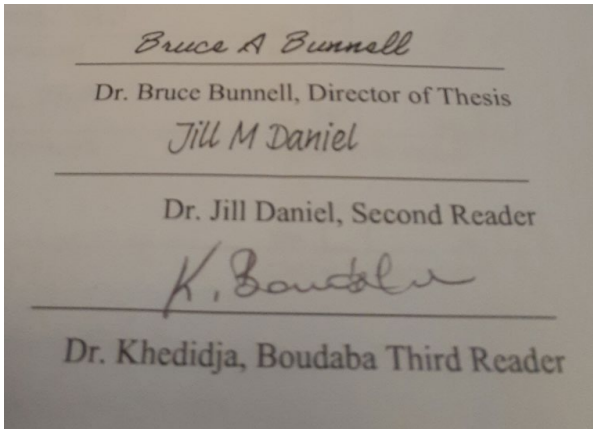

Abstract

Adipose-derived stem cells (ASCs) are adult stem cells that have increasingly been the subject of studies aimed at therapeutic regeneration due to their ability to differentiate into mesenchymal-lineage cells, undergo self-renewal, and modulate inflammatory states. Previous work has determined that there are sex differences between adipose-derived stem cells in regards to pro- and anti-inflammatory cytokines released during differentiation and under stress conditions, potentially because of differences in gene expression. However, there is still a need for a variety of data on the differences between male and female adipose-derived stem cells, especially under a common activator, lipopolysaccharide (LPS).

ASCs derived from male and female adult mice (mASCs) will be treated with acute doses of LPS, alongside their controls. Growth factor and anti-inflammatory cytokine expression will be measured and compared, along with differentiation potential and colony formation. It is hypothesized that there will be a significant difference between male and female mASCs in regard to differentiation ability, growth, and expression of cytokines. This study found significant differences in male and female mASCs' ability to differentiate and remain healthy and viable under an acute inflammatory stimulus.

Acknowledgement

My sincerest thanks to my First Reader Dr. Bruce Bunnell, the Director of the Tulane Center for Stem Cell Research and Regenerative Medicine, whose lab I had the honor of working and learning in. I am very grateful for the opportunity to learn from him and the graduate students in the lab. Specifically, I would like to thank Brianne Sullivan for her patience and hard-work. She was an enormous part of my ability to finish this work.

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Introduction

Adipose-derived stem cells (ASCs) have been characterized by their ability to undergo osteogenic and adipogenic differentiation, making them an attractive option for regenerative therapies. It is well-known that exposing ASCs to a stressful or inflammatory environment is necessary to instigate the most robust therapeutic effect. For example, treating human ASCs with LPS was shown to enhance liver regeneration in mice treated with the hASCs [1]. Therefore, it is important to outline the potential sex dimorphism that exists in ASCs when subject to a common activator, LPS.

Studies on the use of mouse ASCs to combat LPS-induced injuries, and of mASCs reactions to direct LPS treatment, show changes in inflammatory cytokine expression. mASCs used to treat LPS exposure showed decreased levels of pro-inflammatory cytokines, and increased levels of anti-inflammatory cytokines [2]. Human ASCs treated with LPS showed up-regulation of interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNFα), both pro-inflammatory cytokines [1].

mASC expression of cytokines has also been shown to be significantly altered after undergoing osteogenic or adipogenic differentiation, with levels varying according to the type of differentiation and stage [3]. The differences in gene expression of male and female mASCs before and after differentiation is an important part of the outline needed to fully understand the potential effects of sex-dimorphism.

Few studies have explored the sex-dimorphism of mASCs in reaction to LPS. While a sex difference in the ability of ASCs to differentiate and self-renew has been noted [4], and changes in gene expression following differentiation have also been shown, more studies are needed that specifically explore the separate reactions of male

and female mASCs, before and after differentiation. This study demonstrated a significant sex difference in metabolic activity of mASCs, and a significant difference in the differentiation ability of mASCs into adipose cells and osteoblasts.

Materials and Methods

Animals

The inguinal white adipose tissue cells used in this study were obtained from female or male mice (C571b1/6 strain; Jackson Laboratory) at age 8-12 weeks by first washing with phosphate buffered saline (PBS), finely mincing, and digesting in PBS for 1 hour at 37℃ with a solution of 0.1% (w/v) collagenase type I solution and 1.0% (w/v) bovine serum albumin (Sigma-Aldrich). Neutralization of the digested reaction was achieved with a combination solution of complete culture media (CCM) with Alpha Modified Eagle Medium (Thermo, Fisher Scientific), 20% HyClone Characterized Fetal Bovine Serum (FBS) (GE HealthCare Sciences), 100 units per milliliter antimyotic/antibiotic (Thermo Fisher Scientific). For culture, the digested tissue was filtered and resuspended in CCM.

All animal procedures were in compliance and approved by the Institutional Animal Care and Use Committee at Tulane University.

Cell Culture

mASCs were grown to 80% confluency before being treated with LPS. The acute stress condition consisted of 100 ng LPS per ml of media applied to cells for 24 hours. The cells were then washed with PBS and given fresh media. The LPS for the stress

condition was combined with complete culture media (CCM), which consisted of alpha-MEM, 20% heat-inactivated hyclone FBS, and 1% antibiotic/antimycotic solution.

Flow Cytometry

Male and female ASCs for the stress condition and the control were each plated on 15 cm^2 plates in 20 ml of media, with approximately 300,000 cells. At 80% confluency, ASCs were disassociated with trypsin and resuspended at 300,000 cells per 100 µl of PBS. ASCs were stained with 1 µl of each fluorescence-labeled antibody, positive markers stem cell antigen-1 (SCA1) and CD44, and negative markers CD45, CD11b, and CD31. An unstained control tube of 300,000 ASCs was included as a control.

Colony-Forming Units Assay

Colony-forming units (CFUs) were done by plating each condition of ASCs in 3 10 cm^2 plates in 10 ml of media, with approximately 500 cells per plate. They were incubated for 21 days, and media was changed every 3 days. After 21 days, the cells' media was aspirated off and they were washed 3 times with PBS. The 3 plates for each condition were each stained using 3% Crystal Violet for 30 minutes at room temperature. Plates were then washed twice with PBS and once with tap water to remove residual stain. Colonies were counted that were at least 2 mm. Colonies larger than 2 mm were also counted, to allow the calculation of percent CFU's.

Alamar Blue Assay

The rate of proliferation of treated ASCs was determined using Alamar blue. Male and female ASCs for the acute and control conditions were plated in 32 wells per 96-well plates in 100 µl of CCM, with approximately 1,000 cells per well. 24 hours after

seeding, and every other day for 15 days after, four wells of each experimental condition were stained with Alamar blue. Media was aspirated off, and each well received 100 µl of fresh CCM with 10% Alamar blue. As a blank control, four additional wells received 100 µl of fresh CCM with 10% Alamar blue. Each plate was incubated for 4 hours at 37℃ in the light-protected cell culture incubator. The plate was then read with a plate reader at ex545/em590, and the relative fluorescent units of each well were recorded.

