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Investigation of Zinc Chloride & Caffeine and Their ECTS on Bone Homeostasis in a 3D Bone Model

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INVESTIGATION OF ZINC CHLORIDE & CAFFEINE AND THEIR EFFECTS ON BONE HOMEOSTASIS IN A 3D BONE MODEL

By:

Pooja Shah

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College of Arts & Sciences
Department of Biological Sciences

APPROVAL FOR SUCCESSFUL DEFENSE

[student's name] has successfully defended and made the required modifications to the text of the doctoral dissertation for the Master of Biology-Neuroscience during this Fall, 2022.

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Director of Graduate Studies Date

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Abstract

Approximately fourteen percent of the human body is composed of bone. The adult human skeleton is made of 206 bones, which make up the internal framework of the body. Osteoblast and Osteoclast cells are essential in maintaining the structure and function of bone, for bone homeostasis to occur. Previous data shows that caffeine can potentially have a negative effect on bone homeostasis. Whereas, $ZnCl_2$ has been found to have a positive effect on bone homeostasis in controlled doses. Published data for treatments of caffeine and $ZnCl_2$ are inadequate. The goal of this study was to determine the optimal concentration range for these treatments in maintaining bone metabolism. $ZnCl_2$ and caffeine were provided in increasing concentrations to a 3D-bone organoid model (3D-BOM) for 21 days. Treatment concentrations were determined based on WHO/EPA guidelines. Alkaline phosphatase activity and alizarin red staining was completed to assess osteoblast differentiation and function respectively. Data shows that in the concentration range of 3μM-30μM, $ZnCl_2$ maintains alkaline phosphatase activity. 3μM and 10μM $ZnCl_2$ concentrations enhanced calcium deposition while 30μM $ZnCl_2$ showed impairment. Caffeine (0.005μM, 0.01μM and 0.1μM) had negative effects on alkaline phosphatase activity or calcium deposition. Data demonstrates that $ZnCl_2$ and caffeine has concentration-dependent effects on osteoblast differentiation/function. In the future, the effects of trace elements on osteoclast function and gene/protein expression will be evaluated. Determining optimal levels of trace elements for bone metabolism can be used to assess the range of disorders and disease caused by disruption in bone homeostasis.

Introduction

Bone is a specialized connective tissue made up of cells, fibers, and ground substances that makes up the body's skeletal system. Bone contains four main types of cells, osteoblasts, osteocytes, osteoclasts, and bone lining cells. Osteoblasts (OB), osteoclasts (OC) and bone lining cells are derived from mesenchymal cells, known as progenitor cells and lie on the surface of the bone (18). OBs play an important role in skeletal development and remodeling through mineralization and deposition. OBs have regulatory functions in osteoclastogenesis and bone resorption (22). Major regulatory functions include production of constituents that are found in the extracellular matrix. In turn, this allows for the facilitation of mineralization (4). While osteoclast cells are responsible for degradation of the bone matrix, important for carrying out normal bone remodeling and increase resorptive activity. OCs play an essential role in maintaining bone homeostasis via regulation of the differentiation of osteoblast precursors, leading to the regulation of bone mass (4). In addition, OCs regulates bone formation through the detection of mechanical loading. Mechanical loading allows for increased bone formation, a reduction of bone loss and change in bone cell differentiation through the lifespan of the bone (25). Osteocytes are mature bone cells that produce signaling molecules within the endocrine system and regulate mineral deposition. The formation of bone occurs when mesenchymal precursor cells are differentiated into osteoblasts. Once osteoblasts are enclosed in the calcified matrix and differentiate, these cells are known as osteocytes (13).

Bone homeostasis is a dynamic state of equilibrium that is maintained via regulatory action of osteoblasts, osteoclasts and osteocytes. The importance of bone

homeostasis in the skeletal system is to maintain the calcium levels within the body, in addition to bone structure and function. Disruption to bone homeostasis can induce bone disorders, such as osteoporosis. Bone homeostasis is maintained by various components. Osteoblast-derived cells have a large range of cytokines modulate differentiation, including, but not limited to bone-matrix derived TGF-β (transforming growth factor beta), bone morphogenetic protein 2 (BMP-2), BMP-4 and BMP-7. Two essential factors regulating bone homeostasis are osteoclast-derived MCS-F (monocyte-colony stimulating factor) and RANKL (the receptor activator of nuclear factor-κB ligands). Osteocytes serve as master regulators of bone homeostasis, with direct involvement in bone remodeling via sclerostin regulation (1). Osteocytes control functions of OBs and OCs through RANKL and sclerostin (bone cells produced in osteocytes). At the same time, osteocytes have the ability to secrete components involved in phosphate homeostasis within the body (8). When bone homeostasis is abnormal it can result in hypocalcemia or hypercalcemia (abnormally low/high levels of calcium) throughout the skeletal system and lead to various disorders and physiological changes.

Recent studies show that human bone physiology is affected by various components (7). These components vary based on a organisms' sex, size, age, genetic factors, and health. Specific elements that have been found to have impact bone physiology include: calcium, iron, $ZnCl_2$and caffeine (19). Zinc is a trace element that is known for its key functions in immune health and metabolism. The body obtains its main source of zinc via the food sources that are consumed by an organism (27). Zinc is only needed in small amounts; however, it is a necessary for over 100 enzymes to carryout

biological reactions. In addition to playing a large role in growth, DNA and protein synthesis, healing damaged tissue, and supporting immune health. Moreover, zinc is an essential component of skeletal growth and bone homeostasis. Zinc chloride has been found to have a stimulatory effect on osteoblast bone formation and mineralization with the ability to improve bone regeneration. The normally recommended dosage of zinc that has been found to improve human bone health is 8mg for adult women and 11mg for adult men (17). Deficiencies in zinc have been linked to a variety of different concerns in humans and animals, such as impairments in the skeletal system and bone growth (20). Recent research shows that zinc promotes bone growth, homeostasis, and bone regeneration, however, the molecular/cellular pathways that promote these benefits are poorly understood (20). In turn, zinc's bone content has been found to decrease in conditions of aging, skeletal unloading, and menopausal conditions (34).

Caffeine is a methylxanthine class stimulant that blocks the action of adenosine on its own receptor, to achieve a lack of drowsiness. Caffeine stimulates the nervous system and brain activity. The recommended dosage of caffeine for a healthy adult should not exceed 400 mg/day. Recent studies have shown that when 100mg of caffeine ingested, the human body loses 6 mg of calcium (30). Caffeine has been found to significantly decrease the variability of osteoblasts and the mineralization formation in osteoblast cultures under concentrations of 10mM or higher. In addition, the presence of ALP (Alkaline Phosphatase) staining colonies has been shown to decrease significantly under concentrations exceeding 10mM of caffeine (28). However, under lower concentrations (0.005 - 0.1mM), caffeine effectively enhances osteoclastogenesis (formation of osteoclasts from precursor cells). While enhancing COX-2/PGE (2) -

regulated osteoclastgenisis mediated by RANKL, under these low concentrations (16). Recent research shows that caffeine can modulate COX-2 inhibition through energy binding towards the COX-2 acetaminophen complex (14).

Overall, caffeine has a negative effect on bone homeostasis under high concentrations. With the ability to decrease bone density, high caffeine intake results in an increased chance of bone fractures, a negative calcium retention rate and mineral loss (20). Whereas $ZnCl_2$ has a positive effect on bone homeostasis under normal concentrations (below 100 mg/day). In conclusion, bone homeostasis and the functions of osteoblast, osteoclasts, and osteocytes can be affected through different concentrations of essential elements/stimulants.

This study utilizes the development of an in-vitro 3D murine bone model, to investigate the effects of $ZnCl_2$ and caffeine individually on bone homeostasis, at various dosages overtime. The benefits of developing the 3D murine model derived from the ability to study the structure and physiological contents of bone degradation, calcium deposition and bone metabolism from different treatment timepoints. The use of 3D cell culture allows researchers to mimic in-vivo conditions of natural systems in the body (24).

