HYDROGEL ENCAPSULATION OF CELLS MIMICS THE WHOLE BODY RESPONSE TO LMHF VIBRATIONS

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ABSTRACT OF THE THESIS

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Low-magnitude high-frequency (LMHF) vibrations show anabolic effects on bone when applied to the whole body in both animal and human studies. As such, it is being explored as a treatment for osteoporosis and osteopenia in vulnerable populations. In humans, whole-body vibrations have shown modest increases in the bone mineral density of postmenopausal women, children and adolescents, though it had no effect in young adults. These conflicting results prompted long term studies to establish the optimal frequency, magnitude and duration of the vibration. In vitro cellular studies have been carried out to study the physical and biologic mechanisms underlining these outcomes. But there are conflicting results of LMHF vibrations when applied to cell culture as well, with some studies showing no effect when cells are cultured in 2D monolayer as opposed to other studies reporting increased differentiation of progenitor cells towards an osteogenic lineage when cells are cultured in 3D scaffolds. It is worthy of note that the majority of scaffolds used in these studies are from natural sources, which in and of themselves may promote differentiation due to biochemical and microarchitectural cues. This master thesis seeks to explore the effect of Low magnitude high frequency vibrations on human mesenchymal stem cells (hMSCs) encapsulated within a 3D

microsphere structure composed of synthetic polymer polyethylene glycol diacrylate (PEGDA). Synthetic PEGDA has no inherent cues and can serve as a "blank slate" to the entrapped cells. In this study, three different intensity vibrations of 0.3g, 3g and 6g at 100Hz were applied for 24 hours to the encapsulated hMSCs by means of a vibration unit. These cells were subsequently tested for adipocyte, chondrocyte and osteoblast differentiation over a period of 21 days. There was early onset of osteogenic differentiation in 0.3 g and 3 g test samples compared to control samples, while there was no osteogenic differentiation at all observed in 6g test samples. In addition, as the magnitude of acceleration applied increased, the osteogenic differentiation of the encapsulated hMSCs decreased. Thus, LMHF vibrations with low accelerations accelerated the osteogenic differentiation of encapsulated hMSCs, indicating that hydrogel-encapsulated hMSCs may mimic the whole body response to vibration, which paves the way for further *in vitro* LMHF experiments with encapsulated cells.

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Chapter 1

INTRODUCTION

Whole body vibration (WBV) therapy is a noninvasive, non pharmacological therapy for osteoporosis and uses Low magnitude High Frequency (LMHF) vibrations as mechanical stimuli, which are produced by a vibrating platform and in turn load bones and stimulate the bone receptors [1].

Low-magnitude, high-frequency (LMHF) vibrations have been assessed as a means to mimic the mechanical loading (strains) on bones observed during daily activity. LMHF vibrations are helpful in individuals that are confined to bed rest, suffering from complete motor spinal cord injury or astronauts in the micro gravity space that cannot perform normal daily activities and are prone to bone loss, muscle atrophy and in turn developing osteoporosis. Application of LMHF vibration shows anabolic effects on bone when applied to the whole body in both animal and human studies. In animals, increase in both bone area and bone density is seen [2-7], and in humans, there is accelerated fracture healing [8-10]. However, there are contrasting results seen in some randomized clinical trials in humans, where whole-body vibrations have shown modest increases in the bone mineral density of children and adolescents, while showing: (1) no effect in young adults [11] and (2) no change in the bone mineral density (BMD) or bone structure in postmenopausal women [12, 13]. Moreover, even in some animal studies, WBV did not produce a detectable anabolic effect nor did it mitigate bone loss that was induced by a decline in muscle activity in normal mice [14]. WBV when used along with a conventional drug (alendronate) for osteoporosis showed increased prevention in bone

loss and improved trabecular architecture over drug therapy alone, but there was very reduced effect when vibration treatment was given without the drug [15]. Combined applications of LMHF vibrations with the osteoporotic agents estrogen and raloxifene have also shown to improve the healing of osteopenic bones [16]. These studies indicate that this therapy is relatively a new concept – its efficacy and optimal paramters remain uncertain and as such, have prompted large scale long term studies to establish the optimal frequency, magnitude and duration of the vibration [17].

While animal and human studies help in understanding of the effects of vibration in general and during complex challenges in in vivo systems, in vitro cell culture studies helps in having a closer look at the biological (aggressive interplay between the bone cells and their responses) and physical mechanisms that govern the in vivo response. There are conflicting results of LMHF vibrations when applied to cell culture as well, with some studies showing no effect and others showing increased differentiation of progenitor cells towards an osteogenic lineage. There have been differences observed in viability, growth factor expression, response to stimuli, and differentiation when MSCs are cultured in 3D systems as opposed to 2D systems. In some studies, vibrated hMSCs in 2D culture did not show any effect on gene expression of growth factors like BMP -2 and VEGF while MSCs cultured in 3D cultures showed clear expression of these genes [18,19]. These results suggest that the response of cells in 3D culture to vibration might mimic their response in vivo due to the scaffold architecture providing three dimensional cell-cell and cell-scaffold interactions [20]. Moreover, the majority of scaffolds used in such studies are hydrogels made from natural polymers in or are decellularized native ECM [21-24]. These scaffolds often retain their biocompatibility and bioactivity as from

the natural sources of which they are derived [25]. They promote many cellular functions due to their complex microarchitecture and the presence of multiple endogenous factors [26]. Multiple studies have demonstrated that biochemical and microarchitectural cues play a major role in regulating bone functionality [27]. These findings suggest that scaffolds from natural sources may themselves promote differentiation due to biochemical and microarchitectural cues.

In order to isolate the effect of the scaffold from the effect of the LMHF vibrations on entrapped cells, there is a need to develop a 3D model that does not provide an ECM-like microarchitecture or biochemical cues. Here, we examine the effect of three different ranges of LMHF vibrations when applied to human mesenchymal stem cells (hMSCs) encapsulated within polyethylene glycol diacrylate (PEGDA), which is synthetic polymer having no inherent mechanical or biochemical cues that can serve as a "blank slate" to the entrapped cells. In our work, microencapsulated hMSCs are tested for adipocyte, chondrocyte and osteoblast differentiation following exposure to LMHF vibrations

Chapter 2

LITERATURE REVIEW

2.1 Bone Properties and Remodeling

Bone is a rigid and fundamental form of connective tissue and performs four main functions: it protects internal organs and bone marrow soft tissues, maintains mechanical integrity of the body and its motion, is the elementary site of hematopoiesis, and is a storehouse of calcium and phosphorus, which are necessary for the functioning of other body systems.

