Tuning Thermal Dosage to Facilitate Mesenchymal Stem Cell Osteogenesis in Pro-inflammatory Environment

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Abstract

Mesenchymal stem cells (MSCs) are multipotent cells that can replicate and differentiate to different lineages of mesenchymal tissues, potentiating their use in regenerative medicine. Our previous work and other studies have indicated that mild heat shock enhances osteogenesis. However, the influence of pro-inflammatory cytokines on osteogenic differentiation during mildly elevated temperature conditions remains to be fully explored. In this study, human MSCs (hMSCs) were cultured with Tumor Necrosis Factor-alpha (TNF-α), an important mediator of the acute phase response, and Interleukin-6 (IL-6) which plays a role in damaging chronic inflammation, then heat shocked at 39ºC in varying frequencies – 1 hour per week (low), 1 hour every other day (mild), and 1 hour intervals three times per day every other day (high). DNA data showed that periodic mild heating inhibited suppression of cell growth caused by cytokines and induced maximal proliferation of hMSCs while high heating had the opposite effect. Quantitative osteogenesis assays show significantly higher levels of alkaline phosphatase activity and calcium precipitation in osteogenic cultures following mild heating compared to low heating or non-heated controls. These results demonstrate that periodic mild hyperthermia may be used to facilitate bone regeneration using hMSCs, and therefore may influence the design of heat-based therapies *in vivo*.

Key words: heat shock, human mesenchymal stem cells, osteogenic differentiation, cytokines

Introduction

Though bone tissue can regenerate in vivo, the repair of large bone defects and slow bone growth remain unsolved problems despite intensive research in biomaterials, chemical stimuli, and mechanical loading[1]–[3]. Currently, the gold standard for repairing large bone defects is autologous bone grafts[4]. Though they are widely used, there are a number of associated drawbacks including limited donor material, donor site morbidity, and high failure rates[5]–[7]. Allografts are limited by immunogenesis and synthetic grafts usually have poor osteoconductivity[8]. There is a need to develop new therapeutic strategies that produce high-quality bone and enable patients to have a speedy recovery from trauma or pathological bone loss.

Another obstacle to developing improved bone repair therapies is the inflammation that occurs at skeletal injury sites. While inflammation is a normal part of the healing process, chronic inflammation can result in tissue destruction rather than tissue repair. Interleukin-6 (IL-6) and Tumor necrosis factor-alpha (TNF-α) are two inflammatory cytokines that are generally considered to promote pathological tissue degeneration[9]–[12]. TNF-α promotes reduced bone formation by mature osteoblasts, increased osteoclastic resorption, and inhibits differentiation of osteoblasts from precursor cells[13]. It has also been shown to inhibit the synthesis of type I collagen and increase osteoblast resistance to vitamin D[14]–[20]. Studies have also reported that IL-6 may still contribute to tissue degeneration[19]–[21]. Because of their significant role in pathogenesis, potential clinical therapies for bone repair must be shown to be effective in the presence of pro-inflammatory cytokines and be able to decrease the damaging effects of pro-inflammatory cytokines during tissue regeneration.

Human mesenchymal stem cells (hMSCs) have been attracting considerable interest for their potential to restore, maintain, or improve tissue function. They can be easily isolated and cultured and have multipotent capacity for differentiation[22]–[25]. Thus, MSCs can do what current surgical techniques cannot: create a biologically viable tissue that retains functionality in the patient. An early study has indicated that heating 1.5ºC to 3ºC above regular body temperature plays a role in bone growth stimulation in rats and dogs[26]. Our own previous studies revealed that periodic heat shock at 41^{o}C enhanced not only osteogenic differentiation[27] but chondrogenic differentiation of hMSCs as well[28]. We also observed that HSP70 was significantly upregulated by heat shock in differentiated hMSCs[27], which has been shown to promote osteogenesis of hMSCs[29] as well as have anti-inflammatory properties[30]. Regular mild exercise, which several studies show increasing body core temperature to an average temperature of 39ºC[31]–[33], inhibits bone and cartilage degradation in patients with osteoarthritis[34]–[36]. Additionally, pain-relieving effects of heat have also been reported in patients with osteoarthritis[37].

It would be desirable to develop a method to facilitate MSC differentiation and enhance tissue regeneration in the inflammatory environment. Many studies have been performed to determine the properties of MSCs and the factors that lead to their differentiation in order to successfully apply them to achieve the properties of the original tissue. Mild, periodic hyperthermia may be a promising therapy that regulates MSC differentiation, mitigates inflammation, and could be also easily administered. However, no *in vitro* experiments have been conducted which demonstrate the optimal heating protocol that maximizes cell proliferation and facilitates tissue regeneration in the inflammatory environment. The intensity and duration of heat stimulation used for hyperthermia are empirically determined, and its effect has not been

scientifically proven. In order to effectively use mild hyperthermia in future therapies, it is necessary to determine its effect on MSC growth and differentiation as well as the optimal intensity and duration of heat stimulation in human cells first.

This study investigated the effect of different doses of mild heating on human mesenchymal stem cells undergoing osteogenic differentiation when cultured with pro-inflammatory cytokines TNF-α and IL-6. Concentrations of cytokines (4pg/mL TNF-α, 300ng/mL IL-6) were chosen based on the median of a range of cytokine concentrations found in the synovial fluid of inflamed knee joints[38]. Mild heating at 39ºC was chosen to mimic temperature during inflammation and exercise[31]–[33]. Heating intervals were chosen partly based on our previous studies[27] and on the average workout intervals of exercising adults. Osteogenic differentiation was measured by alkaline phosphatase activity and calcium precipitation. The results of this study may benefit further investigations toward thermal treatments of skeletal injury.

Methods and Materials

Cell Isolation and Culture

Human bone marrow explanted from the iliac crest of donors was purchased from AllCells, LLC (Berkeley, CA). Human mesenchymal stem cells were enriched using the RosetteSep MSC enrichment cocktail (StemCell Technologies, Vancouver, Canada) per manufacturer's protocol and expanded in tissue culture flasks with MSC growth medium consisting of Dulbecco's modified Eagle's medium - low glucose, 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) then incubated at 37°C and 5% CO2. Fluorescence-activated cell sorting (FACS) analysis was performed on the hMSCs using a FACSCalibur flow cytometer (BD Biosciences) and tested

positive for surface markers CD146, CD44, CD29, and CD147 and negative for hematopoietic cell markers CD45 and CD34.

Human MSCs were subcultured at a density of 5000 cells/cm^2, and seeded in 24-well plates at Passage 4 with the same density in MSC growth medium (n=4). Growth media was replaced with cytokine-supplemented media the day after seeding to simulate inflammation. Osteogenic differentiation was induced the following day (day 0) with osteogenic medium consisting of MSC growth medium supplemented with 50μM ascorbic acid phosphate (AsAP) (Wako Chemicals USA, Richmond, VA), 0.1μM dexamethasone, and 10mM β-glycerol phosphate, and cytokine-supplemented where needed. Media was changed twice-weekly.