Population-Doubling Time

In order to determine population-doubling time, ASCs from each condition were plated in 4 6-well plates, with approximately 20,000 cells per well, for a total of 21 wells per condition. Each day that the ASCs were counted was conducted in triplicate. Beginning 24 hours after seeding and continuing at the same time every day for 7 days after, each plate had the media removed and was washed with PBS. 300 µl of Trypsin was applied, the cells were incubated for 5 minutes, and then 100 µl of PBS was added. 20 µl of the cell suspension was removed and mixed with trypan blue. The ASCs were counted with a hemocytometer, and recorded.

Population doubling time was calculated using the following equation:

$$PDT = (CT \times \ln 2)/ (\ln(Nf/Ni))$$

Where DT is doubling time, Ct is the culture time in hours, Nf is the final cell number, and Ni is the initial cell number.

RNA Isolation, cDNA Synthesis, Quantitative RT-PCR Analysis

A quantitative reverse transcription PCR (RT-qPCR) was performed to evaluate gene expression of TNFaip6, TGF-β, TNF-α, IL-6, Nanog, Sox2, and Oct4. Forward and reverse primer sequences for the primers used can be found in Table 1. ASCs were plated

on 10 cm^2 plates, with approximately 70,000 cells per plate. Media was changed every 3 to 4 days. Cells were collected for each condition at 24 hours, 48 hours, 72 hours, and 7 days. When collecting, each plate was removed of media and washed twice with PBS. RLT buffer with 1% BME was added to the plates, and cells were lifted using a plate scrapper. Total RNA was extracted from each experimental condition using the Qiagen RNeasy Mini Kit. The RNA was then used to make cDNA with reverse transcripts and random primers, with a cDNA synthesis kit (Invitrogen). Quantitative real-time PCR was run using SYBR Green, with the primers listed in Table 1. β-actin was used as a house-keeping gene.

Table 1

qRT-PCR primer sequences for self-renewal, pro-inflammatory and anti-inflammatory cytokines.

Gene	Forward (5' --> 3')	Reverse (5' --> 3')
β-actin	GTGGGCCGCCCTAGGCACCA	TTAGCACGCACTGTAGTTTCTC
TGF-β	AGCTGCGCTTGCAGAGATTA	AGCCCTGTATTCCGTCTCCT
TNFaip6	GGGATTCAAGAACGGGATCTTT	TCAAATTCACATACGGCCTTGG
TNF-α	ATGGCCTCCCTCTCATCAGTTC	TTGGTGGTTTGCTACGACGTG
IL-6	CCCCAATTTCCAATGCTCTCC	CGCACTAGGTTTGCCGAGTA
Nanog	TCTTCCTGGTCCCCACAGTTT	GCAAGAATAGTTCTCGGGATGAA
Sox2	TAAGTACACGCTTCCCGGAG	AGCCGTTCATGTAGGTCTGC
Oct4	CACCATCTGTCGCTTCGAGG	AGGGTCTCCGATTTGCATATCT

Differentiation Protocols

ASCs were treated with specific media cocktails to induce either osteogenic or adipogenic differentiation, and stained for calcium production and neutral lipid droplets, respectively.

For adipogenic differentiation, ASCs were plated in 6-well plates with approximately 100,000 cells per well for 21 days. Each condition had three wells. After 21 days, the ASCs were treated every three days with alternating adipogenic differentiation media (ADM) and adipogenic maintenance media (AMM). The mASCs of the experimental condition were exposed to 100 ng/ml LPS for the first 24 hours of differentiation. After 21 days, the cells were washed with PBS and fixed with 4% paraformaldehyde (PFA). They were stained with 0.5% Oil-Red-O, made with 0.25 grams of Oil-Red-O and 50 ml of 100% Isopropanol. Using a belly dancer, cells were stained for 30 minutes. The cells were washed first with PBS and then several times with DI water, until there was no residual stain. Pictures were taken using a Nikon Eclipse TE200 (Melville, NY) with a Nikon Digital Camera DXM1200F using the Nikon ACT-1 software version 2.7, at 4x and 10x, to obtain visualization of neutral lipid droplets. The differentiations were destained with 100% isopropanol and read with a plate reader at ex545/em590. For each experimental condition, one plate remained untreated with the differentiation medium before being stained, to determine the total differentiation of the cells.

For osteogenic differentiation, the cells were plated in 6-well plates with approximately 100,000 cells per well for 21 days. Each condition had three wells. The

ASCs were treated with osteogenic differentiation media (ODM), which was changed every 3 days for 21 days. The mASCs of the experimental condition were exposed to 100 ng/ml LPS for the first 24 hours of differentiation. After 21 days, the cells were washed with PBS and fixed with 4% paraformaldehyde (PFA). They were stained with alizarin red, made with 0.5 grams alizarin red and 50 ml of DI water. Using a belly dancer, cells were stained for 30 minutes. The cells were washed first with PBS and then several times with DI water, until there was no residual stain. Pictures were taken using a Nikon Eclipse TE200 (Melville, NY) with a Nikon Digital Camera DXM1200F using the Nikon ACT-1 software version 2.7, at 4x and 10x, to obtain visualization of calcium deposition. The differentiations were destained with 10% Cetylpyridinium Chloride Monohydrate (CPC) and read with a plate reader at ex545/em590. For each experimental condition, one plate remained untreated with the differentiation medium before being stained, to determine the total differentiation of the cells.

Statistics

All values in the data and graphs are presented as means ± SEM. The statistical differences between groups in all data sets were determined using 2-way ANOVA or a two-tailed t-test with a confidence interval at 95%. Statistical significance was set at $p < 0.05$, and all statistics were calculated using Graphpad software. Graphs were generated on Graphpad.

Results

Flow Cytometry

To confirm that the ASCs used in the experiment were a homogenous population without immune and endothelial markers, flow cytometric analysis determined the continued presence of mesenchymal stem cell markers CD44 and SCA1, and the continued negative presence of immune cells markers CD45, CD11b, and CD31 (Fig.1). The male and female acute and control conditions were compared, and statistics determined no significance between any of the conditions within each antibody group.