The purpose of this study is to determine the optimal levels of zinc chloride and caffeine to maintain bone metabolism. For this study, a 3D-BOM was developed, utilizing both OB (MC3T3.E1 cells) and OC (RAW 267.4 cells) cell lines to create a bone organoid under homeostatic conditions. The 3D-BOMs were treated with the trace element ($ZnCl_2$) and stimulant (caffeine) at different concentration groups (low, medium,

and high). Bone homeostasis was then characterized by measuring osteoblast function and associated functions.

Materials and Methods

Cell Culture Preparation of MC3T3 Osteoblast Cells

Plated MC3T3.E1, murine preosteoblasts (atcc.org CRL-2593) were maintained in sterile conditions in a 37ºC per 5% CO_2 incubator. After two days of culture, the media was replaced with fresh media. The osteoblasts reached confluence at 7-8 days. The OB differentiation media was made using Alpha-Mem complete (10%FBS, 1%P/S) supplemented with L-AA (VMR Life Science, PA), Dex (Sigma-Aldrich, MO), BMP-2 (R&D Systems, MN) and BGP (Sigma-Aldrich, MO).

Cell Culture Preparation of RAW 264.7 Cells

Plated primary osteoclasts cells were maintained in sterile conditions in a 37ºC per 5% CO_2 incubator. After two days of culture, the media was replaced with fresh media. The osteoblasts reached confluence at 7-8 days. RAW 264.7 cells were cultured in Alpha-Mem complete media. The osteoclastic differentiation media was made by supplementing Alpha-Mem complete with Recombinant Mouse TRANCE/RANK-L/TNFSF11 (E. Coli expressed) (R&D systems, cat# 462-TEC-010), which induced osteoclast differentiation of RAW 264.7 cells and Recombinant Mouse M-CSF protein (R&D Systems, cat # 416-MP-050/CF), induced cell proliferation. Osteoclastic media was prepared using the “Osteoclastic Media Preparation” protocol.

Characterization of 3D in vitro models

Osteoblast/Osteoclast differentiation and bone mineralization were monitored through microscopic analysis on (Accuscope) using 40x magnification. Several images were captured through the 30-day course of the entire osteogenic differentiation and

mineralization process. MC3T3.E1, murine preosteoblasts (atcc.org CRL-2593) were cultured in Alpha-Mem complete media. The MC3T3.E1 cells were seeded at a density of 60,000 cells per 17 µl cold Matrigel™ growth factor reduced (GFR) (cat #). The MC3T3.E1 and Matrigel™ suspension was maintained on ice until it was pipetted at 17 µl per well into a room temperature 96-well flat bottom plate (Thermo Fischer Scientific, cat#07-200-91). The suspension was pipetted either slowly to the center of the well or by "drop method"—a droplet allowed to fall from the end of the pipette tip to the center of the well—to ensure a droplet or dome shape formed as the suspension solidified. After the suspension was pipetted, the 96-well plate was incubated for 7 minutes at 37°C to allow the Matrigel™ to polymerize. Then, 200ul of osteoblastic differentiation media was gently added to each well, taking care not to disturb the Matrigel™ droplet. After the addition of the osteoblastic media, the plates were incubated at 37°C with 5% CO_2 for 21-30 days, depending on the observed mineralization of the osteoblasts. 50% of the osteoblast media was replaced every 2-3 days. On day 21-30, RAW 264.7 cells were suspended at a density of 60,000 cells per well in 100µl complete bone homeostasis media (1:1 mix of OB differentiation media and OC differentiation media described above). The 3D bone models were incubated at 37°C and 5% CO_2 for 14 days to allow the models to reach a state resembling homeostatic bone. 50% of the media was replaced with the complete bone media every 2-3 days. After day 32, the 3D-BOMs are actively maintaining bone homeostasis and can be used for various studies (Figure 1).

Osteoblastic Media Preparation

Osteoblastic media was prepared using Alpha MEM Complete media (Alpha MEM media with 10% FBS, 1% Penicillin-Streptomycin (P/S)), Bone Morphogenic Protein 2 (BMP2) at a concentration of 100µg/mL, 300M concentration of Bone Growth Protein (BGP), 0.025M concentration of Ascorbic Acid (L-AA), 0.001M concentration of Dexamethasone (DEX) and 49.5 mL Alpha MEM Complete Media. Media was kept sterile and stored at 4°C.

Osteoclastic Media Preparation

Osteoclastic media was prepared using Alpha MEM Complete media (Alpha MEM media with 10% FBS, 1% P/S), 66.66 nm/mL of media Receptor Activator of Nuclear Factor Kappa Beta (RANKL) (R&D Systems, cat# 462TEC010), 33.33 nm/mL Macrophage Colony-Stimulating Factor (M-CSF) (R&D Systems, cat# 416-ML-050/CF), and 25 mL Alpha MEM Complete Media. Media was kept sterile and stored at 4°C.

Treatment Dosage Media Preparation

Media for each treatment group was prepared using the following concentrations for $ZnCl_2$: 3uM(Low), 10uM (Medium), and 30uM (High). Media dosage for the Caffeine treatment group was prepared using the following concentrations: 0.005 uM(Low), 0.01uM (Medium), and 0.1uM (High). Treatment concentrations were determined based on the WHO/EPA guidelines. Basal media was used as Minimum Essential Alpha MEM media (Corning). Preparation for Complete media was made from a 1:1 dilution of osteoblastic media and osteoclastic media.

Alkaline Phosphatase Assay

An Alkaline Phosphatase (ALP) Assay was run to measure osteoblast activity. An alkaline phosphatase assay (Abcam, cat # ab83369) was run on spent media to evaluate alkaline phosphatase activity in the 3D-BOM (3D Bone Organoid Model) samples. Samples were prepared using the manufacturer's instructions and prepared as a 1:1 dilution, using 40 µl of sample to 40 µl ALP Assay Buffer (Abcam). Plates were read immediately using a microplate reader (VarioSkan) at OD 405 nm. The results were processed using GraphPad Prism 5, Excel, and SigmaPlot 13.0.

Alizarin Red S Staining

3D-BOMs were stained using Alizarin Red S Staining to view calcium deposition within samples. Samples were prepared and evaluated using the Alizarian Red S Staining Quantification Assay protocol (ScienCell, cat # 8678). The media was removed from each of the wells of the sample and the plates were washed twice using 200 µl of sterile PBS. Samples were each fixed for 30 minutes in 4% PFA at room temperature. The fixative was removed, and the cells were washed three times with DI water. 40 mM Alizarin Red Stain reagent (ScienCell, cat #8678a) was added at 1x concentration and the plate was shaken at room temperature for 30 minutes. Alizarin red staining was removed, and the plate was washed three times with DI water. Samples were each prepped with 200 µl of 10% acetic acid per well and incubated for 30 minutes on a rocker at room temperature. Samples were collected and vortexed for 30 seconds. Samples were heated at 85°C for 10 minutes and then incubated on ice for 5 minutes. The slurry of each sample was centrifuged at 20,000g for 4 minutes. After centrifugation, 200µl of the supernatant was transferred to a new tube and added to 100µl of 10% ammonium hydroxide to neutralize the acid. 75µl of each sample was

used for reading. Standards 1-8 were prepared using the Alizarin Red Staining protocol. Plates were analyzed through a plate reader at an absorbance of 405nm. Images of 3D BNF were captured using light microscopy imaging (Accuscope).