The skeleton is divided into axial skeleton and appendicular skeleton. The axial skeleton consists of the pelvis, flat bones like skull and sternum and vertebrae while the appendicular skeleton consists of long bones of the upper and lower extremities. These long bones can be distinguished into three different components called the epiphysis, metaphysis and diaphysis. The region at both ends of a long bone is called the epiphysis, and it is separated by a plate from the rest of the bone in a growing skeleton. The diaphysis comprises the central portion of the bone shaft and the part between the epiphysis and diaphysis is called the metaphysis.

Bone tissue is organized into two types called cancellous (trabecular) and compact (cortical) bone. Together, these make a hollow bone structure and help in proving both a light and strong frame to the body. Cortical bone forms the dense and solid outer shell that covers the external part of bones and roughly makes up 75% of the total skeletal mass. Internal to cortical bone is trabecular bone, which consists of fine network of pores and trabeculae, which are aligned along the lines of principal stresses and make up the remaining 25% of skeletal mass. Cortical bone defines the shape of the body while trabecular bone

provides a large surface area for mineral exchange and maintains skeletal strength and integrity [28]. Trabecular bone is thus metabolically more active than cortical bone, but is not as stiff as cortical bone [29, 30].

Bone tissue is 70% inorganic matrix, 22% organic matrix, and the remainder is predominantly water. Crystalline hydroxyapatite and calcium phosphate make up most of the inorganic phase, while highly organized Type I collagen fibers and noncollagenous proteins make up most of organic matrix [29-31]. The remaining part of the organic matrix consists of a variety of cells. There are four types of bone cells, which are responsible for metabolism and turnover of bone and respond to various environmental signals like mechanical loads. Osteoblasts are bone-forming cells and are responsible for the synthesis of osteoid, the unmineralized bone matrix precursor. This newly synthesized osteoid then mineralizes and osteoblasts that are captured in the osteoid matrix at the end of bone formation become osteocytes. Those that are not trapped may become bone-lining cells, which are responsible for activating signals that begin bone resorption and bone remodeling as well as play role in maintenance of ion fluxes between bone fluid and interstitial fluid compartment for mineral homeostasis. In fully mineralized bone, osteocytes consists of more than 90% of the bone cells and they take part in mechanical regulation of bone tissue by receiving mechanical signals and transmitting them to other osteocytes [32-33]. They are able to do this through their connections to one another through cellular processes in a canulicular network that is dispersed throughout the bone matrix. The fourth type of cells is bone-resorbing cells called osteoclasts. They are multinucleated and are derived from a mononuclear line of hematopoietic cells [34].

The osteoblast and osteoclast are involved in a constant remodeling process that gives bone

tissue a natural ability to repair and regenerate itself [35, 36]. This process is activated by an interaction between osteoblastic cells and the osteoclast mononuclear precursors, causing them to merge into multinucleated osteoclast cells. These cells attach to the bone surface and remove bone by dissolving the mineral with acids and the organic phase with proteases, leading to the breakdown of bone in a process called as bone resorption. This phase of osteoclast activation to bone erosion lasts around 1-3 weeks [37]. Once this phase is over, the osteoclast machinery slows and osteoclasts apoptose. Thus, altering the rate of production of osteoclasts or altering their life span can control levels of bone loss. These two phases of quiescence and activation are followed by a brief reversal phase, in which osteoblast precursors become active and migrate towards the site of resorbed bone tissue [36]. At this point, osteoblasts differentiate from mesenchymal stem cells (MSCs) then secrete the previously mentioned osteoid. Osteoid consists of type I collagen, other non-collagenous proteins like osteocalcin, osteopontin, osteonectin, and alkaline phosphatase [36]. Calcium phosphate mineral is deposited on osteoid matrix over time, leading to the formation of the final composite material, bone [37]. This process of complete bone formation and mineralization may take 2-6 months [36].

2.2 Mechanical Adaptation of Bone

Several tissues, including bone need mechanical stimuli to remain healthy [38]. Bone undergoes strain when stress is applied and the resulting deformation can result in a relative change in width, length or angulation [39].

The intrinsic change in bone mass and architecture as a response to the strain induced by mechanical loads is called bone adaptation. Bone adaptation requires a relatively short

duration of loading, is driven by a dynamic stimulus, and involved bone cells become acquainted to daily mechanical loading [40]. Additionally, bone responds to dynamic strain and not to static strain [41]. These properties of whole bone adaption indicate that bone cells are responsive to mechanical inputs, including parameters such as strain frequency and strain magnitude, which may act as important stimuli for evoking an adaptive bone response [42].

Forces like ground reaction forces, muscle forces and gravitational forces are experienced by the skeleton during daily routine activities and lead to bone remodeling. High impact exercise and physical activity like running and weight training have led to bone mineral density growth in athletes and active young adults [43, 44]. In populations where such activities are not possible, such as the elderly or disabled, alternative strategies to load their skeletons may mitigate the bone loss common in these populations. One such strategy is to impart strain from forces generated by low magnitude high frequency (LMHF) vibrations [45].

Whole body vibration elicits skeletal responses similar to physical activity by stimulating osteogenesis through mechanotransduction, the conversion of physical force into a cellular response. Mechanotransduction can be divided into four phases, namely: a) mechanocoupling, the conversion of mechanical force into a local mechanical signal that can be sensed by a mechanoreceptor; b) biochemical coupling, the conversion of a local mechanical signal into a biochemical signal, followed by activation of proteins and expression of genes; c) signal transmission, transmitting a signal from the sensor cell to the cell that will actually form or remove bone; and d) effector response, which takes into account the final tissue level response. Integrins, cadherins and stretch activated Ca^{2+}