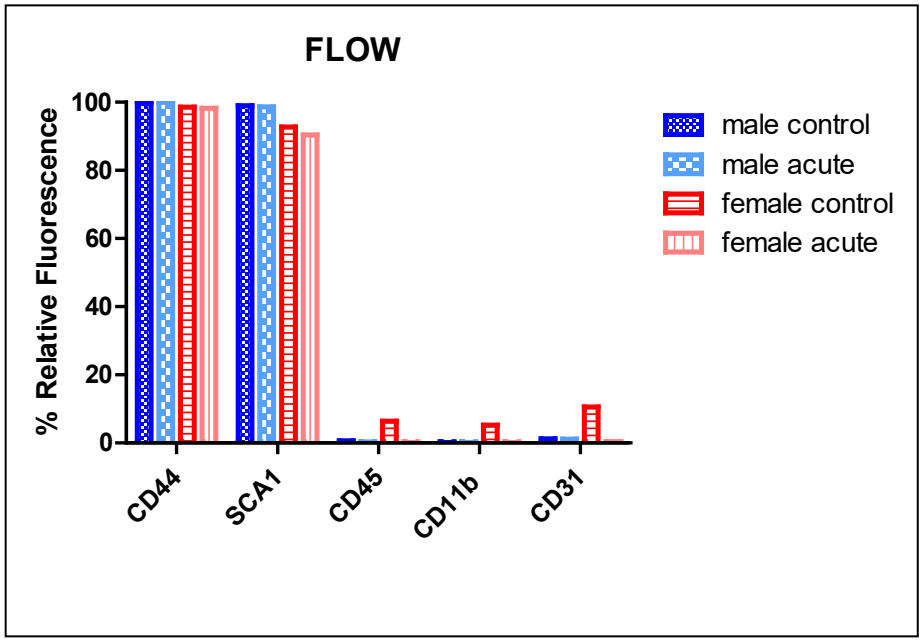


Figure 1: Male and female ASCs that had received 100 ng/ml LPS for 24 hrs. showed no significant difference in the % relative fluorescence of antibodies labeling known stemness and immune cell markers. A 2-way ANOVA and two-tailed t-test showed no significance in any of the conditions

Colony-Forming Units Assay

Male and female ASCs from the control condition, and the acute condition of 100 ng/ml LPS for 24 hours, were incubated for 21 days before staining with 3% Crystal

Violet. After being washed with PBS, the 3 plates for each condition were counted for every colony greater than or equal to 2 mm, and then for each colony exceeding 2 mm (Fig. 2).

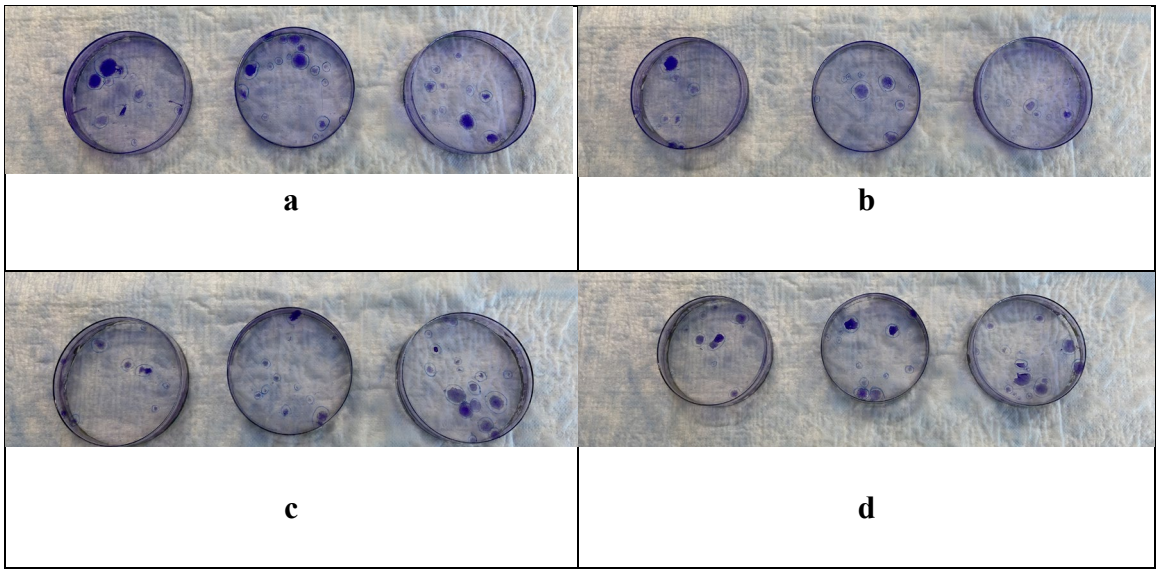


Figure 2: Male and female ASCs either treated with or without 100 ng/ml LPS for 24 hrs. were allowed to form colonies for 21 days, and then stained with 3% Crystal Violet. (a) Female acute ASCs (b) Female control ASCs (c) Male acute ASCs (d) Male control ASCs

The number of colonies for each condition showed no significant difference (Fig. 3A). The percent CFU, calculated by dividing the number of colonies exceeding 2 mm by the total colony count and multiplying by 100, also showed no significant difference between any of the conditions (Fig. 3(b)).

The lack of a significant difference in colony-formation is potentially the result of a lack of replicates. A small biological sample was used, and the counting relied on gross visible trends.

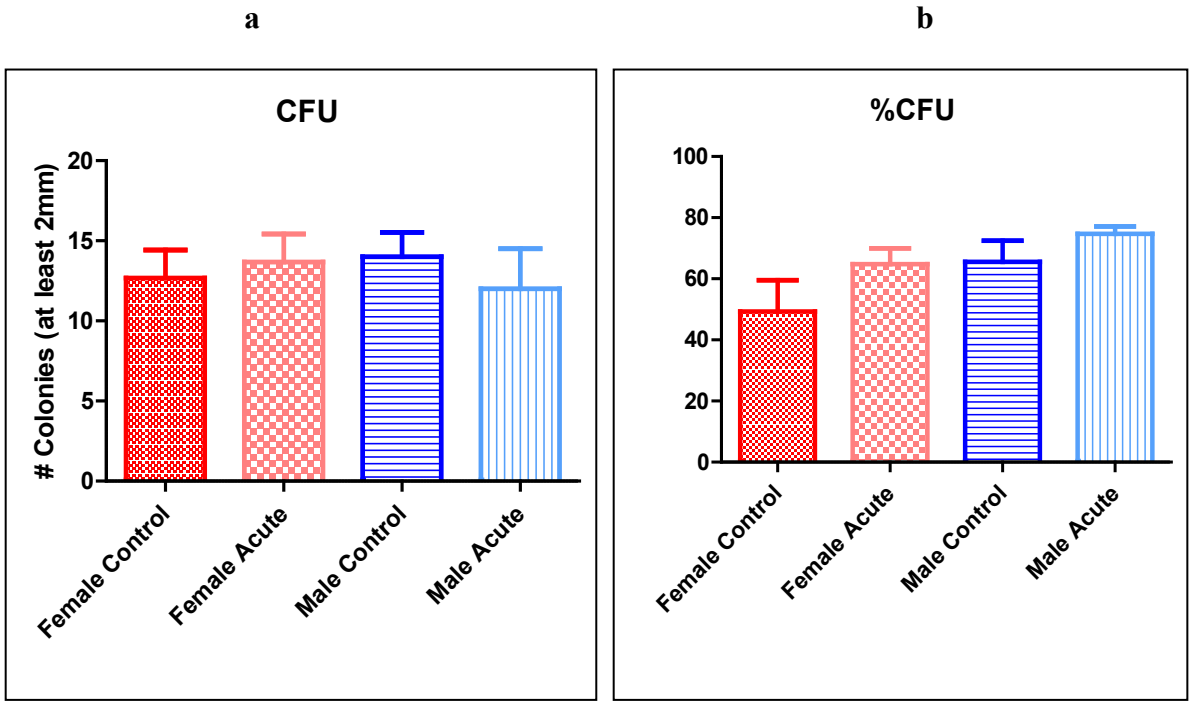


Figure 3: (a) Colonies from the male and female acute and untreated conditions were counted for colonies of 2 mm or greater. (b) % CFU was calculated using the total number of colonies and the number of colonies measuring larger than 2 mm. A 2-way ANOVA and two-tailed t-test showed no significance in any of the conditions

Proliferation Assays

Male and female ASCs from each condition were stained with Alamar blue every other day for 15 days, to determine proliferation. The relative fluorescent units were averaged for each day and condition after being measured at ex545/em590 (Fig. 4). Alamar blue was chosen as an indicator of ASC proliferation because of its use as an

indicator of cell health and viability. Male and female ASCs from both acute and control conditions were compared to each other.