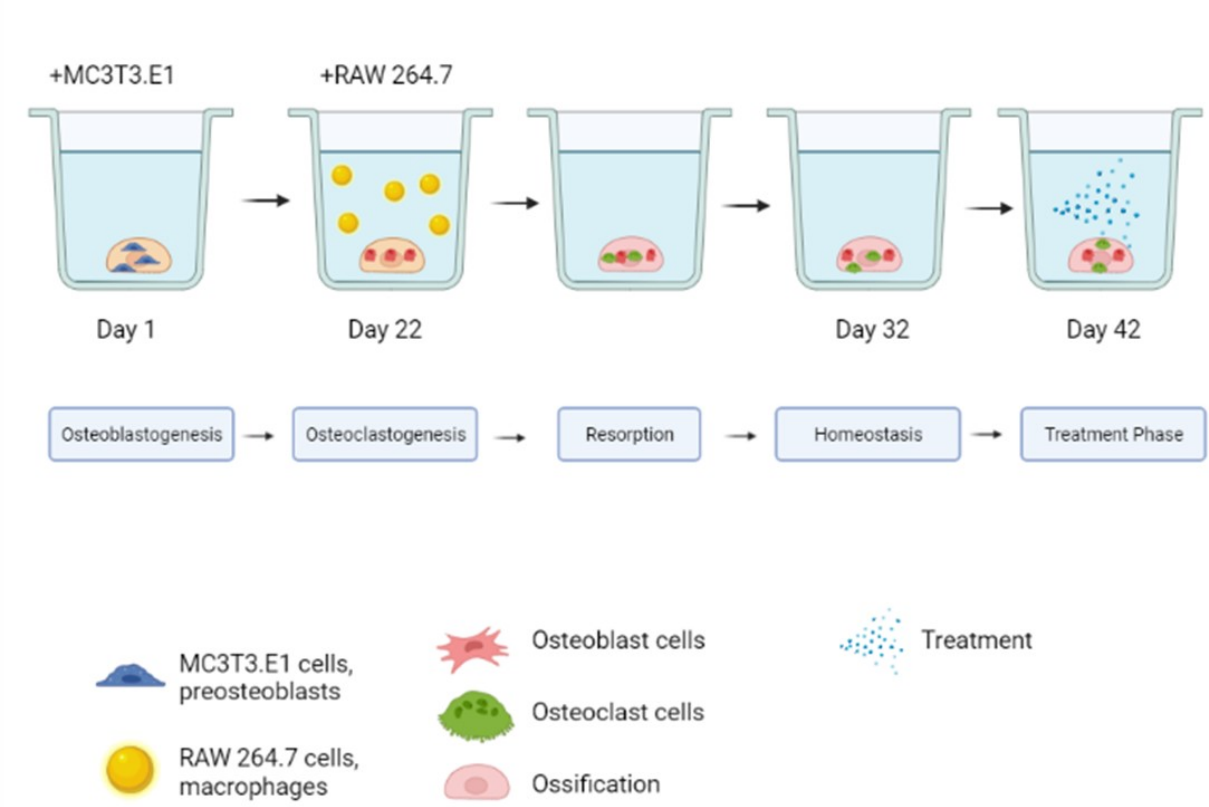
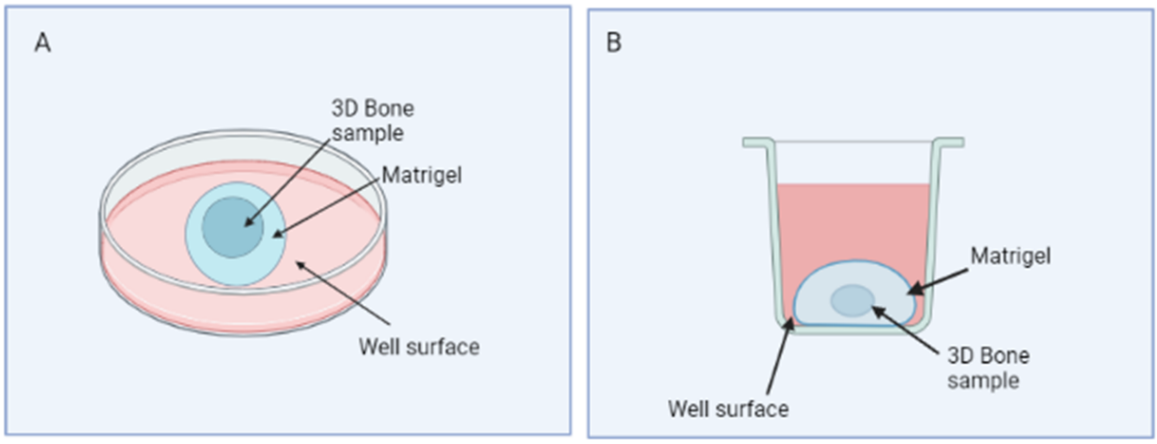
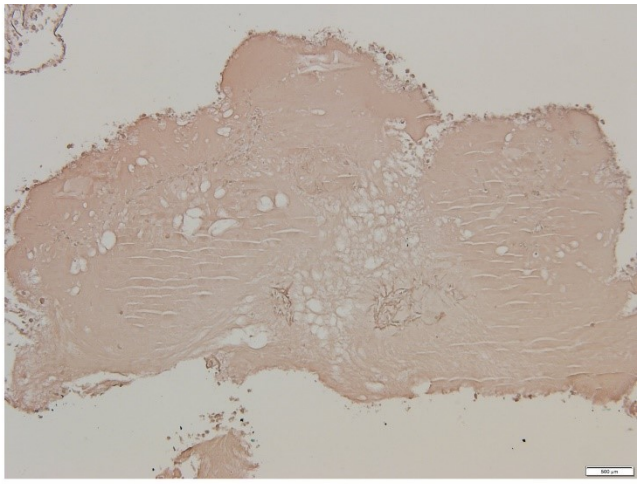


Figure 1: Schematic Timeline of 3D Murine Bone Organoid Model; Three-dimensional in vitro model was developed through osteogenic differentiation of MC3T3.E1 osteoblast stem cells, mineralization of Matrigel and RAW 264.7 Osteoclast cell differentiation over 32 days.

Note: *Data and figures collect and created by Pooja Shah.*


A
3D Bone sample
Matrigel
Well surface
B
Matrigel
3D Bone sample
Well surface

C



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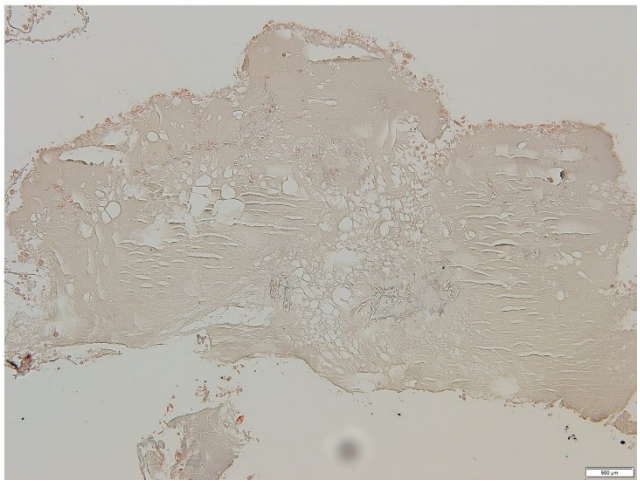


Figure 2: Matrigel-embedded differentiated OBs and OCs grown in Matrigel; Three dimensional 96-well plate 3D-BOM display. (A) Dorsal diagram of matrigel-embedded 3D-BOM illustration with respectful location on the well surface. (B) Lateral diagram of Matrigel-embedded 3D-BOM illustration with respectful location on the well surface. (C) Image of control group 3D-BOM stained with Saffron staining to show cartilage and bone interface. (D) Image of control group 3D-BOM stained with Trichrome staining to show cartilage and bone interface.

Protein Extraction

Protein extraction for the 3D-BOM samples was done following the Thermo Scientific M-PER Mammalian Protein Extraction Reagent Protocol. Samples are removed from -80°C after harvesting and left to thaw on ice. Each sample was rinsed 3X with Phosphate Buffered Saline (PBS) (Corning, NY). All PBS was removed from 3D-BOM samples, 200µl of Mammalian Protein Extraction Reagent (M-PER) Buffer (Thermo Scientific, cat #78501). Samples were left to shake on a rocker for 5 minutes. The lysate was collected and stored in a microcentrifuge tube and spun down at 14,000 RPM for 5 minutes. Supernatant was collected and used for analysis through a BCA Protein Assay. Samples were stored at -80°C.

BCA Protein Assay

BCA Protein Assay was performed using the Pierce BCA Assay Kit protocol and collected spent media samples. Each standard was prepared using the Pierce table for *Preparation of Standards and Working Reagents*. Each 3D BOM supernatants

sample/standard was used at 25 µl /well. Plate was read on a microplate reader at 563nm.