Heat Exposure with Calibration and Conformation of Heating Time

To quantitatively estimate the time it takes for the media in a well of a 24-well plate to heat up from 37^{o}C to 39^{o}C a simple heat transfer computational model was implemented in COMSOL and an analytical calculation was also performed. For both approaches some model parameters are shown in Figure 1A and tabulated in Table S1 in Supplementary Materials. Briefly, the system was modeled as a stack of cylinders with a diameter of 15.6mm, media volume of 0.5ml, a total volumetric capacity of 3.4ml, and polystyrene bottom and top (lid) thicknesses of 1mm each. It was assumed heat transfer only occurred from the top and bottom surfaces and the boundary temperatures at the top and bottom of the system was maintained by the convective incubator at 39^{o}C (boundary conditions). The side wall of the cylindrical model of the well is treated as adiabatic. Computing the heat transfer manually, we model a thermal resistive circuit as shown in Figure 1A. Thermal resistivity can be calculated for each component using the standard formula R = L/kA, where L is the length of the component, A is the cross-sectional area, and k is the thermal conductivity. For a thermal conductivity model, each

component is in series, and the total thermal resistivity is computed by the cumulative sum of individual resistivity. Breaking the thermal conduction into two super-positioned components partitioned across the media layer at node C in Figure 1A, we compute a thermal resistivity across A to C, $R_{A\text{-}C}$ as 3438 ^{o}C/W, and across C to E, $R_{C\text{-}E}$ to be 57.64 ^{o}C/W. Assuming that all the heat transferred is to raise the temperature of the media, more than 98.5% (3438/(3438+57.64)) of the heat conducted will be through the $R_{C\text{-}E}$ path. The following equations are then utilized to solve the problem:

$q = \frac{\Delta T}{R_{C-E}} = \frac{(39℃-T)}{R_{C-E}}$, where q is the rate of heat transfer, and T is the media (water) temperature;

$\frac{dT}{dt} = \frac{Heat\ Transfer\ Rate}{Heat\ Capacity} = \frac{q}{\rho V C_w} = \frac{(39℃-T)}{(R_{C-E})\rho V C_w} = \frac{(39℃-T)}{(R_{C-E})*2.094} = \frac{(39℃-T)}{(120.5\ sec)}$,

where ρ, V, and C_w are the density, volume, and specific heat of media (water), respectively.

Solution of the simple differential equation above gives: $T(in\ ℃) = 39 - 2e^{-t/120.5\ sec}$

The heating incubator was pre-calibrated with an accuracy of ±0.2°C. Using a digital thermometer it was measured that it took about 16 minutes for the temperature of water in the culture well to reach 39°C after a 24-well plate was moved from a 37°C incubator to a 39°C one. Therefore the actual time that culture plates stayed in the heating incubator was 1 hour and 16 minutes for an hour heating at 39°C. Human MSCs were exposed to three patterns of heat shock (HS) at 39°C: 1) low dose at 1 hour once per week on day 2, 9, and 16, 2) mild dose at 1 hour every other day from day 2 to day 18, and 3) high dose at 1 hour heating three times per day with 5 hours "resting" at 37°C between each heating interval, and this process was repeated every other day. Media was changed after heating to account for evaporation then cells were returned

to 37°C. Control cell cultures remained in 37°C had their media changed too to keep the experimental conditions the same for all samples.

Alkaline Phosphatase Activity

Alkaline phosphatase (ALP) activity was quantified using a colormetric assay using *p*-nitrophenol. Medium was removed from MSCs and the cells washed twice with PBS then lysed with 0.5% Triton X-100 lysis buffer (Bio-Rad Laboratories, Hercules, CA). They were incubated with alkaline buffer solution containing 5mM *p*-nitrophenol phosphate (PNP) and ALP substrate solution for 15 min at 37°C. Specific ALP activity expressed as nanomoles of PNP/mL/min, was measured at 405nm using a SpectraMax M2e microplate reader (Molecular Devices, Silicon Valley, CA) and quantified against a standard curve of *p*-nitrophenol.

DNA Content

Some cell lysates prepared for the ALP assay were measured for DNA content using the Quant-iT™ PicoGreen ® dsDNA Reagent kit (Invitrogen, Carlsbad, CA). Briefly, 75µL of 13.22 mg/mL pepsin in 0.05N acetic acid was added to 300µL of cell lysate and incubated at 2-8°C for 24 hours in order to digest excess protein in the lysate. The pepsin was neutralized with 75µL of pH 8.0 Tris buffer following the incubation period. Supernatant was used with PicoGreen fluorescent dye solution. A SpectraMax M2e reader at excitation of 480nm and emission of 520nm was used for fluorescence measurement with a DNA standard curve.

Calcium Deposition for Mineralization

Calcium levels were determined using the StanBio Total Calcium Procedure (Fisher Chemical Co., Los Angeles, CA) on day 19. The MSCs were washed twice with PBS then lysed using a solution of 0.5N HCl in distilled water. Three microliters of lysate was added to a 96-

well plate with 300µL of provided color reagent, then read at 550nm using a SpectraMax M2e microplate reader and quantified against a standard curve.

Visualization of Minerals in Osteogenic MSCs by von Kossa Staining

The morphology changes of hMSCs in different culture conditions were observed by phase microscopy using a Zeiss Axio Observer Z1 Inverted microscope. Samples were stained for minerals by the von Kossa method on Day 6 and 19 to confirm the mineralization. Briefly, hMSCs were rinsed with Tyrode's balanced salt solution (Sigma-Aldrich, St Louis, MO), fixed with 10% buffered formalin (Fisher Scientific, Pittsburgh, PA) for 30 mins, incubated with 2% silver nitrate solution (Sigma-Aldrich, St Louis, MO) for 10 mins in the dark, rinsed thoroughly with distilled water, and then exposed to bright light for 15 mins. The samples were subsequently observed and bright field images were captured with a Zeiss Axiovert 40 CFL inverted microscope with a color camera.

Statistical Analysis

Analysis of variance (ANOVA) with Bonferroni post-hoc testing was used to determine significance with a *p*-value less than 0.05 taken as statistically significant.

Results

Time for Culture Media to Reach 39^{o}C from 37^{o}C

The average temperature of the media (water) volume over time from heat transfer modeling is plotted in Figure 1B, and snapshots of the evolving computational solution are shown in Figure 1C at 0.5, 1, 3 and 5 minutes. Simulation results suggest that the temperature stabilizes to 39^{o}C within 10.5 minutes. On the other hand, using the standard 5 time-constant point at which an exponential reaches ~99.3% of the maximum/minimum value, we evaluate ~603 seconds or just over 10 minutes for the media to heat up from the analytical calculation. A

similar evaluation across R_{A-C} gives about 10 hours, we therefore ignore the thermal contribution across this path. If heating of the polystyrene bottom is considered, using only R_{D-E}, with an average specific heat capacity of 1.4 J/g-K, it adds an additional 0.8 minutes to the heating time to reach 39^{o}C equilibrium. These evaluations are resonant with the simulation results predicting ~10.5 minutes to reach thermal stability. In practice, we find that it takes about 16 minutes for the media to warm up to 39^{o}C in the incubator, as several thermo-physical practical factors (e.g. open the door of an incubator in order to load a sample plate) affect the incubator and dish temperature during and after sample loading.