Statistical analysis showed a significant difference between male acute and both female conditions (Fig. 4(a), $p < 0.001$), and a significant difference between male control and both female conditions (Fig. 4(b), $p < 0.001$), on days 11 and 13.

Although there appears to be a lack of reaction to the LPS in the health of the ASCs, the difference between male and female cells remains regardless of the treatment, indicating that factors connected to the sex of the cells may have a significantly larger impact on viability than the stress factor.

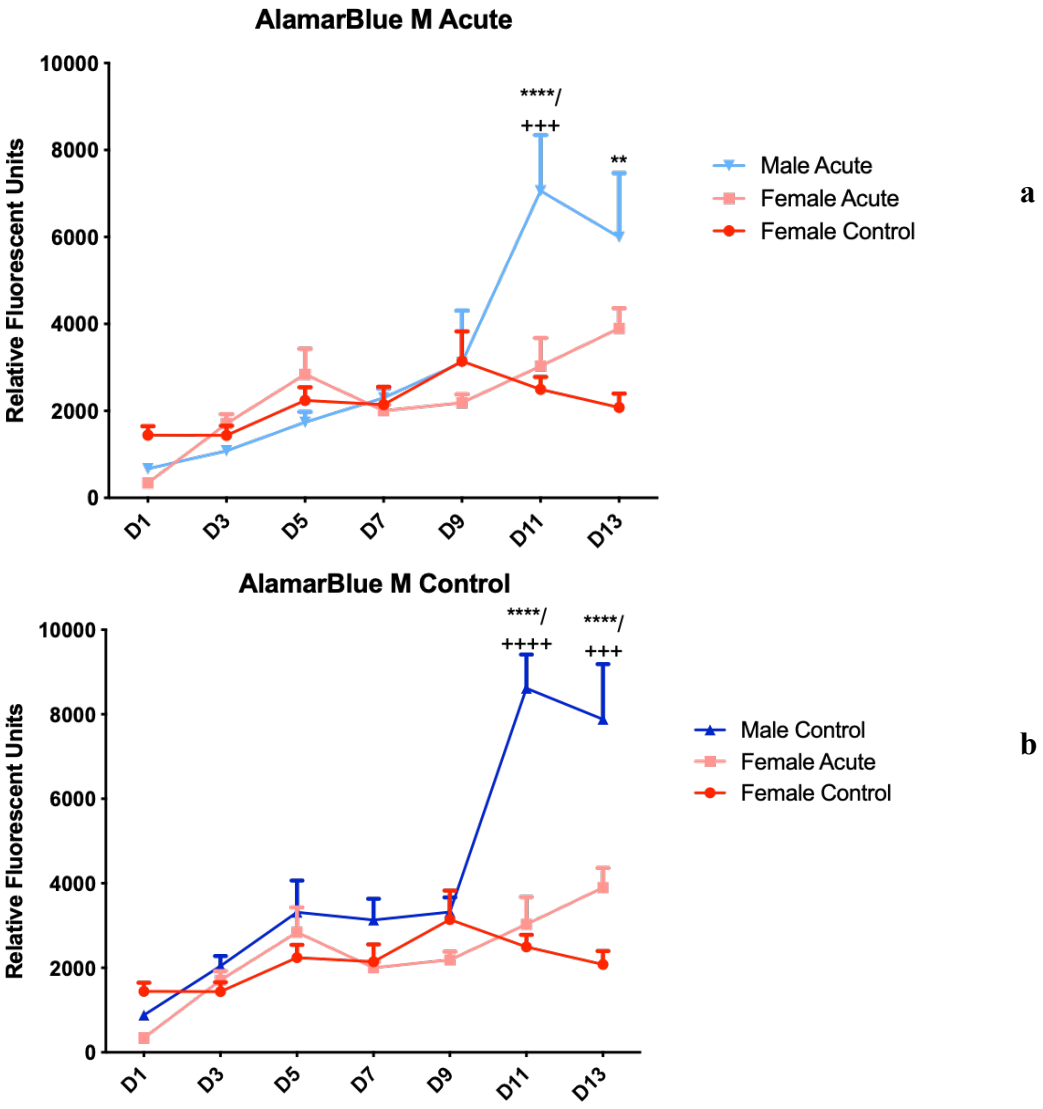


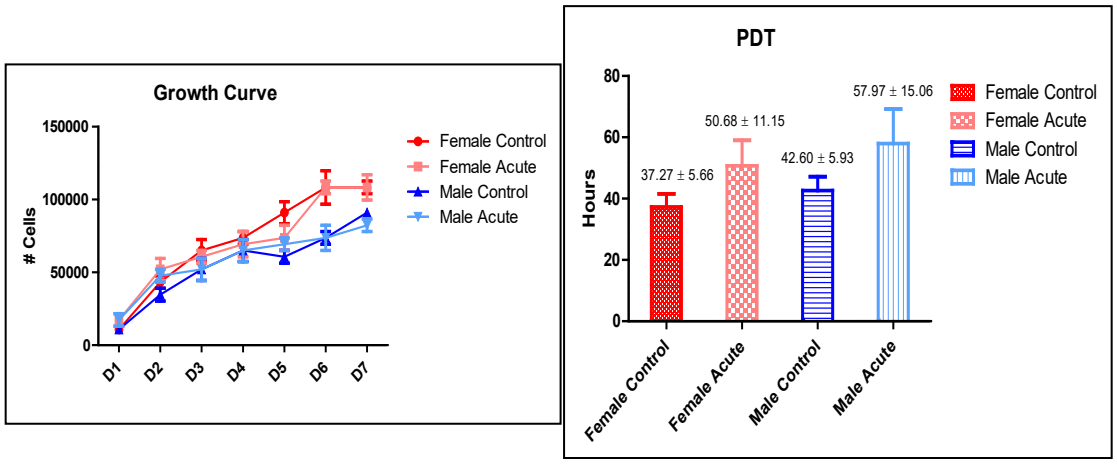
Figure 4: Male and female ASCs in the acute and control conditions were stained with Alamar blue and relative fluorescent units were measured using a plate reader. (a) Male acute ASCs showed a significantly higher rate of metabolic activity than the female acute and control groups (b) Male control ASCs showed a significantly higher rate of metabolic activity than the female acute and control groups. A 2-way ANOVA and two-tailed t-test showed significance. *** $p < 0.001$ relative to female cells.