channels act as mechanoreceptors [46]. Cell membrane deformation is induced by shear stress caused by increased fluid flow through bone canaliculi and lacunae. This fluid flow is usually generated due to mechanical loading and is proportional to the loading frequency of the bone. The induced cell deformation leads to activation of membrane receptors and opening of L-type voltage sensitive Ca^{2+} channels that permits calcium entry into the cell. Increase in cytosolic Ca²⁺ also occurs through phospholipase activity and inositol-1,4,5-trisphosphate (IP3) signaling, also resulting in release of Ca^{2+} from intracellular stores. The calcium dependent Protien kinase C and Ras now activates mitogen-activated protein kinase pathway causing the activation of c-jun kinase, ERK1 and 2, and p38 mitogen-activated protein kinase. This leads to upregulation of c-fos and c-jun expression and the gene product of c-fos with the c-jun forms the activator protein-1 (AP-1) transcription factor, which is involved in MSC differentiation into osteoblasts [47]. Mechanical stimulation also leads to upregulation of growth factors such as bone morphogenetic protein (BMP) 2 and 4, insulin-like growth factor (IGF) I and II, and vascular endothelial growth factor (VEGF). These growth factors in turn stimulate the expression of three major osteogenic transcription factors Runx2, Osterix and Dlx5 [48]. During whole body applications of LMHF vibrations, bone cells are exposed to acceleratory motions in addition to the fluid shear stress resulting from fluid-cell interactions. Certain computational studies suggest that the bone surface in contact with bone marrow is subjected to this stress at vibrations as small as 0.1 g. Changes in fluid shear magnitude during these vibrations influence the mechanical adaptation of bone [49]. LMHF vibrations have been shown to generate enough mechanical loading to increase the fluid flow in bone and result in mechanotransduction [32,50].

2.3 Osteoporosis

The mechanical adaptation of bone can result in bone growth or bone resorption. Osteoporosis is a skeletal disease defined by a decrease in bone density and deterioration of the bone's microarchitecture, resulting in increased bone fragility and susceptibility to fracture [51]. Bone strength is determined by cortical bone porosity, mineral and matrix composition, fine trabecular bone structure, and the presence of microfracture or damage in bone. Trabecular bone provides great strength through a structure of well-connected plates, and so any changes in this microarchitecture impact bone strength. In people with osteoporosis trabeculae are reduced in number and size, resulting in a significant reduction in bone strength. Thus, most frequent fractures in osteoporosis occur at sites where trabecular bone predominates, like the wrist, hip, vertebrae, etc. The condition occurs mainly in older women due to changes in hormonal regulation, drops in physical activity, or physical or neurological impairments that reduce their mobility [53,54] resulting in accelerated bone breakdown with decrease in bone formation. Due to the absence of gravitational loading, astronauts [52] are also at an increased risk of developing osteoporosis.

2.4 Current Treatments for Osteoporosis

Present clinical protocols favor dietary and pharmacological treatments to prevent osteoporosis and related fractures. Calcium and vitamin D intake in the diet as well as in supplements has been recommended since these compounds are known to prevent thinning of bones [55]. The supplement is decided based on the age, condition and gender of the patient. Major pharmacological treatments bisphosphonates, strontium ranelate, estrogen like medications (raloxifene), hormone therapy, calcitonin and denosumab. All of these drugs work to reduce the amount of bone loss. For example, denosumab is an antibody aimed against a factor called RANKL that is involved in the formation of osteoclasts. Of all these drugs, bisphosphonate is the most common pharmacological intervention used for osteoporosis treatment. Unfortunately, bisphosphonates have adverse effects, including osteonecrosis of the jaw, renal and gastrointestinal toxicity, esophageal irritation, minimal trauma atypical fractures and acute-phase reactions [56]. Moreover, these interventions may be onerous to some patients, as some drugs require the patients to sit upright for 30 minutes after taking medications so as to avoid esophageal irritation [57]. Although weight-bearing exercise is also used as an osteoporosis therapy it is not feasible for all patient populations, particularly those with reduced mobility. One intervention that is increasingly explored is whole body vibration therapy (WBV) [58, 59].

2.4.1 Whole Body Vibration

Whole-body vibration delivers mechanical challenges to the skeleton without requiring patient mobility. It is most commonly applied to a person standing on a vibrating plate that produces horizontal, vertical, or pivotal accelerations [60]. This therapy has been shown to direct MSCs towards osteogenic differentiation [61] and to inhibit their adipogenic differentiation [62]. Although very little is known regarding the effects of vibration on osteocytes, research shows that during the application of LMHF vibrations on cultured osteocytes [63], there is a decrease in expression of osteoclast-forming RANKL. In addition, when LMHF vibrations are applied, there is increased cell-cell communication through gap junctions [64]. Whole body vibrations can be delivered as

LMHF vibrations (mimicking muscle contraction), high magnitude high frequency (10-100Hz), vibrations, and low frequency (<10 Hz) high magnitude vibrations (mimicking exercise). The magnitude or acceleration of the vibration (e.g. the 'M' in LMHF) is defined by the frequency [Hz] and amplitude [mm]. These parameters along with the exposure time and their effects have been tested on animals, children, older adults, and postmenopausal women during a variety clinical and preclinical condition.

2.4.2 WBV in Animals

Various animal models have relevant pathological conditions that can produce an osteoporotic-like state including the ovariectomy (OVX) rodent model and the botulinum toxin disuse model. Ovariectomized (OVX) rodents undergoing WBV (90 Hz, 0.3 g, 10 min/day) displayed increases in osteoblast activity. The rates of osteogenesis were 159% greater in 90 Hz rats when compared to 45 Hz rats and the unvibrated controls. Additionally, the bone morphology of the 90 Hz rats had the highest trabecular bone volume and thickest trabeculae [3]. LMHF vibration has also shown promise in improving fracture healing in osteoporotic bone in an 8-week study of OVX rats. Ninemonth-old OVX rats had inferior fracture healing than intact rats, administration of WBV (35 Hz, 0.3 g, 20 min/day) enhanced fracture healing over the non-vibrated OVX rats [6]. Another study showed LMHF vibration treatment (45 Hz, 0.3 g, 5 day/week for 5 weeks, 15 min) resulted in improved femoral and tibial cortical area and thickness and showed higher trabecular bone volume fraction in proximal tibia in osteogenesis imperfecta mouse model compared with sham controls [5]. While in another study, 3 weeks of WBV increased trabecular bone in mice undergoing vibration at 0.3 g while mice undergoing 0.6 g WBV did not demonstrate such increases. This result indicates that the