DNA Content is the Highest from the Mild Heating Dose

MSCs cultured with TNF-α and IL-6 had significantly lower DNA content on day 6 compared to controls (no cytokines) at 37ºC (Fig. 2A). The difference was much greater in TNF-α cultures. Not surprisingly, at day 12, all cultures kept at 37°C have the same cell population most likely due to the limited surface areas for cells to grow in the 2D culture conditions. Heating MSCs at 39ºC for 1 hour once every two days (1x/2days), the mild heating dose, resulted in significant increases in DNA content in the early days of differentiation (days 6 and 12) in cytokine cultures (Fig. 2C, 2D). MSC cultures without cytokines shown similar proliferation to those cultured with IL-6 over time (Fig. 2B, 2C). Heating MSCs at 39ºC for 1 hour once per week (1x/wk) also resulted in significantly higher DNA content on day 6 and day 12 for cultures with IL-6 and TNF-α compared to respective controls at 37ºC. In cultures without cytokines, DNA was indifferent from respective controls at 37ºC following this heat treatment (Fig. 2 B). Compared to other heating and non-heating patterns, heating MSCs at 39ºC for 1 hour three times every two days (3x/2days) resulted in significantly lower DNA content across all time points in cultures without cytokines or with IL-6 (Fig. 2B, 2C), and in cultures with TNF-α

at day 12 (Fig. 2D). This apparent indication of the 3x/2day heating regimen inhibiting cell proliferation precludes its inclusion in further experiments.

ALP Activity is the Highest from the Mild Heating Dose

Alkaline phosphatase (ALP) activity is a marker of early stage osteogenic differentiation[39]. Dynamic ALP activity was first studied with low (300pg/mL) and high (20ng/mL) IL-6 concentrations between days 6 and 14 of differentiation using one heating dose, heating at 39°C for one hour once a week, to identify when ALP activity plateaus. The high cytokine concentration is often used in *in vitro* studies[40]–[42], and is selected here to observe ALP responses compared to the physiological condition with a low cytokine concentration. Figure 3C and 3D show that ALP activity is significantly higher in osteogenic cultures than in cultures in growth media. ALP activity was also significantly higher in MSCs with heating between days 6 and 10, though this varied with IL-6 dosage (Fig. 3C, 3D). 20ng/mL IL-6 in osteogenic cultures significantly increased ALP activity compared to control cultures (no cytokines) at 37°C and after periodic heating at 39°C (Fig. 3A, 3B). 300pg/mL IL-6 in osteogenic cultures significantly increased ALP activity only on day 8 and day 12 at 37°C. ANOVA also showed that ALP activity was significantly higher in high-dose cultures (20ng/mL) than low-dose cultures (300pg/mL) at both 37°C and 39°C, except on day 12 (Fig. 3A, 3B). In general, ALP activity was shown to be correlated with temperature elevation from 37°C to 39°C and IL-6 concentration, plateauing between day 10 and 14. Therefore, in following large and comprehensive study of ALP activities experimental samples was only collected on Day 6 and 12.

Next, we studied the effects of thermal dosing on the total ALP activity in hMSCs while exposed to inflammatory cytokines IL-6 and TNF-α (300pg/mL and 4pg/mL respectively) to

identify under what conditions we can observe maximum total ALP activity. After observing ALP activity peaking around day 12, we chose to observe the difference in total ALP activity at days 6 and 12. Compared to undifferentiated controls in growth medium, differentiated samples in osteogenic medium showed a significant increase in ALP activity at day 6 and even more at day 12 (Fig. 4 and Supp. Table S2, osteogenic conditions) when kept at 37ºC. Periodic mild heating at 39ºC significantly increased ALP activity in all osteogenic culture conditions by day 12 by up to 92% compared to controls at 37ºC, the only exception being cytokine-free culture heated 1x/2days which showed no difference. There was little to no difference in ALP activity between the heating groups at day 6. The 1x/week (low) and 1x/2days (mild) heating patterns increased ALP activity by about 36% and 31% respectively. This upregulation of ALP activity by periodic heat shock is consistent with what we have previously reported[27].

Calcium Deposition is affected by Pro-inflammatory Cytokines

Calcium deposition was used as an indicator of osteogenesis and maturation of osteoblasts differentiated from MSCs[43], [44]. Calcium deposition was negligible in all cultures containing growth media, and is excluded from Figure 5. In osteogenic cultures without cytokines kept at 37ºC, heating 1x/wk (low heating dose) did not appreciably increase calcium content after 19 days. Heating 1x/2days (mild heating dose), however, significantly increased calcium content by 75.2%. Osteogenic cultures supplemented with inflammatory cytokine TNF-α and incubated at 37ºC generated significantly more calcium compared to no-cytokine controls in the same condition, whereas osteogenic cultures with IL-6 contained about the same amount of calcium. We observed less calcium content after heating cytokine-supplemented cultures with our low heating dose (i.e. 1 hour at 39ºC 1x/wk). Interestingly, heating cytokine-supplemented

cultures to our mild heating dose (i.e. 1 hour 39ºC 1x/2days) induced as much calcium content as found in parallel cultures kept at 37ºC.

Morphological and Mineral Appearance of MSCs Changes during Osteogenic Differentiation

Images of Von Kossa stained cultures in Figure 6 show that hMSCs undergo significant changes in morphology during expansion in culture in osteogenic medium compared to the undifferentiated control in growth medium. Cells lose their spindle-like morphology as they differentiate toward an osteogenic pathway, and mineralization is visibly higher in osteogenic cultures compared to controls. Mineralization was also observed to be higher in the no-cytokine osteogenic condition following periodic heat shock compared to osteogenic controls kept at 37°C, as well as heat shocked osteogenic cultures supplemented with pro-inflammatory cytokines.

Discussion

The effects of periodic heat shock at different thermal dosing and frequencies are herein investigated. Human MSCs in osteogenic medium formed mineralized aggregates in 2D culture plates (Fig. 6). A mild periodic heat shock regimen (1 hour 39°C every other day) significantly improved cell proliferation (Fig. 2) in the early stage of differentiation, even when cultured with pro-inflammatory cytokines. Cell differentiation and maturation, represented by ALP activity and calcium content respectively, were also enhanced by periodic mild heat shock including in biochemically stressing (Fig. 3, 4, 5) conditions. Overall, these results demonstrate that our mild periodic heating can mitigate osteogenic differentiation in the IL-6 or TNF- α simulated pro-inflammatory environment.