Western Blot

Protein isolated 3D BOM samples were used to perform a Western Blot to detect protein. Samples were prepared using 4X Nu-Page LDS Sample Buffer (Invitrogen/cat #NP0008) and 1X mercaptoethanol. Each sample was combined with 6.5ul of sample, 2.5ul of Nu-Page LDS Sample Buffer and 1ul of Nu-Page reducing agent. Samples were heated at 70 ℃ for 10 minutes and transferred to ice. The gel was loaded into the cassette in the gel electrophoresis box. Gel electrophoresis box was filled with fresh 1x MOPS buffer with SDS. Each of the wells of the gel were flushed with a 1X MOPS Buffer using a syringe. Samples were loaded into the gel (10ul per sample) and 5ul of Page Ruler Protein Plus ladder (Thermo Scientific/cat #26619) was added to the first well. Gel electrophoresis was run for 50 mins at 200V. For transfer, filter paper and sponges were saturated with transfer buffer until fully hydrated. Polyvinylidene difluoride (PVDF) membrane was placed in methanol for 30 seconds, rinsed immediately with diH2O, and then placed in transfer buffer. The gel was cracked open, and wells/bands are cut off. The sandwich method was used to transfer the membrane in the following order; (top to bottom) blotting pad, blotting pad, filter paper, transfer membrane, gel, filter paper, blotting pad, blotting pad. The base and top of the cassette were kept always hydrated during this process. The sandwich membrane was then placed in the gel electrophoresis box, with the PVDF membrane facing the front of the box. The outside of the box was filled with diH2O. Gel electrophoresis was run for 60 mins at 30V.

PVDF Membrane was removed and placed in 5ul blocking reagent (insert cat/company) overnight on a rocker at 4°C. PVDF Membrane was rinsed 3X in Tris-Buffered Saline with 0.1% Tween (TBS-T) for 5 mins. PVDF was then treated with Cox2 primary antibody; (Cell Signaling/cat # 4842) and Human/Mouse/Rat GAPDH primary antibody; (R&D Systems/cart # MAB5718) for 4 hours at room temperature on a rocker. Membrane was rinsed 3X in TBS-T for 5 mins and placed in Anti-Rabbit IgG HRP Conjugated secondary antibody (R&D/cat #HAF008) and Anti-Mouse HRP Conjugated Secondary antibody (R&D/cat # HAF018) for 1 hour on a rocker at room temperature. Membrane was rinsed 2X in TBS-T for 5 mins and rinsed 1x in TBS. PVDF Membrane was then placed in a luminol solution of reagent 1 ((insert company/cat) and reagent 2 (Pierce/cat # PI32106) from ECL Western Blotting Substrate and placed on a rocker for 5 minutes, covered from light. PVDF Membrane was examined under Chemiluminescence setting using the Fluorochem setting.

MTT (Cell Proliferation) Assay

MTT Cell Proliferation Assay was performed using the GOLDBIO protocol. MC3T3.E1 cells and RAW 264.7 cells were grown for 6-8 days prior to the start of the assay. Both cell lines were grown and split using the basic cell preparation protocol. Cells were ready to use at 80% confluency. In a 96 well plate, 100ul of cells were added to each well (24 wells were plated with MC3T3.E1 cells and 24 wells were plated with RAW 264.7 cells). Cells were plated at a density of 6,000 cells per well. Each well was treated with the media of the appropriate treatment group. Plated cells were incubated at 37°C for 48 hours for cell growth. The MTT stock solution 5mg MTT in 1ml 1X PBS) was prepared at the completion of the 48 hours incubation and sterilized by filtration. All

wells containing cells were treated with 10ul of MTT stock solution per well and incubated for 3 hours at 37℃. Once incubated, the cell media is carefully removed without disturbing the cells. Each well was treated with 100ul of DMSO and pipetted up and down for mixing. To create a blank, 100ul of DMSO is added to a well without cells. Plated cells are incubated at 37℃ for 15 minutes. Plate was read immediately using a microplate reader (VarioSkan) at 570 nm.

Histology

All media was carefully aspirated off 3D BOM samples in a 96-well plate. The 96-well plate was carefully tilted for 5 minutes to facilitate all media off the sample; the remainder of the media was aspirated. Samples are fixed with 200ul of 4% Paraformaldehyde for 48 hours at room temperature and covered with parafilm. After 48 hours, the paraformaldehyde was aspirated off each sample, the 96-well plate was carefully tilted for 5 minutes to facilitate all media off the sample, and the remainder of the media was aspirated. Samples are rinsed 3x with 100ul PBS for 5 mins. Wells with samples were treated with 200 ul of 10% filter sterile EDTA solution (Ph 8) to decalcify the samples for 24 hours at 4℃. After 24 hours, samples are left to reach room temperature, 10% filter sterile EDTA solution is carefully aspirated. The plate was tilted for 5 minutes to facilitate all media off the sample; the remainder of the media was aspirated. Samples are rinsed 3x with 100 ul PBS for 5 mins. 3D BOM samples are treated with 200ul of 70% ETOH and stored at room temperature on a rocker to decalcify. ETOH can be replaced as needed in order to prevent dehydration of the samples.

Saffron Staining

3D BOM samples were prepared for Saffron staining using the decalcification histology protocol. Saffron staining was performed by Marc Teitelbaum at Rutgers University Medical School.

Trichrome Staining

3D BOM samples were prepared for Trichrome staining using the decalcification histology protocol. Trichrome staining was performed by Marc Teitelbaum at Rutgers University Medical School.

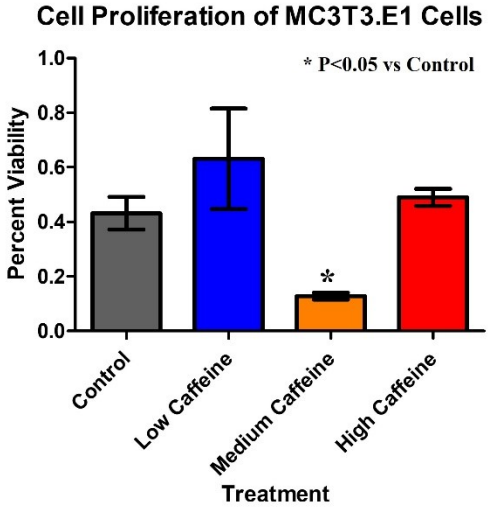
Results

Matrigel-embedded MC3T3.E1 cells and RAW 264.7 cells (Figure 1 & 2) in the presence of Alpha MEM Complete medium, growth factors; BGP, BMP2, L-AA, DEX, RANKL and M-CSF showed mineralization and resulted in bone formation. The functional ability of OBs was present on Day 21, the functional ability of OC's was present Day 42. Both OB and OC cells were maintained to reach homeostasis prior to the addition of each treatment group (Figure 1). Histological analysis was completed on BOMs on day 21 and through tri-chrome and saffron staining (Figure 2C & D). Stained images of the control group are captured to show bone interface and cartilage.

I. Effects of Caffeine and $ZnCl_2$ on Osteoblast and Osteoclast Cell Proliferation.

The MTT Cell Proliferation Assay was used to determine the effect of increasing concentrations of caffeine on cell proliferation in the MC3t3 cell line and the Raw 264.7 cell line. After 24 hours of exposure, 0.01μM caffeine treatment reduced MC3t3 cell proliferation by 12.76% percent (P value=0.029), when compared to complete media (Figure 3A). Caffeine concentrations of 0.005μM and 0.01μM significantly reduced Raw 264.7 cell proliferation by 34.85% ($P \leq 0.05$) and 6.97% ($P \leq 0.05$) percent, respectively. Interestingly, 0.1μM caffeine was found to significantly increased cell proliferation in the Raw 264.7 cell line by 8-fold ($P \leq 0.05$). Our data shows that $ZnCl_2$ concentrations between 3μM and 30μM significantly reduce cell proliferation in both MC3T3.E1 and RAW 264.7 cell populations by 9.2% ($P \leq 0.050$) and 8.04%, Figure 4.