anabolic response decreases with increases in the magnitude of acceleration [65]. Moreover, vibration therapy has been shown to be effective in improving low bone mass when used in combination with pharmacological interventions. In a study where alendronate treatment was given in combination with LMHF vibration (45-55 Hz, 0.3 g, five times/week for 20 min/day) treatment, alendronate and LMHF combined produced greatest anabolic response while LMHF alone showed less improvement in trabecular bone compared to the drug alone [15]. In another OVX rat model, LMHF vibration treatment combined with either raloxifene or estrogen resulted in increased endosteal and trabecular bone densities compared to vibration alone [16]. Contradictory results have also been reported where LMHF vibrations show no anabolic effect on bone in animal models. In a botulinum toxin type A (BTX) muscle disuse mice model, LMHF (0.6 g, 45 Hz) did not prevent the disuse-induced bone loss [14]. In addition, various studies show that different short term (30 s/day, 2 days) and long term (30 s/day, 3 days/week for 4 weeks) LMHF vibration (0-50 Hz) signals did not enhance cortical bone formation at these frequencies in mice [66]. LMHF vibrations (0. 5g, 45 Hz) [67] and (0.3 g, 90 Hz, 20 mins/day, 5 days/week for 6 weeks) [68] had no effect on cortical bone volume or trabecular volume fraction after 10 week and 12 week follow up respectively in OVX rats [67, 68].

2.4.3 WBV in Humans

Current human studies are perplexing as multiple vibration parameters are considered, from high (3-6 g) to low intensity (< 1 g) accelerations. High intensity devices are usually marketed as exercise machines and are not recommended for patients with weak bones. A 1-year randomized, double blind placebo controlled study was carried out in 70 women,

3-8 years after onset of menopause. LMHF vibrations (0.2 g, 30 Hz, 20 mins) were given to one group and an inactive sham plate to the other. Loss of 2% femoral neck bone mineral density (BMD) was observed in the sham group while the treatment gained 0.04% BMD, a 2.17% relative BMD increase [69]. Another randomized trial showed the effects of 24 weeks of whole body vibrations on the hip density of 70 (58-74 years old) postmenopausal women volunteers. High intensity accelerations (2.28–5.09 g, 35–40 Hz) led to significant increases in the hip BMD (0.93%) compared to resistance training controls (-0.60%), suggesting that vibrations delivering 3-5 g acceleration can be useful in osteoporosis prevention [70]. A dose response relation was investigated in a study using LMHF vibrations (0.3 g, 30 Hz, 20 mins) in postmenopausal women. These vibrations led to a 34.6% reduction in bone resorption markers detected in urine (NTx/Cr) compared to the control group [71]. Moreover, an 18-month study in 150 postmenopausal women demonstrated that by combining vibration therapy with low impact activity increased lumbar BMD, while vibration alone did not [8]. Another long term 18 month study involving 710 elderly patients was carried out in which LMHF vibration (0.3 g, 35 Hz, 20 min/day, 5 days/week) treatment was given for 18 months. There was an effective reduction in falls observed in the patients with no significant difference in BMD [72]. In another randomized controlled trial, 202 postmenopausal women with osteopenia were given LMHF vibrations (0.3 g, 37 or 90 Hz, 20mins/day) for 1 year and no significant differences were found in volumetric trabecular BMD, or in tibial, lumbar spine, or total hip femoral neck BMD [12]. Moreover, there was no beneficial effect of short term WBV (16 g, 35 Hz) on bone mass and its structure in elderly patients (men and women) of both test (trained squat 3 times/week for 11 weeks) and control groups (receiving no vibration A 6-month study that tested the ability of LMHF vibrations in protecting the bone quality in children (4–19 years) with cerebral palsy showed that the volumetric trabecular BMD (vTBMD) increased by 6 % in vibration (0.3 g, 90 Hz, 10 min/day) treated patients and decreased by 12 % in sham control patients [74]. A followup study using the same 6 month LMHF protocol showed strengthening of the cortical bone structure in children with cerebral palsy (6–12 years) [75]. The effects of LMHF have also been explored in disabled children with reduced mobility. Disabled children were randomized to LMHF (0.3 g, 90 Hz, 10 min/day) and treated groups showed a 6.3% increase in BMD compared to a 12% decrease in control groups [76].

2.4.4 WBV Limitations

Varied study design and different parameters of the choice of intervention may account for these discrepancies seen in the outcomes following exposure to whole body vibrations. The contributing factors include the range of vibration parameters selected, the direction in which vibration is applied, the time (minutes- hours) and duration (daysmonths) of WBV exposure, the frequency and amplitude of the vibration applied, the demographics and health of the participants, the type of platform used, and the differing designs of research and outcome measures. Thus, many researchers have indicated the need for additional research [77-79] to overcome these inconsistencies. An *in vitro* model for WBV therapy may facilitate larger numbers of studies, establish that vibration alone acts as mechanical stimuli for the differentiation of mesechymal stem cells into the osteogenic lineage, and may more rapidly enable researchers to determine the most appropriate, safe and effective vibration parameters.

2.4.5 WBV and LMHF Vibrations in Cells

Recent studies have shown that low magnitude, high frequency (LMHF) vibrations act as a stimulus for directing MSC differentiation towards osteogenesis [85]. The influence of mechanical stimulation on bone morphology and mass through its effect on bone cell populations has resulted in many researchers to explore the effect of mechanical stimulation on the proliferation and differentiation of osteoclasts and osteoblasts [80]. However, it is important to note that bone marrow stem cells (BMSC) are another important group of cells present within the trabecular spaces and central canal of bone. BMSCs are further divided into hematopoietic stem cell (HSC) and MSCs. These cells are known to respond to the LMHF vibrations as they share the bone marrow environment and influence the bone remodeling process by interacting with each other. HSCs are the progenitor cells for osteoclasts, while MSCs are the precursors of osteoblast cells. MSCs are a promising cells source for regenerative medicine and tissueengineering purposes as they are self-renewing, possess immunosuppressive properties, and are multipotent, having the capacity to differentiate into osteoblasts, chondrocytes, adipocytes and also myocytes, tenocytes and neuronal cells [81]. The fate of MSCs is dependent on activation of many transcription factors and cytokines like bone morphogenetic proteins (BMP-2, -4, and -6), which play a major role in stimulating differentiation of MSCs into osteoblasts [82]. After stimulation by these cytokines, the runt-related transcription factor 2 (Runx2) and Osterix (Osx) transcription factors are activated and are early markers of MSC differentiation into osteoblasts. It has also been found that Runx2 manages the transcription of osteoblast markers like osteocalcin and type I collagen [83]

Moreover, in *in vitro* studies, MSCs differentiating into osteoblasts express alkaline phosphatase (ALP) after approximately 1-2 weeks of culture in osteogenic media and this is followed by formation of calcium deposits approximately 3-4 weeks later. From these studies is understood that ALP is the early marker of differentiation while calcium deposits are late marker [84]. Such markers are used to determine the effect of LMHF vibrations on the cells.