Both IL-6 and TNF-α depressed cell growth up to day 6 (Fig. 2A). Previously, a 10ng/mL dose of TNF-α was found to significantly inhibit MSC growth in low-serum culture[45]. Another

study showed lower MSC content in osteogenic cultures with 10pg/mL TNF-α compared to control but the difference was not significant[46]. Studies performed with IL-6 show conflicting results. A 5ng/mL dose of IL-6 did not inhibit MSC growth until day 7[47], but a 10ng/mL dose significantly enhanced MSC proliferation[48]. In the present study, heating was shown to mitigate suppression of cell growth, evidenced by higher DNA content following heat shock at day 6 (Fig. 2C, 2D). Interestingly, cell mass in all cultures were approximately the same by day 12 at 37°C (Fig. 2A). Given the near-confluency of cell cultures by day 6 as shown in Fig. 6, we believe cell growth is limited by available space and contact inhibition sets in by day 12. Previous studies have shown that TNF-α can stimulate hMSC proliferation (at 3ng/mL)[49], possibly via activation of IκB kinase 2 (IKK-2)[50]. IL-6 was also shown to enhance MSC proliferation (at 10ng/mL)[48]. It is worth noting that these studies used higher concentrations of TNF-α and IL-6, sometimes with different media formulations, than the present study. At the time of this writing, the present study is the only one to observe hMSC osteogenesis using physiological concentrations of pro-inflammatory cytokines.

Exposing MSCs to 39ºC for 1 hour every two days (i.e. mild heating dose) had the most significant effect on proliferation in the early days of differentiation. This 1x/2days heating pattern maximized cell proliferation on day 6 in cultures with IL-6 (Fig. 2C) and TNF-α (Fig. 2D). The highest cell content was observed on day 12 in osteogenic cultures with IL-6 and cultures without cytokines exposed to the same thermal dosage. This is partly corroborated by a previous report demonstrating that heat shock induces proliferation of hMSCs[51], though not significantly. And Shui *et al.*'s study does not include the effects of pro-inflammatory cytokines. We observed cell mass significantly decrease after 19 days in all cultures heated 1x/wk as well as IL-6 (Fig. 2C) and no-cytokine cultures (Fig. 2B) heated 1x/2days. This may be because cell

mass begins to decrease following a period of over-confluence around day 12[52]. At the highest thermal dosing, 3x/2days, we observed minimal cell mass at day 6 followed by gradual cell growth through day 19. Heating at 39°C 3x/2days significantly depressed cell proliferation in this study. Shui *et al*. found a 1 hr exposure of hMSCs at high temperature (42.5-45°C) inhibited cell growth as well as a 96 hr exposure at 40-41°C[51]. Other studies have shown that heat shock at 45°C can lead to premature senescence and even apoptosis[53, 54]. In those studies, Alekseenko *et al.* used shorter intervals (10 and 30 minutes) than the present study, but this nonetheless corroborates our observation that high thermal dosing inhibits cell growth over time. Overall, our mild heating protocol appears to inhibit cytokine-induced apoptosis during the early days of differentiation and promote proliferation, potentially having a significant impact on future *in vivo* applications.

ALP activity and calcium deposition observed following periodic heat shock are consistent with previous studies done by this lab and others[27] [51]. Briefly, Shui *et al.* showed that ALP activity and calcium content increased linearly after exposing cells to temperatures ranging from 33°C to 41°C for 1 hour every 3 days up to 21 days. Chen *et al.* also showed increased ALP activity and calcium content in hMSC cultures at day 6 following 1 hour heat shock at 41°C. ALP activity is a dynamic process that usually peaks between day 9 and 12 during osteogenic differentiation depending on the donor[55]. Heat shock enhances differentiation by shifting peak ALP activity earlier than normal[51], meaning ALP activities peaked earlier than day 9 under 41°C stimulation. However, our current study observing the effects of heat shock at 39°C 1x/week on ALP activity in hMSCs cultured with IL-6 (Fig. 3) showed ALP activity peaking between days 10 and 14. As shown in Fig. 5 and Supp. Table S2, ALP activity increased with thermal dosing during osteogenesis. Other studies conducted heat

shock at 41°C but our current study was performed at 39°C. Because ALP activity increases with increased thermal doses, this may explain a slight shift of ALP peaks between those findings and ours. We observed significantly more ALP activity in cultures with high-dose IL-6 (20ng/mL) compared to low-dose (300pg/mL) all else being equal (Fig. 3), demonstrating that ALP activity is sensitive to IL-6 concentrations. While the function of IL-6 in osteoblastic differentiation is not clear, evidence suggests it can upregulate ALP activity[56].

Calcium content after 19 days in osteogenic culture was highest after heating to 39ºC for 1 hour 1x/2days (Fig. 5). Our previous findings did show that 1 hour heating at 41°C once a week increased calcium mass on day 19[27], but this is the first study that attempts to find an optimal thermal dosage using a physiological temperature. Periodic heating is thought to increase mineralization and enhance osteogenesis overall by upregulating heat shock proteins which activate the ERK signaling pathway[57], and our previous findings showed a correlation between upregulated heat shock protein 70 (Hsp70) and upregulated transcription of osteogenic markers[27]. The exact role of heat shock proteins in heat-enhanced hMSC osteogenesis was also investigated in our study using shRNA knockdown, and results demonstrated downregulation of HSP70 impaired hMSC osteogenic differentiation and inhibited the enhancement of MSC differentiation by a mild thermal treatment [67].

Pro-inflammatory cytokines like TNF-α are known to inhibit osteogenic differentiation via activation of NF-κB[58]. However studies have also reported that TNF-α can stimulate ALP activity and mineralization despite inhibiting osteogenic transcription factors[59]–[61], which might explain why we observed higher mineral content in cultures containing TNF-α compared to parallel cultures that contained IL-6 or were absent of cytokines (Fig. 5). It is unknown why there was no difference in calcium mass between cytokine-free cultures kept at 37ºC and those

heated to 39ºC 1x/wk, but it is possible that the heat stimulation was too weak to generate an appreciable difference. By comparison, Shui *et al.* heated to 39°C for 1hr every 3 days, and Chen *et al.* heated to 41°C once per week. There is also the question of why heating cytokine-supplemented cultures to 39ºC 1x/wk generated less calcium than parallel cultures kept at 37ºC. These data imply that very weak heat stimulation may in fact inhibit mineralization in an inflammatory milieu. The mechanisms for heat-induced osteogenesis are still not completely understood, especially in the inflammatory conditions.

Human MSCs in osteogenic media formed small mineralized nodules as early as day 6 in heat shocked cultures (Fig. 6). Using a mild heating pattern to increase mineral content in an inflammatory milieu may have important implications for a thermal-based stem cell therapy for bone regeneration *in vivo*.