Population-Doubling Time

Male and female ASCs from the acute and control conditions were plated and counted every day for 7 days to determine the growth curve (Fig. 5(a)). The numbers of cells were used to calculate population-doubling time (Fig. 5(b)).

Statistical analysis determined no significant differences between population doubling times of any of the conditions.

Visibly, the growth curves show little deviation between the control and acute cells, once again indicating that the ASCs may have been able to rebound from the acute LPS treatment.



a **b**

Figure 5: Male and female ASCs in the acute and control conditions were counted every day for 7 days. (a) The growth curve shows the number of cells for each day and condition (b) The population-doubling time was calculated using the numbers of cells counted. 2-way ANOVA and a two-tailed t-test determined no significance.

Expression of Pro- and Anti-Inflammatory Cytokines in Male and Female mASC mRNA

Several genes have been identified in previous studies to be upregulated in ASCs during the same stress conditions as this study proposes. In order to collect data on the expression of pro- and anti-inflammatory cytokines, mRNA expression of pro and anti-inflammatory cytokines and self-renewing markers was measured from male and female ASCs in acute and control conditions. Cells were harvested at 24 hours, 48 hours, 72 hours, and 7 days, and the difference in the assessed genes in the ASCs treated with LPS was compared to the control condition, shown as the green lines in Figure 6.

Statistical analysis revealed no significance in any of the conditions and genes analyzed, potentially because too few replicates were used.

However, visually, some pro-inflammatory cytokines showed the potential for upregulation in the male acute ASCs, as seen in Tumor Necrosis Factor alpha (TNF-alpha, Fig. 6(c)) at the 24-hour mark, or the increased expression of interleukin-6 after up to 72 hours (IL-6, Fig. 6(d)). Female ASCs showed no significant upregulation of pro-inflammatory cytokines at any time point measured. None of the acute ASCs showed significant up-regulation of pro-inflammatory cytokines at the 7-day mark.

Acute female ASCs showed the potential for up-regulation of anti-inflammatory cytokine transforming growth factor beta, that surpassed the male acute ASCs up to the 7-day mark (TGF-beta, Fig. 6(b)).

Male acute ASCs showed potential for upregulation of homeobox protein Nanog (mNanog1, Fig.6(e)), and SRY-box 2 (mSox2, Fig 6(f)), both of which are involved in

maintaining pluripotency and self-renewing capability, at the 24-hour mark. Female acute ASCs showed no significant difference when compared to the control.

Male acute ASCs showed a steady increase in the upregulation of octamer-binding transcription factor 4 (mOct4 1, Fig. 6(g)).

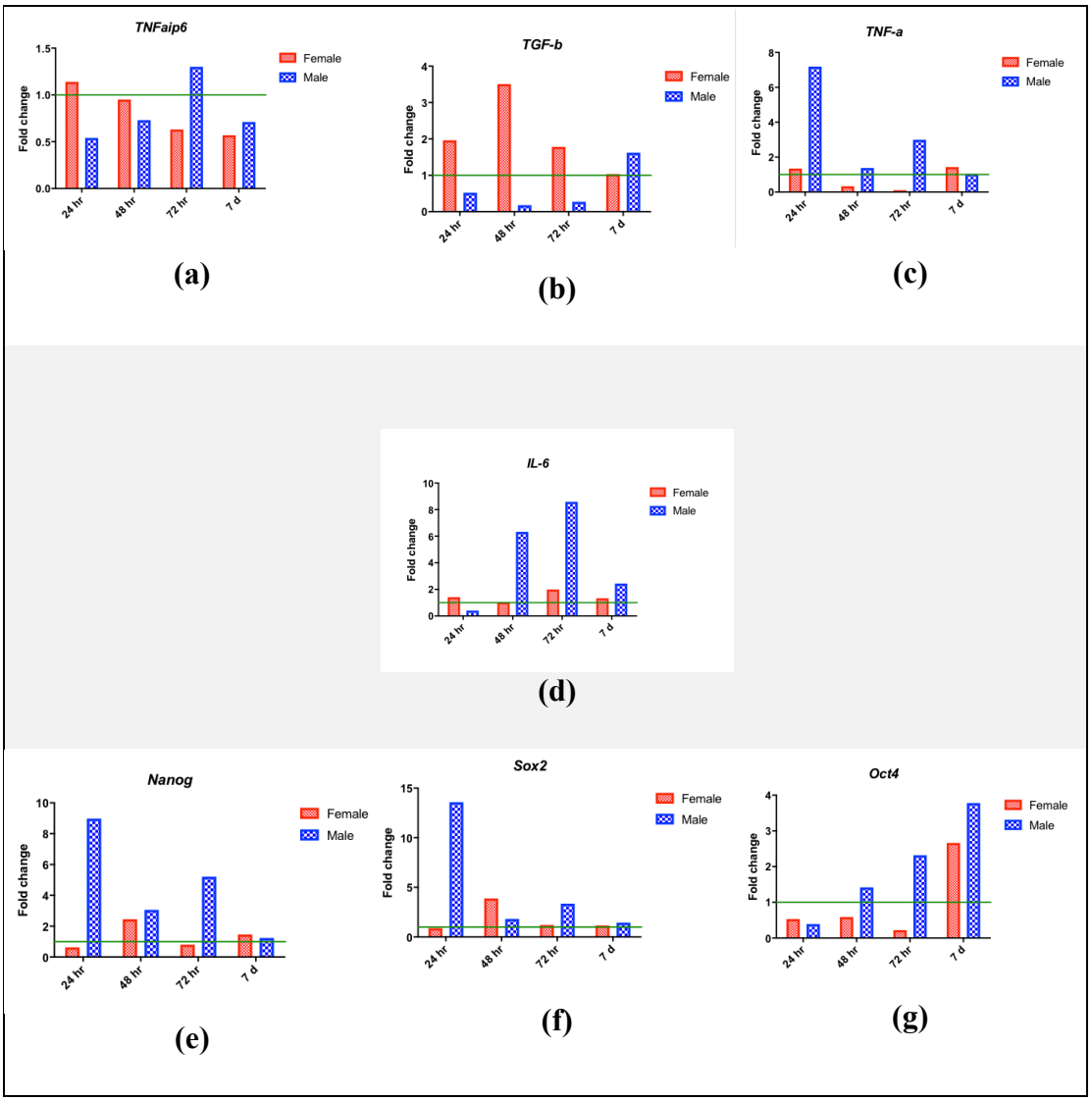


Figure 6: Male and female ASCs were harvested at 24 hours, 48 hours, 72 hours, and 7 days. A 2-way ANOVA and two-tailed t-test showed no significance in any of the conditions.

Adipogenic Differentiation Shows Significant Differences Between Acute and Control Cells

Male and female ASCs from the acute stress condition and the control condition were treated with alternative doses of ADM and OMM to induce adipogenic differentiation. The ASCs were allowed to differentiate for 21 days before being stained and photographed. 3 additional wells from each condition were stained without being differentiated. Figure 7 shows the undifferentiated control and acute cells next the differentiated ones. The stain used, Oil-Red-O, adheres to neutral lipid droplets, a component of adipose cells. Visually, the ASCs that had been differentiated picked up the stain, indicating the clear presence of neutral lipid droplets and therefore adipose cells.