The potential influence of LMHF vibrations on MSCs were studied in disuse male C57BL/6 J mice. LMHF vibration signal (0.2 g, 90 Hz, 15 min/day for 3 weeks) was applied and a 30% greater osteogenic bone marrow stem cell population was seen in the vibration treated groups, suggesting that these mechanical signals conserved the osteogenic potential of progenitor cells during disuse [86]. Another study with dietinduced obesity in mice demonstrated that LMHF vibrations bias the MSC differentiation towards the osteogenic pathway and reduce their commitment towards adipogenesis. LMHF (0.2 g, 90 Hz, 15 min/day) for 6 weeks increased the MSC population up to 37% and biased their differentiation towards osteoblasts [87]. LMHF vibrations have also been used to stimulate cells in 3D scaffolds. Human MSCs in presence of dextran were exposed to vibrations (15–60 Hz, 45 min) and in the 60 Hz group there was an increase in ALP production [88]. However, other studies by Lau et al. and Smith et al. did not find upregulation of ALP at any other frequency tested. In addition, no effect was seen on rat MSCs after exposing them to LMHF (1 hr/day 60 Hz). These studies suggest that results may depend on both the frequency and the induced acceleration [89].

The response of MSCs to LMHF vibrations have also been shown to be dependent on whether vibrations are applied in 3D (in scaffolds or multilayer culture) or 2D (monolayer culture) and there have been observed differences between cells cultured in 2D and 3D in terms of response to stimuli, viability, differentiation, gene and protein expression, matrix adhesion, cellular morphology and migration, and the cultured cells' response to fluid flow [90].

In a study where hMSCs were vibrated (0.1-0.6 g, 10-40 Hz, 10 min/day, 5 days/week) in 2D and 3D culture, the vibrated hMSCs in 2D culture showed no change in the expression of genes such as ECM factor collagen type I, osteoprotegerin (OPG; an osteoclast differentiation-inhibitory factor) and growth factors (BMP-2, VEGF). Conversely, when vibrated in a 3D culture, there were 74.7%, 52.5%, and 61.9% increases in of OPG, Col I and VEGF expression, respectively [21]. MSCs were grown as 3D microaggregates and in a 2D culture and induced by osteogenic medium demonstrated that there was greater upregulation of BMP-2 autocrine signaling, more intense calcium staining and collagen I immunoreactivity (measures of MSC osteogenesis) in the microaggregates compared to the monolayers. In another study, hMSCs were assessed for electrical stimulation (ES) induced bone regeneration by growing them in 3D collagen sponge and comparing their response with the monolayer. There was enhanced expression of growth factors (BMP-2), chemokines (CXCL2) and chemokine receptors (CXCR4) in the 3D culture compared to the 2D monolayer culture [18]. This behavior was explained by the fact that the cytokine expression signaling pathways are efficiently activated in 3D environments due to increased cell-to-cell contact and cell-to-ECM interactions over 2D monolayers [18, 21]. Thus, the use of 3D culture is thought to better mimic the *in vivo* environment and would be an improved

alternative to 2D monolayer culture for the study of cellular responses to mechanical vibrations.

2.5 Hydrogels in *In Vitro* Studies

As discussed in the previous section, studies of applied stimuli in 2D culture of MSCs do not result in responses comparable to their behavior *in vivo*. It has been noted that ECMcell and intracellular interactions are dependent on different ligands and receptors present in the cell and in the ECM. In addition to this, ECM composition and structure have also shown to play important roles in the differentiation of stem cells [91]. Therefore, a variety of scaffold materials with different properties have been used to construct different microenvironments for cells.

Hydrogels have been attractive biomaterials for their use as scaffolds for tissue engineering purposes. Hydrogels are hydrophilic crosslinked networks of polymeric chains that swell greatly in water but are water insoluble, exhibiting high water content that impart tissue like viscoelastic properties to it. Hydrogels have been used to encapsulate cells in 3D microenvironments by mild gelation conditions or thermal or photo polymerization [92]. Hydrogels can be characterized into natural (made from natural ECM components) or synthetic hydrogels [96].

2.5.1. Natural Hydrogels

Several naturally occurring polymers suitable for stem cell differentiation possess properties like biocompatibility, biodegradability, are bioactive and advocate cell adhesion and posses cell responsive components that are used in biological signaling pathways [93]. This is because they are obtained from ECM proteins (fibrin, collagen) and polysaccharides (alginate, hyaluronic acid, chitosan etc.). Fibrin and collagen are FDA approved materials and collagen is often used as matrix for MSCs and has been shown to promote their differentiation into chondrocytes [94]. hMSCs seeded in alginate microbeads differentiate into osteoblast-like cells in presence of growth factors [91]. Alginates are isolated form brown seaweeds and are used as a scaffold for many cells due to their providing increased adhesion, proliferation and differentiation of encapsulated cells. Alginate microspheres were shown to promote calcification *in vivo* without the need for any chemical or biological additive [95]. Thus, these polysaccharides used as natural hydrogels, possess innate biological and biochemical cues that help in the differentiation of encapsulated stem cells. Unfortunately, these intrinsic cues are not desired when investigating the effects of LMHF vibrations as they can confound the results.

2.5.2 Synthetic Hydrogels

Synthetic hydrogels have been studied and used for many biomedical applications. They possess high purity and reproducibility, can be tailored to have specific degradation rates, and possess controlled chemical compositions and mechanical properties. Some frequently used synthetic hydrogels include Poly(ϵ -caprolactone) (PCL), poly(hydroxyethyl methacrylate) (polyHEMA), poly(vinyl alcohol) (PVA), and poly(ethylene glycol) (PEG) [91]. Electrospun PCL fibers, the most common poly(α -hydroxy esters), are used widely in stem cell engineering [91]. Poly(ethylene glycol) (PEG) is also widely used as cell scaffolds or for cell encapsulation [92]. Moreover, PEG hydrogels are biocompatible, bioinert polymers that can be crosslinked by photo-

polymerization with low toxicity and lack cell adhesion sites, unlike natural polymers [96]. These qualities make them ideal to provide a "blank slate" on which to study the effects of LMHF vibrations on MSCs.