In most osteogenic cultures at day 19, the cell culture area appears discontinuous as differentiating cells contract and aggregate. It is known that cells sense the rigidity of their supporting substrates by exerting contractile forces through integrin adhesion complexes - forces that are generated by the polymerization of actin fibers[62], [63]. The connection between integrin adhesion complexes to actin filaments and the rest of the cytoskeleton forms the cell's mechano-receptive network. Osteogenic differentiation affects the mechanobiology of hMSCs, inducing changes in the cytoskeletal structure, specifically, shifting from numerous thin actin microfilament bundles to a few thick actin bundles[64], [65], concomitant with a decrease in elasticity. This may explain the appearance of our 2D osteogenic hMSC cultures. One study showed that dexamethasone increases cell stiffness of alveolar epithelial cells by influencing polymerization of actin microfilaments[66], and may have a similar effect in hMSCs due to the fact that osteogenic culture medium has dexamethasone.

Conclusions

In this study, the effects of different intervals of periodic hyperthermia on hMSC osteogenesis during inflammation were studied. Heat generally appears to be more influential within the first two weeks of the culture. Mild periodic hyperthermia (i.e. 1hr 39℃/2days) improved proliferation and osteogenesis of MSCs, and mitigated the inhibition of pro-inflammatory cytokine effects on MSC growth in the early stage of differentiation. The finding may potentially impact future *in vivo* applications, and this thermal dosing will be selected for future studies. Our high-dose heating pattern at 3 times a day on alternating days resulted in suppressed cell growth over time, suggesting this heating pattern as a poor choice for potential tissue regeneration therapies.

Future investigations will seek to verify this heating optimization in a 3D culture and translate these findings to develop a practical heat-based therapy *in vivo*. Hyperthermia is commonly applied to the rehabilitation of musculoskeletal disorders, and our results demonstrate the potential to mitigate inflammation and improve bone tissue regeneration.

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The authors declare no conflict of interest regarding the publication of this paper. No competing financial interests exist.

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Figure Legends

Figure 1. Heating model for simulation and analytic evaluation. **A)** (Left) Shows the 3D model generated from the physical dimensions of a 24-well culture plate, with appropriate depths of the polystyrene bottom, media, air, and polystyrene lid. (Right) Each interface is considered a node in a conductive thermal resistive model, with the end points (A and E) being the incubator environment conditions maintained at 39^{o}C. The thermal resistances of each component has been computed according to their thermo-physical characteristics. **B)** Shows the COMSOL results of the average media temperature from the time-dependent thermal model from (A), with initial conditions of 37^{o}C of all components except at the boundary. The average temperature reaches the boundary temperature of 39^{o}C within 10.5 minutes. **C)** Left to right: Cross section at the mid-plane of the cylindrical well showing the time-evolving temperature profile along 0.5, 1, 3 and 5 minutes, respectively. The media warms up to over 38.5^{o}C at the coolest regions within the first 5 minutes.

Figure 2. DNA content of differentiating hMSC cultures supplemented with cytokines IL-6 and TNF-α heat shocked at 39°C for 1hr at varying frequencies – once per week (1x/wk), once every 2 days (1x/2days), and 3 times every 2 days (3x/2days), (n=4). **A)** Cells cultured at 37°C with cytokines **B)** Cells exposed to heat treatments without cytokines **C)** Cells exposed to heat treatments cultured with IL-6 **D)** Cells exposed to heat treatments cultured with TNF-α. Dashed lines = growth culture, solid lines = osteogenic culture, * = significant difference compared to respective controls at 37°C (green lines) ($p < 0.05$)

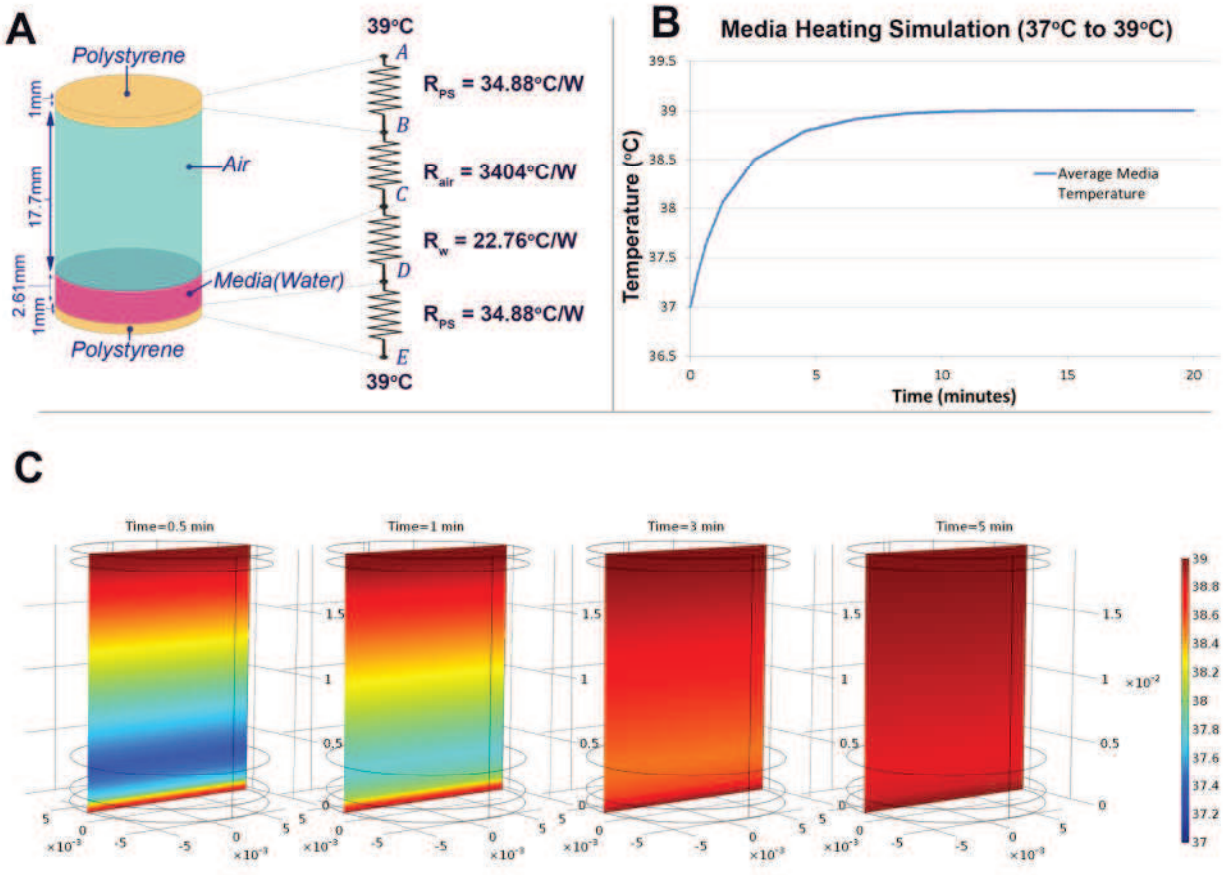
Figure 3. Alkaline phosphatase activity in differentiating hMSC cultures supplemented with IL-6 over time, (n=4) **A)** Cells cultured at 37°C with IL-6 doses **B)** Cells exposed to 39°C for 1 hr