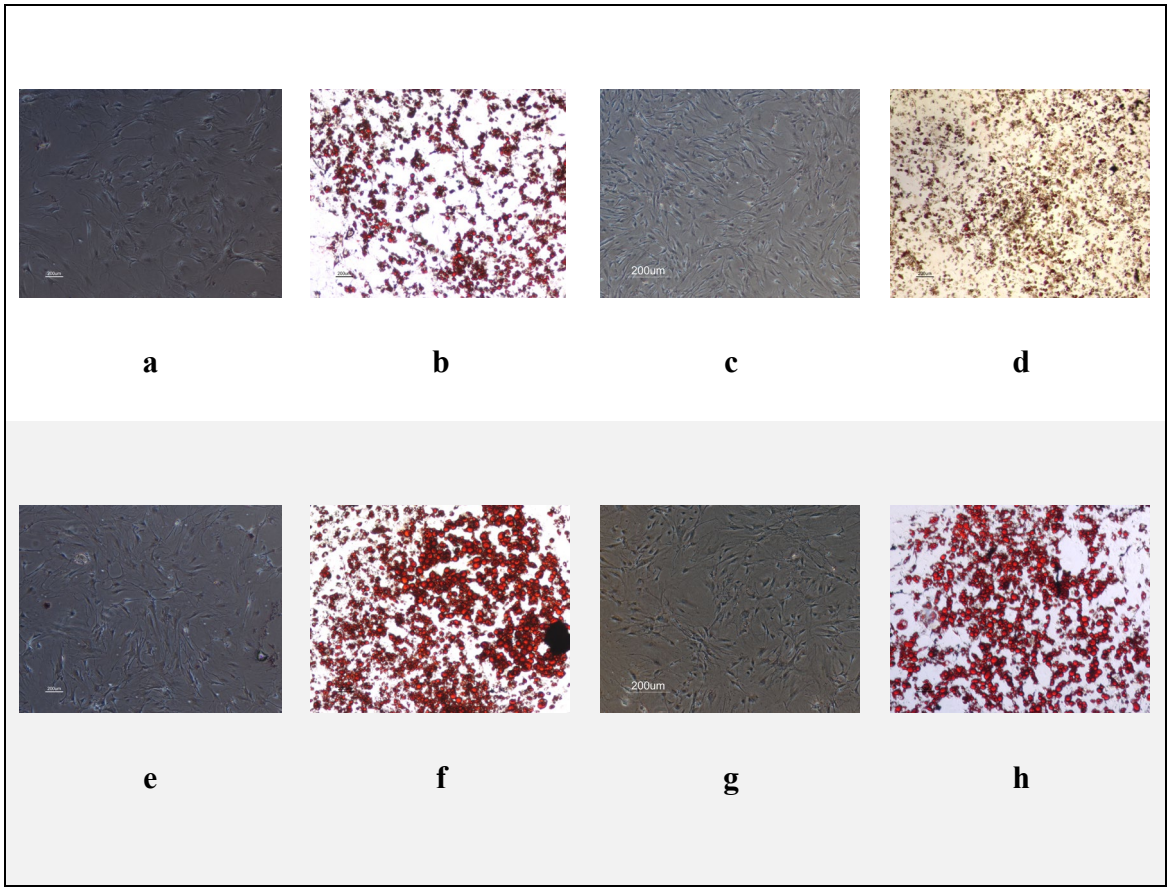


Figure 7: Male and female ASCs from the control and acute conditions were treated alternatively with ADM and AMM for 21 before being stained with Oil-Red-O and photographed, and compared to the undifferentiated cells. Images were acquired at 10x magnification for adipogenic differentiation. (a) Acute female ASCs, undifferentiated (b) Acute female ASCs, adipogenic diff. (c) Control female ASCs, undifferentiated (d) Control female ASCs, adipogenic diff. (e) Acute male ASCs, undifferentiated (f) Acute male ASCs, adipogenic diff. (g) Control male ASCs, undifferentiated (h) Control male ASCs, adipogenic diff.

After being photographed, the ASCs were destained and read in a plate reader, which gave information about the level of stain on each plate, and therefore the amount

of differentiated cells. The absorbance values from the undifferentiated and differentiated cells from each condition were used to determine the total differentiation (Fig. 8). Statistical analysis showed a significant difference between the male and female ASCs that had received the acute LPS treatment ($p < 0.001$). There was also a significant difference in absorption values between the female control ASCs that had not received any LPS treatment, and the female ASCs that received acute LPS treatment ($p < 0.05$), and well as between female acute ASCs and the male control ASCs ($p< 0.05$). There was no significant difference between male acute and male control ASCs.

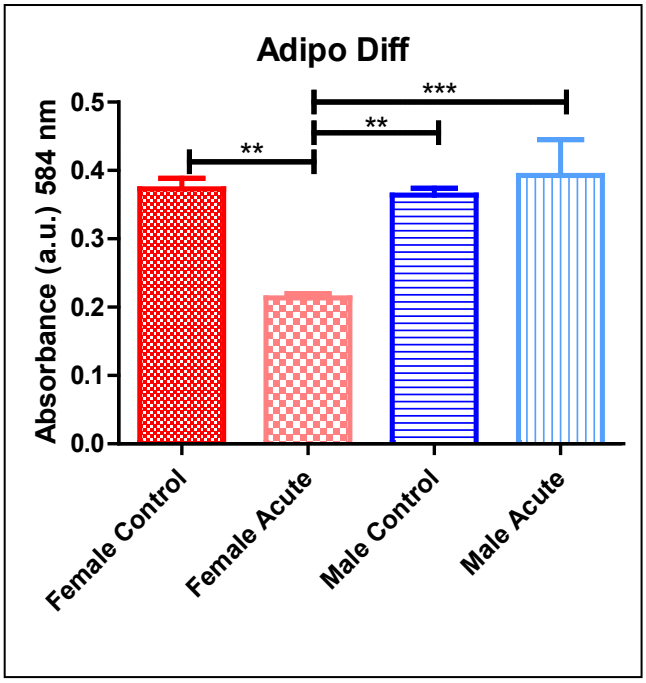


Figure 8: Male and female ASCs from the acute and control conditions that had been treated with ADM and AMM to induce adipogenic differentiation were destained and read with a plate reader (ex545/em590). 2-way ANOVA with multiple comparisons by a post-hoc Bonferonni test revealed significant differences. ** $p < 0.05$, *** $p < 0.001$

These findings indicate a significantly decreased ability for female ASCs to differentiate in the presence of acute LPS, in comparison to untreated female ASCs ($p < 0.05$). The sex difference in this perceived decreased ability is evident in the lack of a similar reaction by male ASCs treated with LPS, even though there was no significant difference in the differentiation ability of male and female control ASCs. There was a significant difference between female acute ASCs and male acute ASCs ($p < 0.001$). The sex difference in the reactions of male and female ASCs of genes controlling

inflammatory response, or genes controlling self-renewing and pluripotency, to the stress caused by LPS may be an explanation for the female acute ASCs' inability to differentiation as fully as the male acute ASCs.