Polyethylene glycol diacrylate (PEGDA) is composed of PEG with two acrylate groups on either side of the PEG chain (Fig. 1) which acts as a crosslinking agent between the molecular chain polymers.



Figure 1: Structure of PEG and PEGDA [98]

There are three major categories for forming covalently crosslinked PEG hydrogels, namely: 1) free-radical photopolymerization (chain growth); 2) step-growth polymerization; and 3) mixed-mode polymerization (combination of reactions 1 and 2). PEGDA is photopolymerizable and follows chain growth polymerization. In the presence of the visible light, there is cleavage of the initiator molecules that give rise to free radical species that in turn initiate the reactions in chain growth polymerization. The free radicals interact with the acrylate groups on the PEG macromers, inducing the covalent crosslinking of PEGDA as shown in Fig 2. Complete gelation within seconds is achieved in such systems. Photopolymerization of PEGDA hydrogels has thus been deeply

explored in tissue engineering and regenerative applications since it is suitable for *in situ* encapsulation of cells and other agents [96, 97].



Figure 2: Structure of PEG hydrogel during chain growth polymerization [98]

hMSCs have successfully been encapsulated in PEGDA and multithiol containing peptides by using a mixed step reaction to promote differentiation of MSC towards the chondrogenic pathway [99]. Other studies have photopolymerized PEGDA and PEG modified with peptide sequences to facilitate chondrogenesis of hMSC *in vitro* [91]. In a study of mineralization, hMSC were encapsulated in a PEGDA hydrogel modified with phosphate groups and RGD sequences, which led to increased mineralization of the hydrogel network over unmodified hydrogels [100].

In this thesis the "blank slate" of the PEGDA hydrogel is exploited to investigate the effects of LMHF vibrations on encapsulated hMSCs, without any contributing intrinsic cues that would be imparted by natural hydrogels. hMSCs are photoencapsulated within 10 kDa PEGDA hydrogel microspheres subjected to 3 different LMHF vibrations, then evaluated for markers of differentiation.

Chapter 3

MATERIALS AND METHODS

3.1 Experimental Design

hMSCs were encapsulated into non-biodegradable PEGDA hydrogel microspheres and these microspheres were then exposed to different ranges of low magnitude high frequency vibrations to see its sole effect on the differentiation of hMSCs. After a single 24hour exposure to vibration, the viability of encapsulated cells is assessed from Day 1-21. Moreover, as hMSCs have the potential to mainly differentiate into chondrocytes, adipocytes and osteoblasts; three different staining methods are used to detect it's differentiation on day 1,4,7,14 and 21 as well as alkaline phosphatase (ALP) assay is performed on day 4. All the differentiation assays including ALP assay are performed in triplicates and the data is tested for their statistical significance.

3.2 hMSCs Culture

hTERT-immortalized human mesenchymal stem cells (hTERT-hMSCs) were obtained as a gift (Glackin Lab; City of Hope, Duarte, CA). Cryopreserved hMScs were defrosted then seeded in a 75 cm² corning cell culture flask at a density of 2500 cells/cm². Cells were incubated with MSC culture medium: Alpha-Minimum Essential Medium (containing nucleosides, Phenol Red and L-glutamine), 15% fetal bovine serum (Sigma), 1% penicillin-streptomycin (Sigma) and were maintained at 37°C and at 5% CO₂ in a humidified incubator. After 2-3 days, hTERT-hMSCs were harvested using 0.25% trypsin-EDTA solution (Sigma) and expanded in T225 culture flasks at 4000 cells/cm² upon reaching 70–80% confluency.

3.3 Cell Microencapsulation

Hydrogel precursor solution was prepared by combining 0.1 g/mL 10 kDa PEGDA (10% w/v), 37 mM 1-vinyl-2-pyrrolidinone with hydrophilic photoinitiators (1.5% (v/v) triethanolamine and 0.1 mM eosin Y) in HEPES-buffered saline (pH 7.4). hTERT-hMSC cells with a final concentration of 5000 cells/µl were later mixed with the hydrogel precursor solution. A hydrophobic photoinitiator solution (2,2-dimethoxy-2- phenyl acetophenone in 1-vinyl-2-pyrrolidinone; 300 mg/mL) was combined with mineral oil (3 μ l/ml, sterile filtered; Sigma-Aldrich). Microspheres were formed after adding the hydrogel precursor solution containing cells into the mineral oil by first vortexing the combination for 4s under ambient light then vortexing for an addition 3s under white light from a Metal Halide Illuminator (MH-100, Edmund optics). After vortexing was complete, microspheres were exposed to white light for an additional 20s (Fig. 3.1). Lastly, microspheres were isolated by two medium washes followed by 5 min centrifugation at 1400 rpm. These isolated microspheres were finally placed in a 25cm² flask with 5ml MSC culture medium, before the application of vibration.



Figure 3.1: Microencapsulation via vortex-induced emulsion. Harvested hTERT-hMSCs are combined with PEGDA precursor solution; vortexed in oil and resulting microdroplets are photopolymerized with white light to form microspheres.

3.4 Mechanical Stimulation through LMHF Vibrations

LMHF vibrations were achieved using a BOSE ElectroForce 3100 mechanical testing machine (Fig. 3.2) that can apply accelerations up to 20 g acceleration and frequencies up to 100 Hz.



Figure 3.2: Mechanical stimulation. a-c) Stimulation set up: Microencapsulated hTERThMSCs are placed in a 25cm² flask and mounted on the vibrating machine.

A custom clamp was built to secure the microsphere flasks (Fig 3.2c). This was attached to the machine's moving actuator, which applied the vibration. The frequency and displacement parameters were selected in the machine's WinTest software. All vibrated flasks were exposed to vibration frequencies of 100 Hz for 24 hours at room temperature. Vibrated flasks were divided into to three different accelerations: 0.3 g, 3 g, and 6 g, with a displacement amplitudes of 0.00745 mm, 0.0745 mm, and 0.149 mm, respectively (Table 1.). The acceleration (g) can be determined by the relation below,

$$a=\frac{D(2\pi f)^2}{9.8},$$

where a is acceleration, f is the frequency, and D is peak-to-peak displacement [102].