once per week with IL-6 doses **C)** Cells cultured with 20ng/mL IL-6 and exposed to 39°C heat once per week **D)** Cells cultured with 300pg/mL IL-6 and exposed to 39°C heat once per week. Dashed lines = growth culture, solid lines = osteogenic culture, * = significant difference between osteogenic and growth conditions ($p < 0.05$), @ = significant difference between heating and non-heating osteogenic conditions ($p < 0.05$), # = significant difference between 20ng/mL IL-6 cultures and cultures without cytokines in osteogenic conditions ($p < 0.05$), + = significant difference between 300pg/mL IL-6 cultures and cultures without cytokines in osteogenic conditions ($p < 0.05$), ^ = significant difference between two IL-6 concentrations in osteogenic conditions ($p < 0.05$).

Figure 4. Heat map of alkaline phosphatase activity (nmol/mL/min) in differentiating hMSC cultures supplemented with cytokines IL-6 and TNF-α and heat shocked at 39°C for 1hr at varying frequencies, (n=4). All right-side comparisons are between osteogenic conditions. * = significant difference at day 6 ($p < 0.05$), ^ = significant difference at day 12 ($p < 0.05$).

Figure 5. Calcium content of differentiating hMSC cultures supplemented with cytokines IL-6 and TNF-α heat shocked at 39°C for 1hr and varying frequencies through day 19, (n=4). OM = Osteogenic medium without cytokines, * = significant difference between marked groups ($p < 0.05$).

Figure 6. Phase contrast images of hMSCs in culture following von Kossa staining. Positive black stain indicates mineral content in culture. Scale bar = 100μm.



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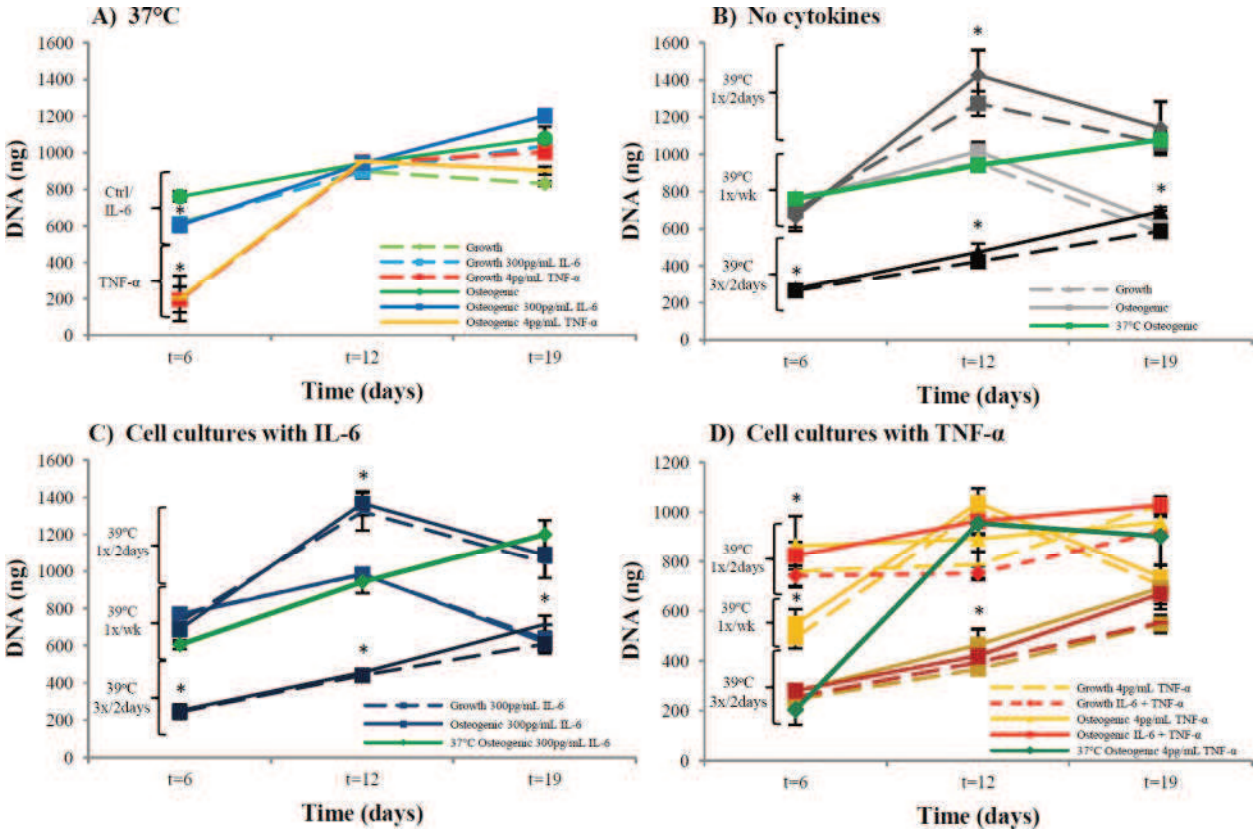