A



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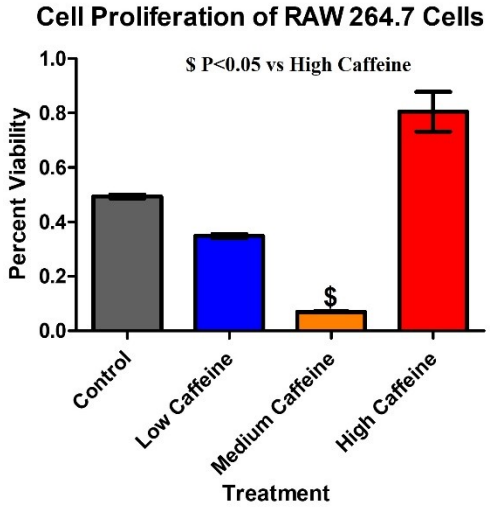


Figure 3: Quantitation of Cell Proliferation 24 hours post-Caffeine treatment. A) MC3t3 Cell Line B) Raw 264.7 Cell Line. Low caffeine dose is 0.005uM, medium caffeine dose is 0.01uM and high caffeine dose is 0.1uM. n=3. Proliferation was quantified using the MTT assay and statistical analysis was completed using Prism.

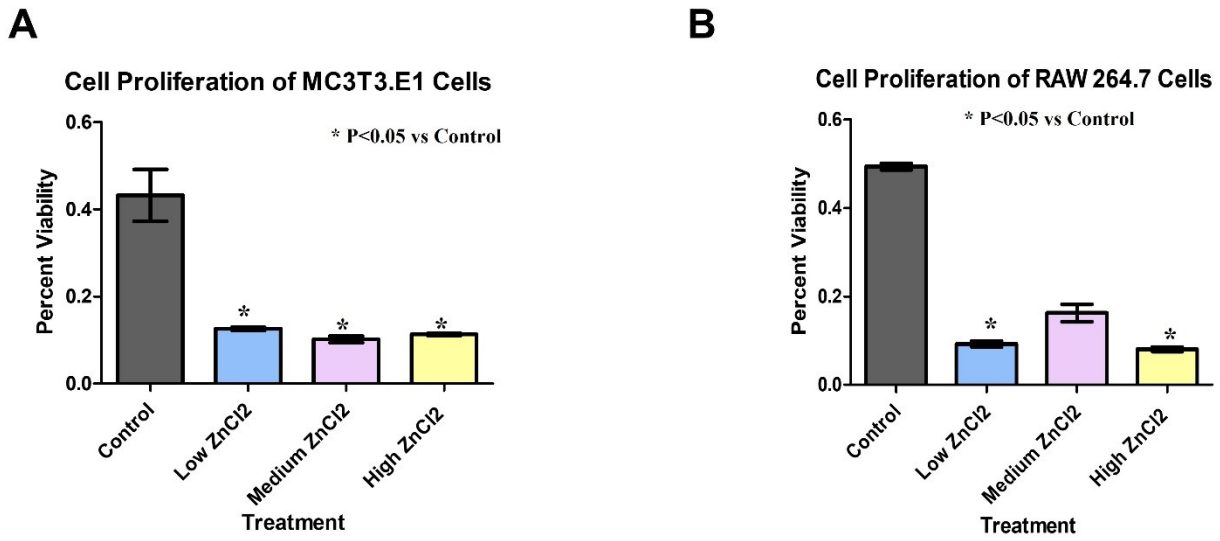


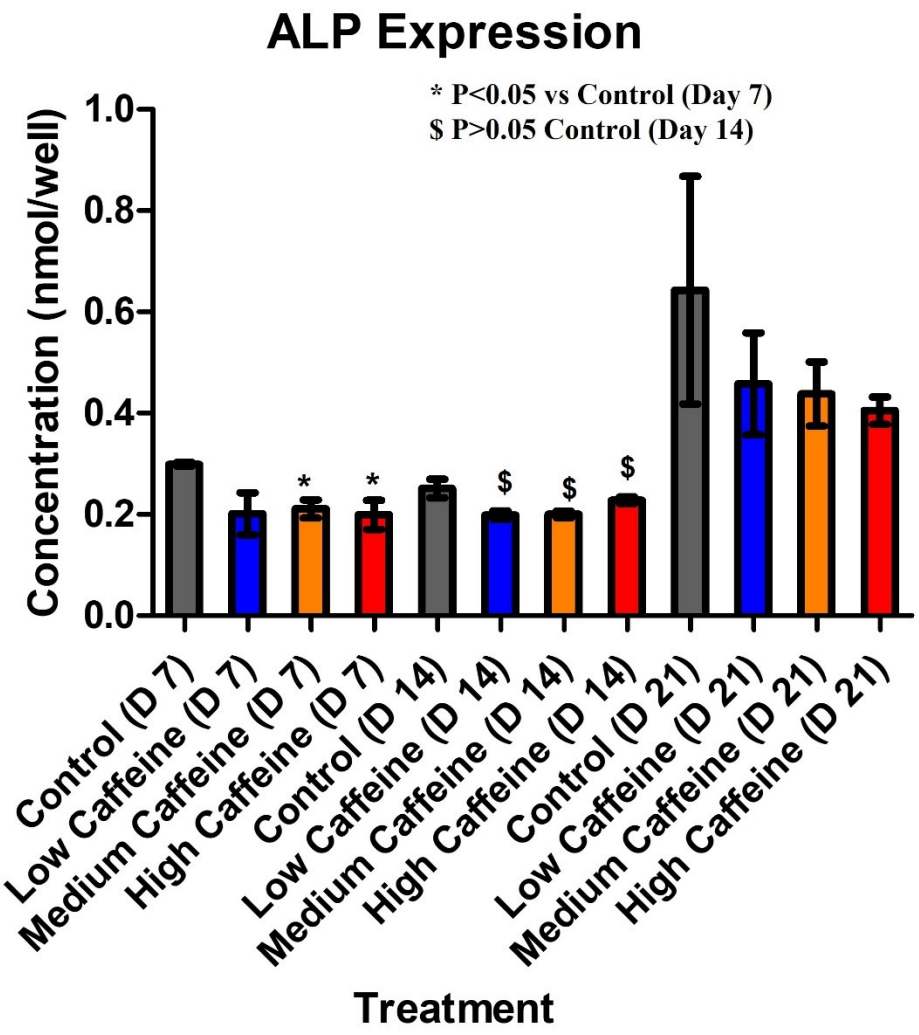
Figure 4: Quantitation of Cell Proliferation 24 hours post-$ZnCl_2$ treatment. A) MC3t3 Cell Line B) Raw 264.7 Cell Line. Low $ZnCl_2$ dose is 3uM, medium $ZnCl_2$ dose is 10uM and high $ZnCl_2$ dose is 30uM. n=3. Proliferation was quantified using the MTT assay and statistical analysis was completed using Prism.

II. *Quantification of ALP activity in 3D-BOM organoids Post-Caffeine and $ZnCL_2$ treatment.*

ALP activity was measured using the spent media samples collected on Day 7, 14 and 21. Caffeine treatment on Day 7 and 14 showed a significant decrease of ≥10% in ALP activity for all concentrations (Figure 5A, P≤0.003) when compared to control. By day 21, caffeine treatment still reduced ALP activity compared to control but only the high dose was found to be statistically different.

Only the medium $ZnCl_2$ dose was found to significantly increase ALP activity by 46% on day 7 (P<0.005) when compared to the control and other $ZnCl_2$ treatment groups (Figure 5B). The low and high $ZnCl_2$ doses were found to slightly increase ALP activity on day 14 when compared to control (P<0.05). The medium $ZnCl_2$ trended was also found increase ALP on day 14 but was not statistically different. No statistical differences were found for the high $ZnCl_2$ treatment group at any timepoint.