Frequency (Hz)	Displacement (mm)	Displacement (mm)	Acceleration (g)
	Peak	Peak-peak	
100	0.00745	0.0149	0.3
100	0.0745	0.149	3
100	0.149	0.298	6

Table 1: LMHF vibration parameters.

Control samples were not vibrated and kept at on an isolated bench at room temperature for 24 hrs. To avoid contamination, fully closed flask caps for both test and control groups were used for the duration outside the incubator. After 24-hour vibration, test and control microspheres were placed in the top well of Transwells (74µm membrane size Corning Netwell inserts; Sigma) in 6-well plates with 5 mL of culture medium and kept in an incubator with a humidified, 5% CO₂ atmosphere.

3.5 Cell viability study

hTERT-hMSCs in both test and control microsphere samples were assessed for cell viability on days 1, 4, 7, 14 and 21. Microspheres were incubated with media and 2 mM calcein acetoxymethyl ester and 4 mM ethidium homodimer (LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells, Life technologies) [101] for 10 min in a 37°C, 5% CO₂ incubator. The stained microspheres were then observed under an epifluorescent microscope (Axio Observer Z1, Zeiss) to see labeled live (green; ex/em ~495 nm/~515 nm) and dead (red; ex/em ~495 nm/~635 nm) hTERT-hMSCs. For each sample, 3-6 pictures were thresholded and labeled cells were counted using Image J to get percentage viability.

3.6 Histochemistry

Several stains were used to observe the effect of LMHF vibrations on the differentiation of the encapsulated hTERT-hMSCs. ALP stains were used as marker of osteogenic differentiation. Alizarin Red S stain was used to detect the presence of mineralization by staining calcium deposits. Oil red O staining was used to identify mature adipocytes by staining lipid droplets. Finally, saffranin O staining was used to identify chondrocytes by staining glycosaminoglycans (GAGs) [103]. All test and control microsphere samples are placed in the top well of 12 well plate Transwells (74µm membrane size Corning Netwell inserts; Sigma) in order to perform the assays.

3.6.1 ALP assay

This assay was conducted four days after the initial vibration using a Fluorescence Alkaline Phosphatase Detection Kit (Sigma). Briefly, microspheres were washed with phosphate buffer saline (pH 7.4, containing TWEEN[®]) (PBST) followed by fixing cells with a proprietary fixation buffer for 5 min, and finally staining the microspheres with the staining solution for 30 min after washing again with PBST.

ALP positive cells stain purple and the stained cells were observed in phosphate buffered saline (PBS) in a 12 well plate under bright field microscopy. Images of ALP-positive cells were taken and positive cells were counted using Image J counter.

3.6.2 Alizarin Red S Assay

Briefly, microsphere samples were washed with PBS and fixed with 10% neutral buffered formalin solution for 15 mins, and then washed with distilled water. Microsphere samples were then stained with 2% Alizarin Red S stain (certified by biological stain commission, Sigma) for 30 mins, followed by two distilled water washes. Calcium deposits stain red in color and these were observed in distilled water under color microscopy. Representative images of positively stained microspheres were taken. Images were analyzed with Image J software

3.6.3 Oil red O Stain

Microsphere samples were washed with PBS and fixed with 10% neutral buffered formalin solution for 15 min, washed with distilled water and then later washed with .60% isopropanol. Microsphere samples were then stained with 0.3% Oil red O solution (certified by biological stain commission, Sigma) for 15 mins, followed by 2-3 washes

with distilled water. The lipid droplets if stained, appear red in color. These were observed in distilled water under color microscopy. Representative images of positively stained microspheres were taken. Images were analyzed with Image J software.

3.6.4 Safranin O Stain

Microsphere samples were washed with PBS and fixed with 10% neutral buffered formalin solution for 15 min and then later washed with distilled water. They were then stained with 0.6% safranin O solution (Sigma) for 10-12 mins and following staining underwent two distill water washes. The sulphated GAGs, if stained, appear orange. Stained microspheres were observed in distilled water under color microscopy. Images were analyzed with Image J software.

3.7 Statistical analysis

For all assays, data were taken in triplicate as number of positively stained microspheres. A Student's t-test was performed for all measurements between controls and each test condition. P values of less than 0.05 were considered statistically significant.

Chapter 4

RESULTS

4.1 Cell Viability

The viability of test (vibrated) and control microencapsulated hTERT-hMSC samples ranged from 70-80% on Day 1 and reduced to 48-52% on Day 21 (Fig. 4.1, Fig 4.2, Fig 4.3). A maximum viability of 80% was observed on Day 1 in the 0.3 g group and a minimum viability of 48% was observed in the 6 g group on Day 21. All groups decreased in viability over time. There was no statistical difference between samples on any days.





Figure: 4.1: Cell viability from Day 1 to Day 21 of the test (vibrated) sample with live (green; ex/em ~495 nm/~515 nm) and dead (red; ex/em ~495 nm/~635 nm) cells.



Figure: 4.2: Cell viability of test (vibrated) samples from Day 1 to Day 21. The maximum viability of 80% is seen in 0.3 g sample at Day 1 and minimum viability of 49% is seen in 6 g sample at Day 21. Error bars indicate standard deviation.



Figure: 4.3: Cell viability of control (non vibrated) samples from Day 1 to Day 21. The maximum viability of 78% is seen in 0.3 g sample at Day 1 and minimum viability of 48% is seen in 6 g sample at Day 21. Error bars indicate standard deviation.

4.2 Alkaline Phosphatase Expression

After the application of LMHF vibrations, ALP levels expressed by hTERT-hMSC (Fig. 4.4) increased significantly in vibrated groups over control for 0.3 and 3 g groups, while ALP expression decreased compared to control in the 6 g group (Fig. 4.5) ALP activity was greatest for 0.3 g and decreased with increasing vibration magnitude.



Figure: 4.4: ALP staining on Day 4 for test (vibrated) and control (non-vibrated) groups. The red arrow shows the ALP positive purple stained cells. There was no ALP activity detected in 6 g groups.



Figure: 4.5: Levels of ALP staining on Day 4 for test (vibrated) and control (nonvibrated) samples. ALP expression decreases with increase in the magnitude of vibration. Asterisks denote statistical significance (p < 0.05). Error bars indicate standard deviation.

4.3 Differentiation

LMHF vibrated hTERT-hMSCs did not differentiate into chondrocyte or adipocyte but were only induced to differentiate down the osteogenic pathway.