Osteogenic Differentiation Shows Significant Differences Between Acute and Control Cells

Male and female ASCs in the acute and control conditions were treated with ODM for 21 days to induce osteogenic differentiation. The cells were then stained and photographed (Fig. 9). 3 additional plates of undifferentiated ASCs from each condition provided a control. The stain used, alizarin red, adheres to calcium deposition, which is seen in bone cells. The red stain present on the differentiated cells in Figure 9 is indicative of the presence of calcium deposition, and therefore osteoblasts. All conditions were able to differentiate successfully.

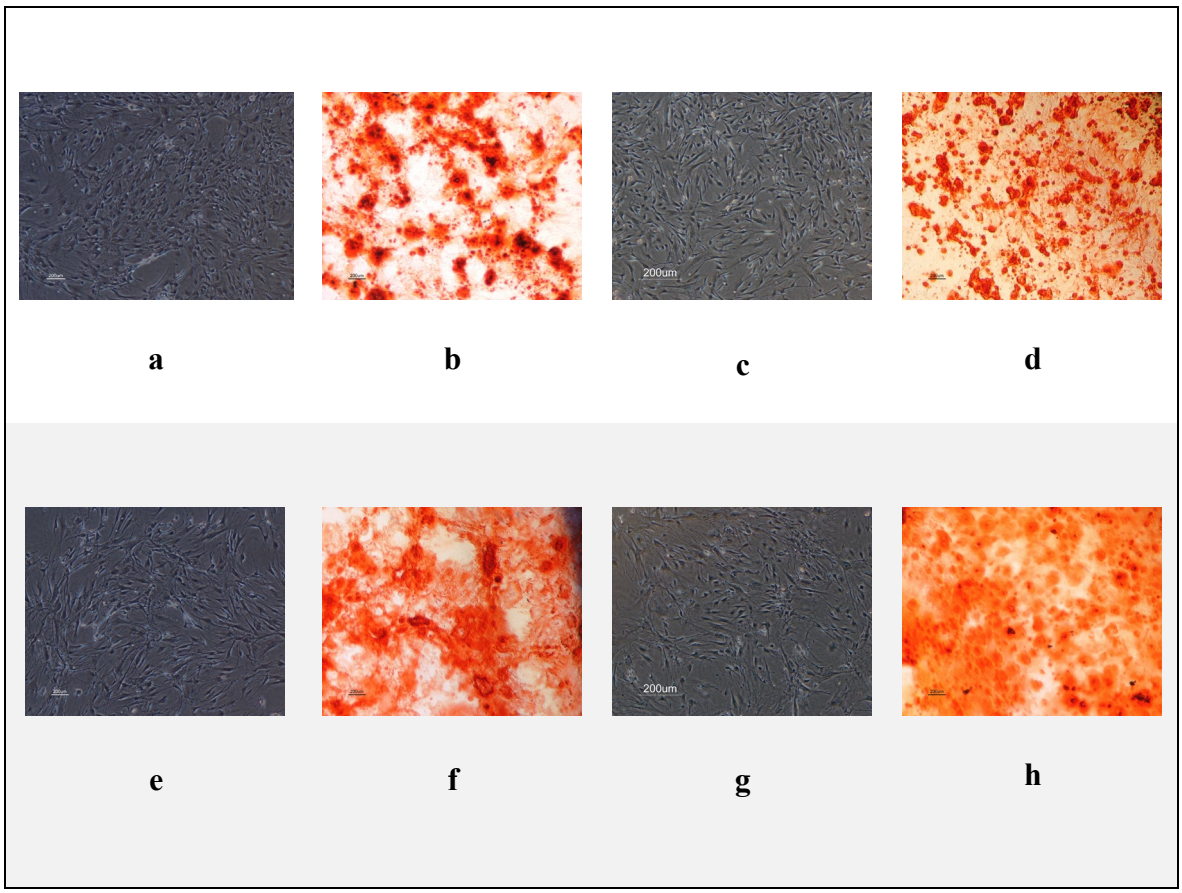


Figure 9: Male and female ASCs from the control and acute conditions were treated with ODM for 21 before being stained and photographed, and compared to the undifferentiated cells. Images were acquired at 4x magnification for osteogenic differentiation. (a) Acute female ASCs, undifferentiated (b) Acute female ASCs, osteogenic diff. (c) Control female ASCs, undifferentiated (d) Control female ASCs, osteogenic diff. (e) Acute male ASCs, undifferentiated (f) Acute male ASCs, osteogenic diff. (g) Control male ASCs, undifferentiated (h) Control male ASCs, osteogenic diff.

After being photographed, the cells were destained and read in a plate reader. The level of stain present was measured as the absorbance value, which allowed a

quantification of the amount of differentiated cells. The absorbance values from the undifferentiated and differentiated cells from each condition were used to determine the total differentiation (Fig. 10). Statistical analysis showed a significant difference between the male and female ASCs that had received the acute LPS treatment ($p < 0.001$). There was also a significant difference in absorption values between the female control ASCs that had not received any LPS treatment, and the male control ASCs ($p < 0.001$), and well as between female acute ASCs and the male control ASCs ($p < 0.001$). There was a smaller significant difference between female control and male acute ASCs ($p < 0.05$), and between male control and male acute ASCs ($p < 0.01$). There was no significant difference between female control and female acute ASCs.

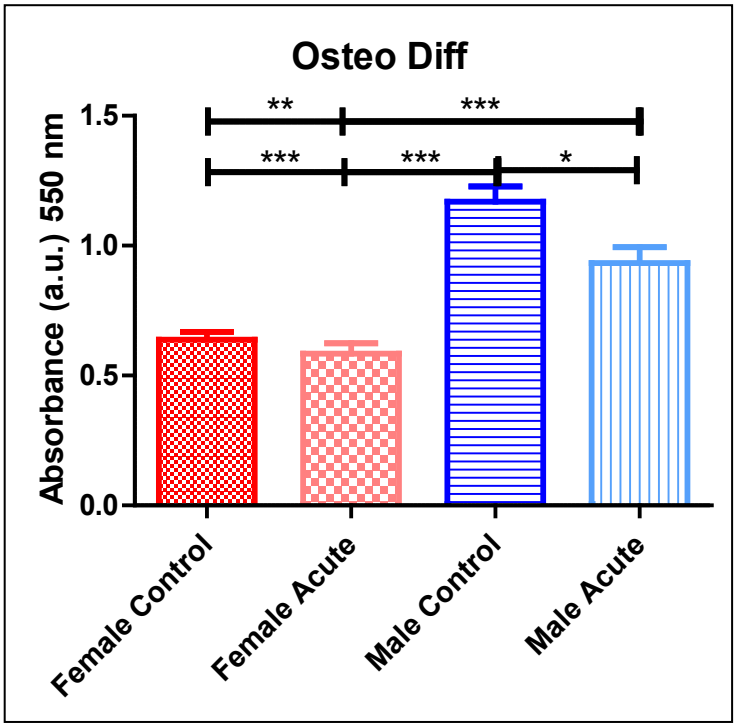


Figure 10: Male and female ASCs from the acute and control conditions that had been treated with ODM to induce osteogenic differentiation were destained and read in a plate reader (ex545/em590). 2-way ANOVA with multiple comparisons by a post-hoc Bonferonni test revealed significant differences. * $p < 0.01$, ** $p < 0.05$, *** $p < 0.001$

These findings indicate a significant difference between male and female ASCs both in the control group and the acute LPS group ($p < 0.001$, $p < 0.001$, respectively). Female ASCs did not see a significant decrease in differentiation ability in reaction to LPS treatment. Male ASCs did show a slightly significant decrease in differentiation ability compared to male control ASCs ($p < 0.01$). This data stands in contrast to the data from adipogenic differentiation, but if female ASCs already show a decreased ability to

differentiate into osteogenic cells, the effects of LPS may be less obvious than the more readily pluripotent male ASCs. The expression of inflammatory cytokines and self-renewing genes could play a role in the difference seen between male and female ASCs in osteogenic differentiation, in regards to both untreated and LPS-treated cells.