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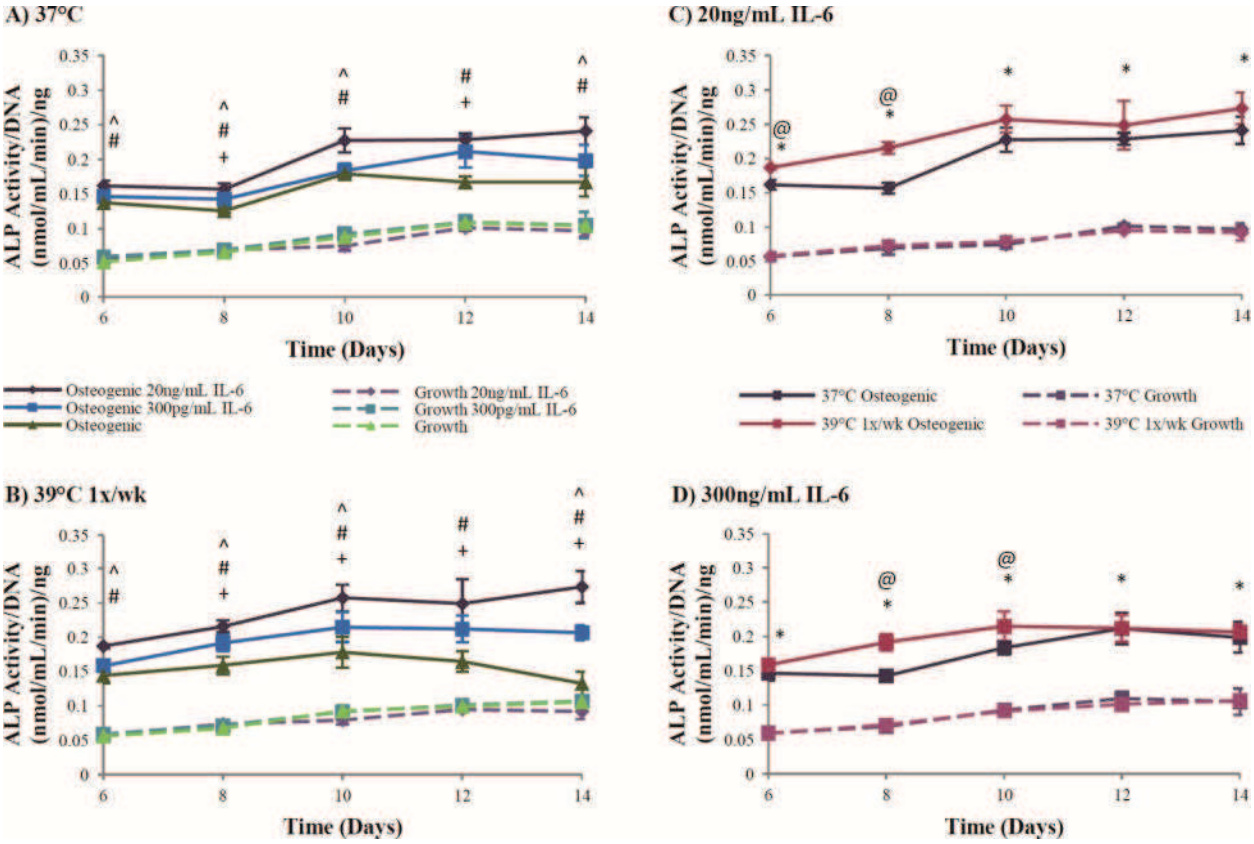

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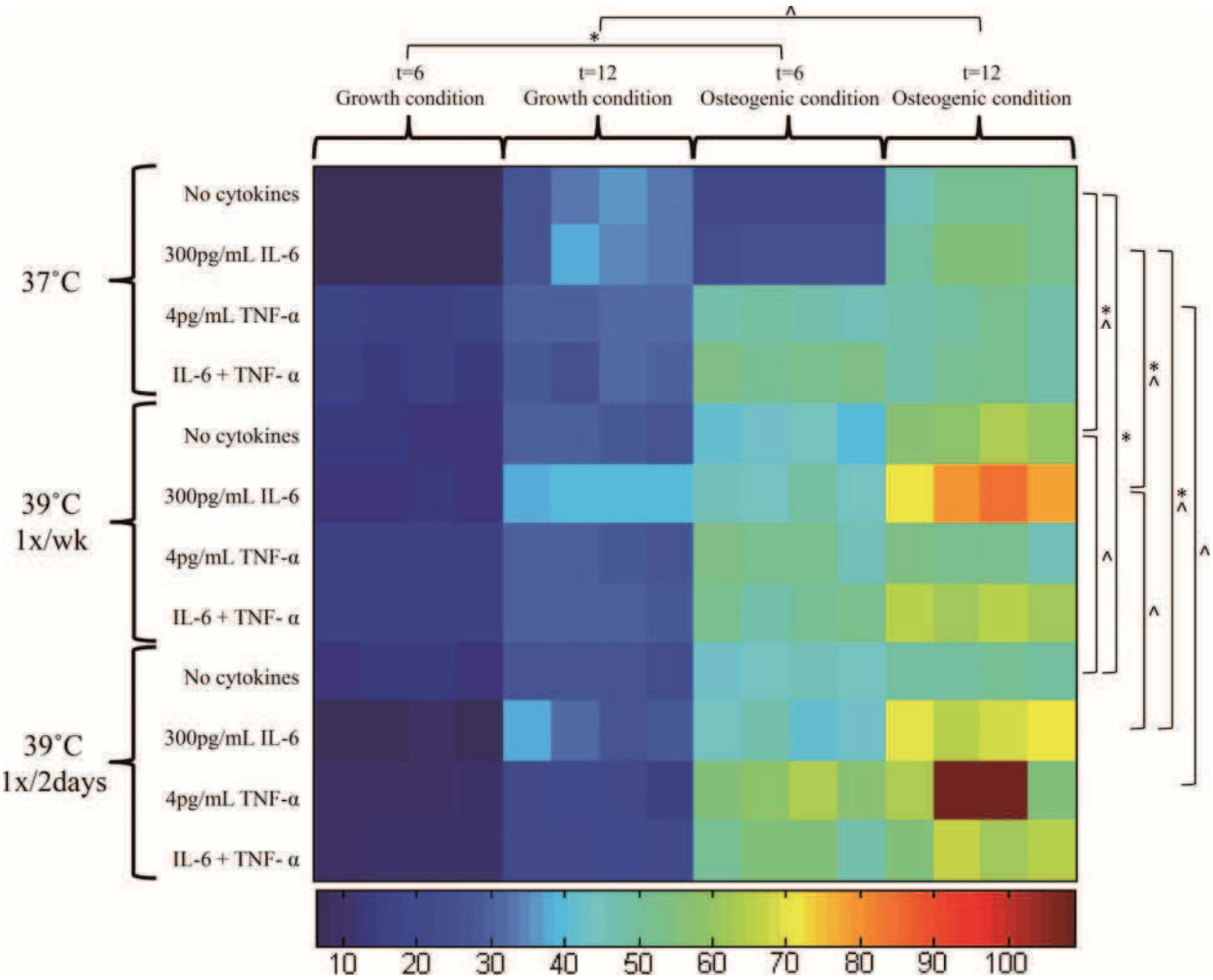

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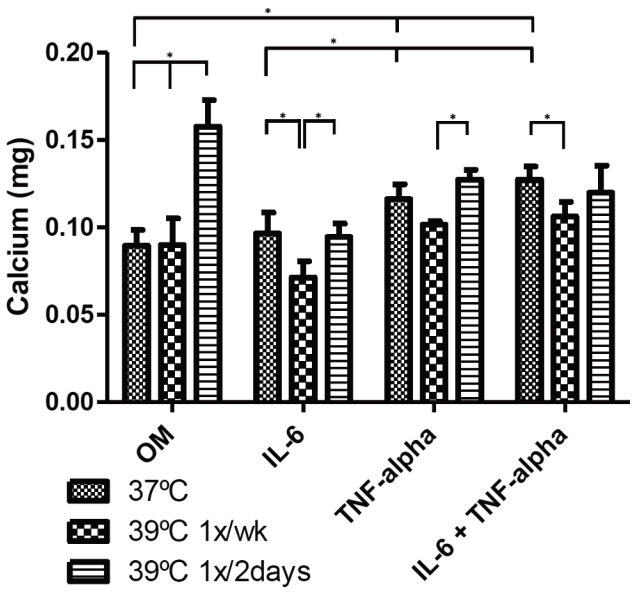