A


ALP Expression
* P<0.05 vs Control (Day 7)
$ P>0.05 Control (Day 14)
Concentration (nmol/well)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
$
$
$
Control (D 7)
Low Caffeine (D 7)
Medium Caffeine (D 7)
High Caffeine (D 7)
Control (D 14)
Low Caffeine (D 14)
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High Caffeine (D 21)
Treatment

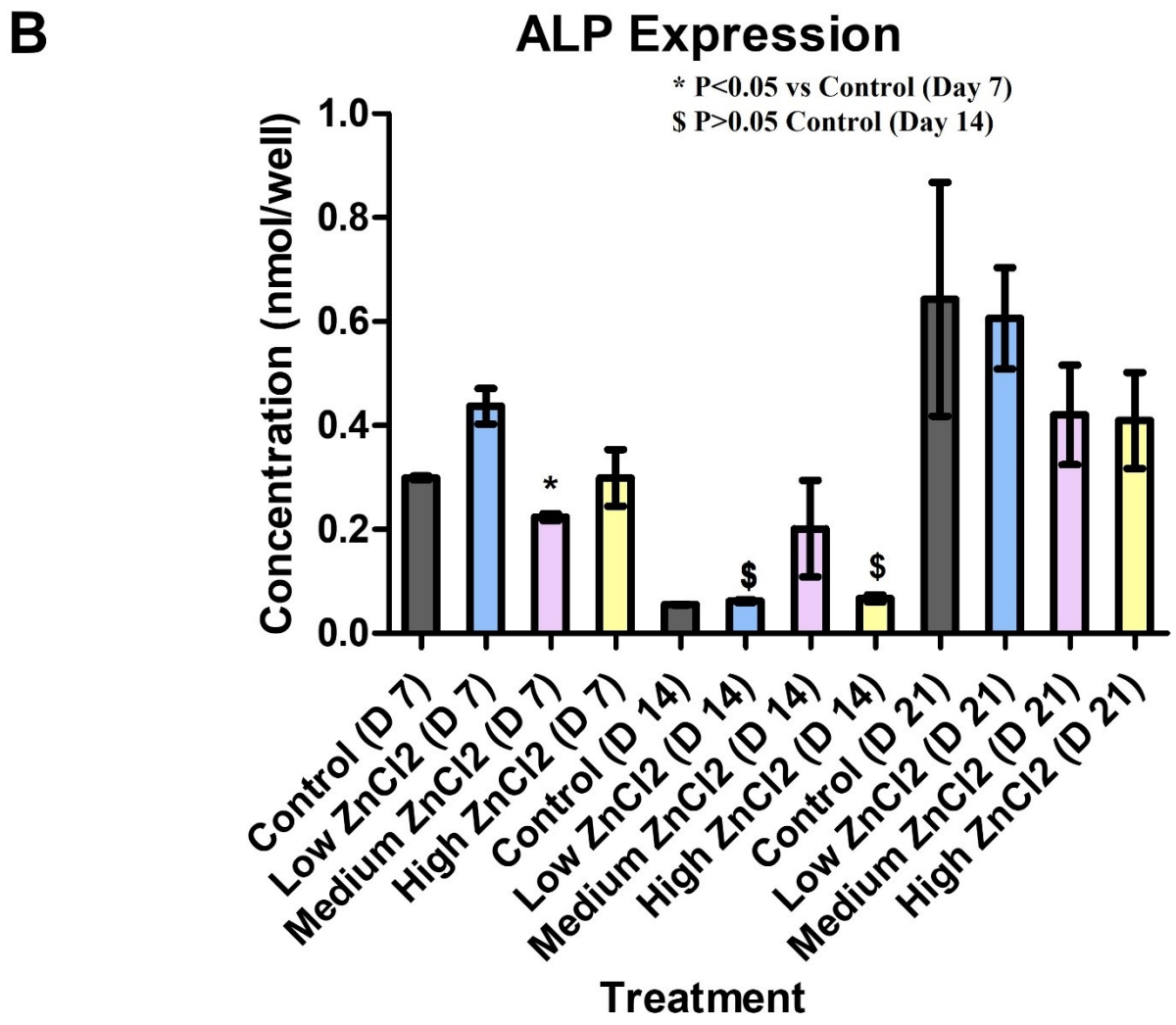


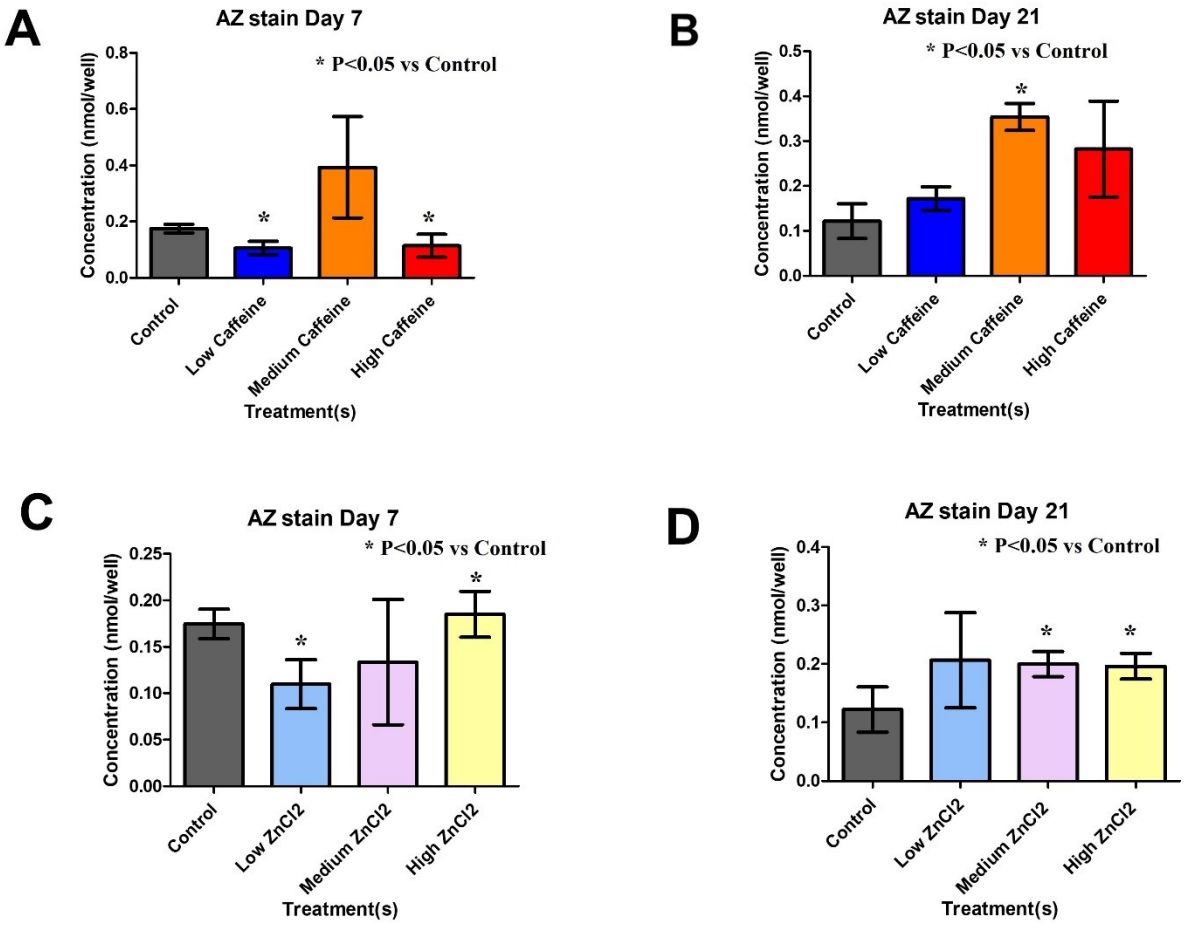
Figure 5: Alkaline Phosphatase Expression Levels were Measured on Day 7, 14 and 21 After Treatment A) Caffeine treatment; B) $ZnCl_2$ treatment. n=3. Statistical analysis was completed using Prism.