Discussion

ASCs are an increasingly attractive candidate for use in regenerative therapies because of how easy they are to obtain, their ability to differentiate into any mesenchymal lineage cells, and their immunomodulatory capabilities. It is therefore important for researchers to understand the behavior of ASCs under stress, and the impact of biological sex on the ASCs' fundamental phenotypic characteristics.

This study evaluated and compared the response of male and female ASCs following an acute exposure to the inflammatory stimuli LPS. Flow cytometry revealed no changes to the stemness of the ASCs themselves, meaning there were no marked changes that would make comparison between the LPS-treated ASCs and the control ASCs inaccurate. Several assays were performed and compared between sexes and treatment conditions to determine any significant differences.

Indicators of growth were determined by counting colonies and calculating population doubling time. The numbers of colonies for each of the condition was counted after 21 days, and showed no significant difference. It is possible that 21 days gave the LPS-treated ASCs time to recover from the acute treatment of 100 ng for 24 hours, and that by the time they were stained and counted, the cells had been able to rebound to control levels. The growth curve graphed during the population-doubling time assay, in

which ASCs were counted each day for 7 days, revealed a steady increase in growth, with no significant differences in acute and control conditions at any day. Previous studies have indicated increases in growth factors in response to LPS in mesenchymal stem cells, which leads to an increase in proliferation [5]. The lack of significant differences in growth rates and colony formation could be a result of a low number of replicates, or be an expression unique to adipose-derived stem cells.

Alamar blue stains in reaction to metabolic reduction, which allows measurement of the levels of oxidation during cell respiration. Beyond simply showing the growth of cells, this assay provides a measurement of cell viability and health. The levels of oxidation measured in relative fluorescent units showed no differences in acute and control conditions for the sexes, but it did reveal significant differences between male and female ASCs. Female ASCs maintained a steady rate of cell respiration despite the calculated growth curve suggesting growth (Fig. 10 (a)), while male ASCs increased cellular respiration during growth. This could indicate that male ASCs have a higher metabolic function than female ASCs, regardless of the presence of LPS.

RT-qPCR showed no significant up-regulation in any of the targeted genes, most likely because of the small biological sample. The visual trends of the graphs do, however, show interesting differences between male and female ASCs, with female acute ASCs trending more toward up-regulation of anti-inflammatory cytokines (TGF-β) and male acute ASCs trending toward up-regulation of pro-inflammatory cytokines (TNF-α, IL-6). The data was dependent on the time that the mRNA was extracted and analyzed. In order to obtain a fuller, more significant understanding of these genes, a higher n is likely needed. Information on the differences in gene expression between male and female

ASCs would help in understanding the reasons behind the significant differences in metabolic function seen in the Alamar blue assay. Visually, there appears to be a larger up-regulation of Nanog and Sox2 in male ASCs (Fig. 5(e, f)), genes that deal in self-renewing capacity. A statistically significant difference in male and female expression of these genes would offer some explanation as to why the male ASCs in the Alamar blue assay appeared healthier and more viable.

Interestingly, significant differences were observed between male and female ASCs from both the control and acute condition while undergoing either adipogenic or osteogenic differentiation. Adipogenic differentiation was measured using absorbance values and indicated a much lower number of stained cells in the female acute condition than in any other condition (Fig. 7). Because there was not a significant difference between male and female control ASCs, this finding indicates that it is the ASCs' reaction to LPS that distinguishes male from female. Previous studies have found greater expression of pro-inflammatory cytokines in LPS-stressed female mesenchymal stem cells (MSCs) [6], but more research should be done in female adipose-derived stem cells to see if the same is true. Other studies have identified unique gene expression patterns in cells undergoing differentiation [1]. Further studies are needed to identify whether the sex-dimorphism of gene expression is connected to the difference in differentiation ability.

Osteogenic differentiation showed a different pattern. There were significant differences between male and female ASCs of both conditions, but only male ASCs showed a slight difference between the control and acute condition (Fig. 9). The clear difference in the number of osteoblasts stained for female and male indicates that

differentiation ability differs between the sexes even before the introduction of a stressor. The slight difference between the male ASCs treated with acute LPS and the untreated male ASCs indicates that LPS has some effect on the differentiation ability of male ASCs. The effect on female ASCs may be difficult to see because of the lessened pluripotency. To be able to determine whether sex-dimorphism in the up or down regulation of certain genes plays a role in determining differentiation ability, future studies should analyze gene expression in differentiated cells for males and females separately.

Conclusion

There are clear differences between male and female ASCs during osteogenic and adipogenic differentiation. In adipogenic differentiation, untreated male and female ASCs showed similar ability to differentiate, but female ASCs treated with LPS showed significantly less stained adipose cells. In osteogenic differentiation, the only significant difference was between sexes, with male ASCs showing greater differentiation ability than female ASCs, regardless of LPS treatment. Proliferation assays showed steady growth in both male and female ASCs, but Alamar blue revealed a difference in cell viability, with male ASCs showing higher levels of oxidation. The larger amount of cellular metabolic reduction could be a contributing factor to male ASCs greater osteogenic differentiation ability, or their ability to maintain adipogenic differentiation in the presence of LPS.

ASCs are being researched for use in regenerative medicine, which relies on activation in an inflammatory or stressor environment to exert the most effective

therapeutic results. Therefore, it is crucial to have an understanding of the sex dimorphisms that exist between ASCs when subject to stress. Further studies are necessary to determine whether or not the differences between sexes during differentiation is significant enough to have a clinical impact, but this study indicates that the sex of cells determines their ability to differentiate to some extent.

The lack of significance in the PCR data is likely due to the small n, and further studies should look at a larger biological sample to determine the differences in gene expression for male and female ASCs, both before differentiation and after. The trends seen in this study do parallel those seen in previous studies [1, 2] that attempt to quantify sex-dimorphism in stem cells. It indicates that the differences seen in the differentiations here are the result of different levels of expression of inflammatory cytokines, and growth and self-renewing factors. Understanding the inter-play between gene expression and differentiation ability in male and female ASCs will be invaluable in their therapeutic use.

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