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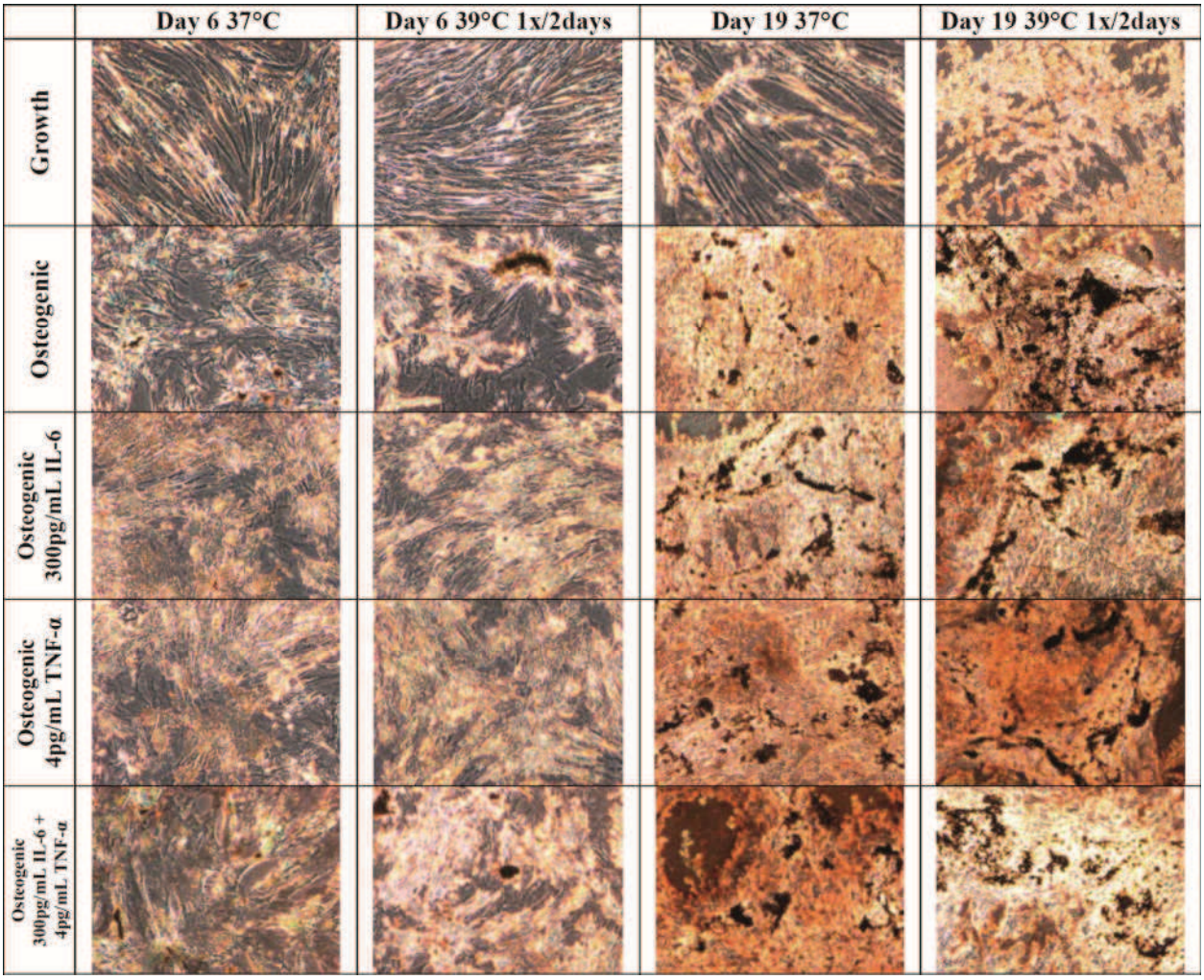

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Supplementary Materials

Table S1. Model Parameters (for simulation and manual analysis):

Material	**Property**	**Value**
Polystyrene	Thickness, top or bottom (L_{PS})	1 mm
	Thermal conductivity (k_{PS})	0.15 W/m-K
	Specific Heat Capacity	1.3-1.5 J/g-K(1.4 in simulation)
	Density	1.05 g/cm^3
Media (water)	Thickness (L_{water})	2.61mm
	Thermal conductivity (k_{water})	0.6 W/m-K *
	Specific Heat Capacity	4.184 J/g-K *
	Density	1g/cm^3 *
Air	Thickness (L_{air})	17.7mm
	Thermal conductivity (k_{air})	0.02721 W/m-K *
All	Area (A_{well})	1.911E-4 m^2

Table S1. Material properties and dimensional parameters utilized for computational simulation and manual estimation of heating profile for media in a 24-well culture plate.

Table S2. Alkaline phosphate activity (nmol/mL/min) of heat shocked hMSCs during osteogenic differentiation					
		t=6	t=12	t=6	t=12
		Growth condition	Growth condition	Osteogenic cond.	Osteogenic cond.
37°C	No cytokines	6.75 ± 0.18	32.05 ± 4.31	20.34 ± 0.78	50.44 ± 2.66
	300pg/mL IL-6	6.87 ± 0.18	33.21 ± 4.46	24.71 ± 0.63	52.61 ± 2.98
	4pg/mL TNF-α	16.12 ± 0.6	30.88 ± 1.21	47.86 ± 1.47	52.43 ± 3.28
	IL-6 + TNF-α	14.83 ± 0.87	28.21 ± 3.0	52.41 ± 1.83	49.73 ± 2.55
39˚C 1x/wk	No cytokines	12.6 ± 0.55	28.65 ± 1.48	41.74 ± 1.4	59.78 ± 2.26
	300pg/mL IL-6	12.67 ± 0.99	39.28 ± 0.76	45.85 ± 1.86	78.39 ± 5.32
	4pg/mL TNF-α	15.28 ± 0.54	28.65 ± 1.48	50.86 ± 3.59	68.71 ± 2.89
	IL-6 + TNF-α	15.36 ± 0.45	29.61 ± 0.78	50.97 ± 2.02	63.8 ± 2.27
39˚C 1x/2days	No cytokines	12.82 ± 0.32	25.74 ± 2.03	43.93 ± 1.4	49.38 ± 0.6
	300pg/mL IL-6	7.76 ± 0.36	31.35 ± 4.74	43.56 ± 3.0	68.76 ± 3.47
	4pg/mL TNF-α	9.57 ± 0.34	18.69 ± 2.17	58.47 ± 3.68	72.39 ± 17.09
	IL-6 + TNF-α	9.98 ± 0.29	20.36 ± 1.49	52.25 ± 4.08	61.94 ± 6.28

Table S2. Summary of alkaline phosphatase activity (nmol/mL/min) of differentiated and undifferentiated hMSCs cultured with and without pro-inflammatory cytokines while variably heat shocked at 39°C, (n=4).