III. *Characterization of Calcium Deposition for 3D-BOMs Post Caffeine and $ZnCL_2$ Treatment.*

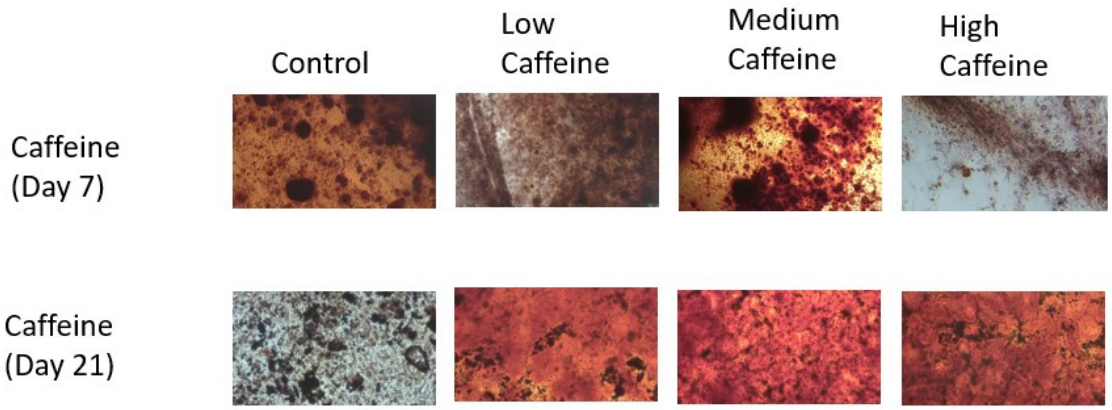
Calcium Deposition of the 3D-BOMs was completed on Day 7 by Alizarin Red Staining. The data show that on Day 7 when samples were treated with low and high caffeine (Figure 6A & 6E), calcium deposition decreased by 6.7%($P<0.05$) and 16.84% ($P<0.05$),

respectively. Conversely, the medium concentration of caffeine showed a 3.92% significant increase in calcium deposition ($P \leq 0.055$) on day 7. On Day 21, our results show a statistical of 23% and 16% increase, respectively, in calcium deposition when medium and high caffeine were compared to the control ($P<0.05$, Figure 6B&F).

When analyzing the effect of $ZnCl_2$ on calcium deposition in the 3D-BOM on day 7, our results showed a significant decrease in calcium deposition when low $ZnCl_2$ was compared to controls while high concentrations of $ZnCl_2$ were found to be significantly increased ($P<0.05$, Figure 6C&F). By Day 21, our results showed that all $ZnCl_2$ treatment increases calcium deposition, but it was not found to be significant (Figure 6D&F).


A
AZ stain Day 7
* P<0.05 vs Control
Concentration (nmol/well)
0.0
0.2
0.4
0.6
0.8
*
*
Control
Low Caffeine
Medium Caffeine
High Caffeine
Treatment(s)
B
AZ stain Day 21
* P<0.05 vs Control
Concentration (nmol/well)
0.0
0.1
0.2
0.3
0.4
0.5
*
Control
Low Caffeine
Medium Caffeine
High Caffeine
Treatment(s)
C
AZ stain Day 7
* P<0.05 vs Control
Concentration (nmol/well)
0.00
0.05
0.10
0.15
0.20
0.25
*
*
Control
Low ZnCl2
Medium ZnCl2
High ZnCl2
Treatment(s)
D
AZ stain Day 21
* P<0.05 vs Control
Concentration (nmol/well)
0.0
0.1
0.2
0.3
0.4
*
*
Control
Low ZnCl2
Medium ZnCl2
High ZnCl2
Treatment(s)

E



F

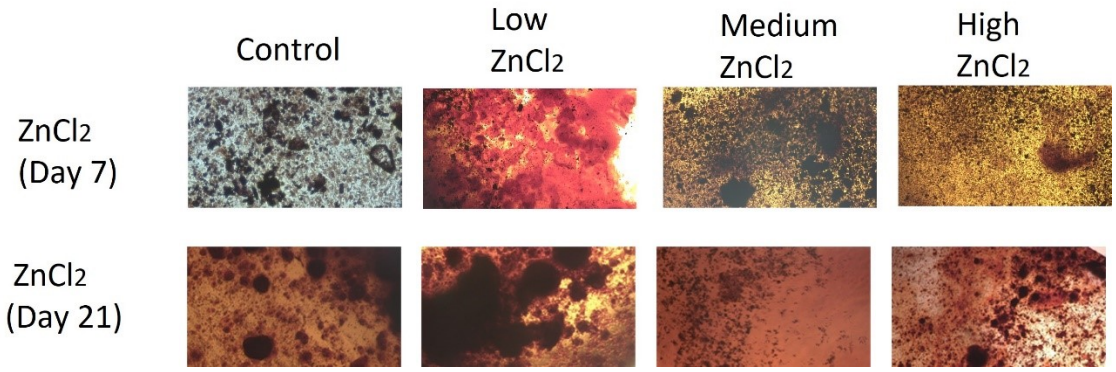
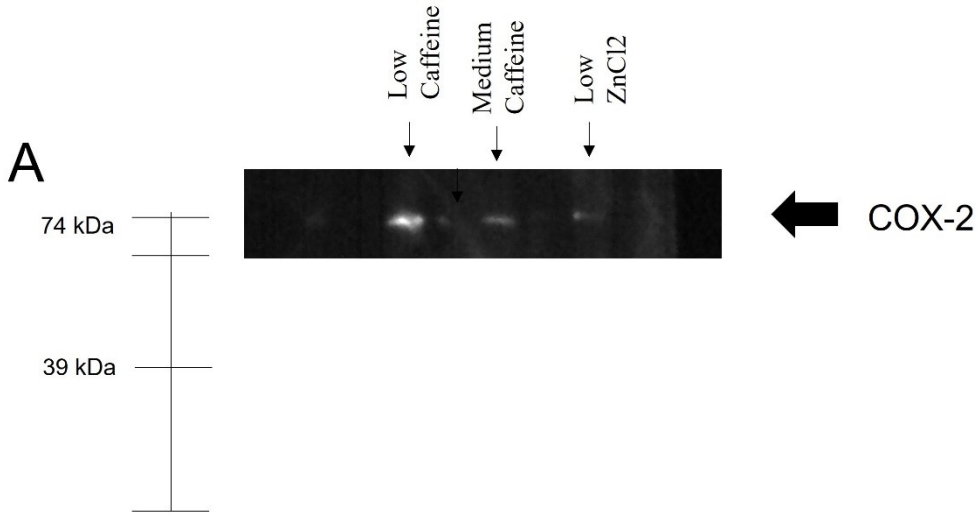


Figure 6: Calcium Deposition of 3D BOMs at Day 7 and 21. Quantification: A) Day 7, post-caffeine treatment. B) Day 21, post-caffeine treatment C) Day 7, post-$ZnCl_2$ treatment D) Day 21, post-$ZnCl_2$. Staining: E) Day 7 and Day 21 post-caffeine treatment (F) Day 7 and Day 21 post- $ZnCl_2$ treatment. n=3. Statistical analysis was completed using Prism.

IV. *Quantification of Protein for 3D-BOMs Post Caffeine and $ZnCL_2$ Treatment.*

3D-BOMs were evaluated for protein concentration using Western Blot analysis and BCA Assay using COX-2 and GAPDH primary antibody. Results show that low caffeine promotes COX-2 expression (Figure 7A). Low levels of COX-2 expression were also found in treatment doses of medium caffeine and low $ZnCl_2$. Figure 7B represents that protein concentration found at Day 21 through BCA analysis. When the low caffeine treatment group COX-2 expression is normalized to protein levels it was evident that the low caffeine group increases COX-2 expression.



V. Figure 7: Protein Quantification Post-caffeine and $ZnCl_2$ of treatment on Day 21. (A) Western Blot results of COX-2 expression.

Discussion

Bone is a remodeling tissue that continues to regenerate itself through its lifetime, utilizing both OB, OC, and osteocyte cells. Osteoblasts and osteoclasts work as one unit to remodel bone and continue to maintain bone homeostasis. Osteoblasts work as the bone reabsorption unit for aged/damaged bone cells, and osteoclasts work as the formation sector for new bones. The role of osteoblast cells allows for them to differentiate into osteocytes (osteoblastogenesis), although OBs are not terminally differentiated. Differentiation of osteoclast cells (osteoclastogenesis) is a product of several different secretary molecules including: M-CSF and RANKL. Together these molecules work with osteoblasts and osteocytes to initiate osteoclast differentiation (11). Without the constant commitment of OB, OC and osteocytes, bone homeostasis cannot be maintained.