4.3.1 Osteogenesis

There was no Alizarin Red S staining detected in either vibrated or control groups from Days 1-7 after exposure to LMHF vibrations On Days 14 and 21, however, Alizarin Red S positive microspheres were seen in both vibrated and control groups (Figs. 4.6, 4.7). Alizarin Red S positive microspheres increased in all groups from Day 14 to Day 21, but significantly higher staining was observed in vibrated groups when compared to control (Figs. 4.8, Fig, 4.9). The 6 g group did not stain positive on Day 14. Alizarin Red S positive microspheres were highest in the 0.3 g vibration group on Days 14 and 21. Positive staining decreased with an increase in the intensity of vibration.



Figure: 4.6: Alizarin Red S staining on Day 14 for test (vibrated) and control groups. Arrows show the uniquitous positive red color of stained calcium deposits. The onset of mineralization was accelerated in 0.3 and 3.0 g vibrated groups compared to non-vibrated controls. There was no positive staining detected in 6 g vibrated groups.



Figure: 4.7: Alizarin Red S staining on Day 21 for test (vibrated) and control groups. Arrows show the abundant positive red color stained calcium deposits. Maximum staining can be observed in 0.3 g test sample at Day 21.



Figure: 4.7: Alizarin Red S staining on Day 14 for test (vibrated) and control groups. Alizarin Red S positive microspheres were highest for 0.3 g vibrations at Day 14 and decreased with increases in vibration magnitude. Asterisks denote statistical significance (p < 0.05). Error bars show standard deviation.



Figure: 4.8: Alizarin Red S staining on Day 21 for test (vibrated) and control groups. Alizarin Red S positive microspheres are also highest in 0.3 g at Day 21 and decreased with increases in vibration magnitude. There was an increase in positively stained control cells compared to Day 14. Asterisks denote statistical significance (p < 0.05). Error bars indicate standard deviation.

4.3.2 Adipogenesis

There were no Oil Red O stained cells observed in either vibrated or control groups from Days 1-21 after exposure to LMHF vibration (Fig. 4.9).



Figure: 4.9: Oil Red O staining on Day 21 for test (vibrated) and control samples. There was no positive staining detected at any time point in any group.

4.3.3 Chondrogenesis

There was no positive Safranin O staining observed in either vibrated or control groups from Day 1-21 after exposure to LMHF vibration (Fig. 4.10).



Figure: 4.10: Safranin O staining on Day 21 for test (vibrated) and control samples. There was no positive staining detected at any time point in any group.

Chapter 5

DISCUSSIONS, CONCLUSIONS, FUTURE DIRECTIONS

This study investigated the effect of range of LMHF vibrations on PEGDA-encapsulated hTERT-hMSCs. Experiments were performed to broadly assess whether these conditions can mimic the osteogenic whole body response to LMHF vibrations reported in the literature. Of the conditions tested, it was observed that LMHF vibrations with low accelerations (0.3 g) enhanced the osteogenic differentiation of encapsulated hTERT-hMSCs the most. This was assessed by ALP activity and matrix mineralization. It was also observed that LMHF vibration did not differentiate encapsulated hTERT-hMSCs into chondrogenic or adipogenic lineages as determined by negative results when staining for lipid droplets and cartilage ECM specific GAGs.

The use of the synthetic PEGDA to encapsulate hTERT-hMSCs served as a "blank slate" for these cells due to the polymer's lack of cell adhesion sites and non ECM-like structure [96, 104]. This implies that these blank slate-encapsulated cells receive no microarchitectural or biochemical cues that can stimulate hMSC differentiation like cells encapsulated in natural polymers [93, 95]. Moreover, the PEGDA-microencapsulated hTERT-hMSCs were kept maintained in MSC culture medium and not in an osteogenic medium, which contains the chemical stimulators (dexamethasone, ascorbic acid, and beta-glycerophosphate). These conditions permitted as the study to determine whether LMHF vibration alone is a sufficient stimulus to drive the differentiation of hTERT-hMSCs. A positive result would further the development of a 3D *in vitro* model of whole body vibration. Such a model might avoid the inconsistences reported with human and animal whole body vibration studies [8, 12, 67, 68, 72].

The range of LMHF vibrations with low and high accelerations (0.3 g, 3 g and 6 g) at 100 Hz that were applied to encapsulated cells fall within the same ranges of LMHF vibrations performed on various animal and human whole body vibration studies [3, 6, 8, 67-71]. The 100 Hz frequency was selected because various animal models have reported osteogenesis at accelerations within the 90-100 Hz frequency range [3, 105, 106].

Declining cell viability occurred over time, but this seemed correlated to mineralization levels; cell viability appeared to decrease with increases in calcification. ALP activity was higher in 0.3 g and 3 g vibrated groups compared to control, indicating that these cells are differentiating down an osteogenic pathway [27]. Positive Alizarin red S staining starting on Day 14 confirmed osteogenesis in those groups. There was a level of positive alizarin red stain in control groups. The mineralization observed in control samples might be the effect of the vibration induced in the vortexing step during the cell microencapsulation process, predisposing the hTERT-hMSCs towards the osteogenic lineage. A future investigation to determine the contribution of the vortex would repeat this study with cells encapsulated into polymer structures requiring no vortexing.

The inhibition of mineralization observed in 6 g groups may be partly explained by the fact that in some animal studies it has been shown that higher acceleration (approximately 5 g and above) decreases bone resorption, but has no effect on bone formation [107]. While this does not explain the inhibition effect observed at 6 g, it is important to note that *in vivo* studies do not expose subjects to 24 hours of LMHF vibrations as in the present study. Future studies will explore the effect of varying LMHF vibration duration on encapsulated cells.

Since all groups stained negative for Saffranin O, and Oil red O stains at every time point, it is possible to conclude that LMHF vibrations do not induce encapsulated hTERT-hMSCs towards chondrogenic or adipogenic pathways, but preferentially induce them towards osteogenic pathways.

LMHF vibrations accelerated osteogenic differentiation of PEGDA encapsulated hTERThMSCs, conflicting with reports from many studies of LMHF vibrated monolayers while confirming results obtained with MSCs encapsulated in natural materials. This work confirms that the LMHF vibrations alone can induce osteogenesis without input from the encapsulating biomaterial. Thus, this study demonstrated the osteogenic effect of LMHF vibrations and paved the way for further *in vitro* LMHF experiments with PEGDAmicroencapsulated cells.

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