The present study demonstrates that caffeine and $ZnCl_2$ have concentration dependent effects on OB and OC differentiation and function. OB activity was evaluated by measuring cell proliferation/viability while bone composition was evaluated by measuring calcium deposition and observing the 3D BOMs post-treatment histologically. Proliferation of caffeine-treated OBs (low dose) and OCs (high dose) increased when compared to controls (Figure 3A/3B). However, the medium levels of caffeine negatively affected both OB- and OC-treatment. Overall, our data shows that the effects of caffeine in these cell types is dose dependent. Similarly, Bode et al (3) found that caffeine concentrations had differential effects on cell proliferation. In this study, increasing caffeine dosages (0.25Mm, 0.5mM, 0.7mM, 1mM, 2mM, 4mM, 5mM and 80mM) given to JB6 cells to assess cell proliferation and apoptosis. The results found

that most concentrations of caffeine resulted in decreased cell proliferation, but an increase in cell proliferation was found specifically at 0.7 mM. This data supports our observation that caffeine concentration dose dependently affects cell proliferation.

Overall, caffeine treatment appeared to decrease osteoblast activity as measured through the ALP assay at all time points (Figure 5A&B). Conversely, $ZnCl_2$ treatment increased osteoblast activity at low dose and decreased activity high dose on Day 7. At other time points, $ZnCl_2$ appeared to have less of a significant effect on osteoblast function. Salesa et al (26) found that $ZnCl_2$ has diametric effects on cell proliferation. Using HaCA T cells and increasing concentrations of $ZnCl_2$, Salesa observed the effects of cell viability over a period of 3, 12, and 24 hours. Results showed that after 72 hours, cytotoxic effects were found in concentrations as low as 5 µg/mL of $ZnCl_2$. Whereas, positive effects were observed at $ZnCl_2$ concentrations of 10uM in RAW 264.7 cells (Figure 4B). Interestingly, the data found in this study coincides with the results of diametric effects found in Salesa's study.

Park et al (22) researched the effects of zinc on the differentiation of OB and results showed that zinc does promote OB differentiation though the activation of cAMP-PKA-CREB signaling pathway. In this study, Park et al (22) used human bone marrow samples of male and female donors, to study the effects of $ZnSO_4$ at 100 µM for 72 hours. Confirmation of the enhanced expression of zinc on OB, confirmed 100 µM $ZnSO_4$ to be the maximal noncytotoxic concentration to hBMSCs. The data found in this study coincides with the findings of Park et al, OB activity increases at day 21 in all groups treated with $ZnCl_2$, however as the concentration of $ZnCl_2$ increases, OB activity decreases (Figure 5B).

In support of Salesa et al's (26) study and the results of this study, Li et al (15) found similar results when measuring cell proliferation of Endometrial Epithelial Cells (EEC) of laying hens. Li et al (15) studied the effects of zinc on cell proliferation at 24 and 48 hours, when treated with 50 μM $ZnSO_4$. Results of this study coincide with those found by Salesa et al (26), Park et al (22) and the results of this experiment.

Development of the 3D BOM allowed for the evaluation of bone composition and calcium deposition. An early view of calcium deposition post-caffeine treatment on day 7, found that the low and high doses were detrimental to OBs. These results are further supported by Heaney et al, this data showed that caffeine consumption reduce bone mass in women aged 20-40 years old, at a total amount of 680mL of caffeine. Whereas, conditions of high caffeine in the 3D BOMs, results showed an increase in calcium deposition at 3 weeks (Figure 6F). In Heaney's study, bone consumption was measured by utilizing 7 beverages: white milk, chocolate milk, Mountain dew, Sprite, Coke, Coke-Free, Sprite-Free and water, as a negative control. $ZnCl_2$ treatment showed a similar trend with abilities to promote bone regeneration (20). On day 7, $ZnCl_2$ decreased calcium deposition when compared to control but by day 21 $ZnCl_2$-treated groups has significantly more calcium deposition. Results found in this study support those found by Togari et al, who found that higher doses of zinc (50 and 100mM) MC3T3-E1 cell cultures inhibit calcium deposition.

In support of high zinc dosages inhibiting calcium deposition, Voelkl et al (30) at investigated the effects of zinc through TNFAIP3-Mediated Suppression of NF-ƙB. This experiment was conducted using primary human aortic vascular smooth muscle cells (VSMCs) and serum samples from patients with Chronic Kidney Disease (CKD). The

results of this study differ from those found by Togari et al (29) and this experiment. Voelkl et al (30) found that cultures treated with $ZnSO_4$ blunted phosphate induced calcification.

As an essential component of bone, proteins make up approximately 50% of the volume of bone (29). Our preliminary data, shows the presence of COX-2 (prostaglandin -endoperoxide synthase 2) in the low and medium concentration caffeine group as well as the $ZnCl_2$ treatment groups (Figure 7A). The presence of COX-2 in low caffeine treatment indicated that it could stimulate COX-2 expression. Results of COX-2 expression in low caffeine doses counter the results found in *Kamagata-Kiyoura* study (14). Kamagata-Kiyoura et al (14) found that low caffeine did not stimulate COX-2 expression in a mouse model of ATDC5 cells. However, in this study a 3D model was utilized through the aide of OBs and OCs cell lines. Difference in the results of these studies could be explained by model and cell line differences between the studies.

It is well-established that COX-2 is an enzyme which plays a leading role in inflammation (7). As an element zinc participates in the regulation of proinflammatory responses (9). Preliminary results may suggest a role for COX-2 in caffeine or $ZnCl_2$-treated 3D-BOMS. In support of the findings, Zhang et al (35) demonstrated that increases of COX-2 expression can support the differentiation of OBs. Zhang et al (35) utilized three in-vivo models using wild-type mice to study bone healing and focused specifically on COX-1 and COX-2 expression patterns. The results of this experiment found that elevated levels of COX-2 can be related to increased OB differentiation. It is important to study the effects of COX-2 under conditions of caffeine or $ZnCl_2$. COX-2 inhibition is detrimental to bone healing, is an enzyme, however, under conditions of

$ZnCl_2$ effects can be positive. COX-2 has been studied to be an inflammatory marker with abilities to accelerate the formation of products that cause pain and inflammation (7). By studying the effects of these molecules, we can gain a further understanding of the human body in conditions of pain and inflammation. This study, along with numerous others that focus on inhibition of the COX-2 inflammatory marker can affect the process of bone healing for the field of medicine.

Future directions for this study could include measuring CTX1 levels, TRAP staining, and histology to observe bone composition. CTX1 levels will be utilized on the in-vitro 3D Murine Model to study osteoclast mediated bone resorption. TRAP staining is utilized to stain tartrate-resistant acid phosphatase in OC. Accessing osteoclast activity in the 3D-BOMs will provide us a more complete understanding of $ZnCl_2$ and caffeine effects on bone metabolism. Inflammatory marker, COX-2 will be further investigated to evaluate caffeine induced COX-2 expression in bone homeostasis. As well as the investigation of other inflammatory markers including TNF-α (Tumour Necrosis Factor), IL-1β, and IL-6 to better understand the effect of inflammation has on bone metabolism.

Conclusion

This study utilized a 3D Murine Bone Model to investigate the effects of caffeine and $ZnCl_2$ on bone homeostasis. In this study, results showed that $ZnCl_2$ has dose dependent effects on bone homeostasis. Whereas, caffeine is detrimental to bone homeostasis even in low doses (0.005 μM). In addition to negative effects on bone health, treatments of low caffeine were found to inhibit inflammatory marker; COX-2. Trace of COX-2 inhibition was evaluated under treatments of medium caffeine and low $ZnCl_2$. Results of this study aide to the science industry by allowing scientist to evaluate and study the effects of caffeine and $ZnCl_2$, while utilizing the 3D bone model to replicate conditions of homeostasis.

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