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School of Medicine

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Master's degree in medical biotechnologies

Master Thesis

Multi-stimuli bioreactor inducing shear stress and hydrostatic pressure into 3D bone models for healing studies

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Abstract

Bone tissue engineering (BTE) represents a promising strategy for addressing critical-size bone defects that surpass the natural regenerative capacity of bone. This study aimed to investigate the influence of mechanical stimuli on the osteogenic differentiation of immortalized human bone marrow-derived mesenchymal stem cells (BM-MSCs) cultured within biomimetic 3D-printed polylactic acid (PLA) scaffolds. A custom-designed bioreactor system was developed to apply two culture regimens: continuous perfusion and a rehabilitation program combining perfusion with cyclic hydrostatic pressure (The condition designated as D refers to constructs maintained under continuous direct perfusion at a constant flow rate of 0.3 mL/min. The condition designated as RP1 represents constructs cultured under the same perfusion parameters as in D, with the additional application of hydrostatic pressure.) Cellular metabolic activity was assessed using the resazurin reduction assay on day 14. Results indicated comparable viability in both groups. Osteogenic differentiation was evaluated by reverse transcription-quantitative PCR (RT-qPCR) targeting key osteogenic markers (RUNX2, ALP, OPN, OCN, MMP3, and MMP9). demonstrated significant upregulation of both ALP and MMP3, with MMP3 showing the highest expression among all markers. Early osteogenic marker RUNX2 also showed a considerable increase, while late markers OPN and OCN displayed limited expression, indicating the culture period primarily supported early-to-mid stage differentiation. These findings underscore the potential of integrating D and RP1 into bioreactor systems to promote osteogenesis within engineered scaffolds. This approach provides valuable insights for optimizing scaffold design and mechanical stimulation protocols in BTE applications.

1. Introduction

1.1. Bone tissue

Bone performs a diverse range of functions and responds to various metabolic, physical, and endocrine stimuli. It serves as the foundation for bodily movement, provides load-bearing capacity to the skeleton while protecting internal organs, houses essential biological components for hematopoiesis, sequesters harmful metals such as lead, and regulates the balance of key electrolytes through calcium and phosphate storage (Amini, Laurencin and Nukavarapu, 2012). In the human body, bones play a crucial role by serving several essential functions. They form a structural framework that supports the attachment of muscles and various tissues, facilitating body movements. Additionally, bones provide a protective barrier for internal organs, safeguarding them from potential injuries (Biomaterials *for bone tissue engineering*, 2008). Bone possesses a natural ability for regeneration, especially in younger individuals, with most fractures or bone defects healing on their own without the need for significant medical intervention. However, when bone defects exceed a certain threshold, known as critical-size defects (CSDs), they lose this regenerative capacity and require surgical treatment. A CSD is defined as a segmental bone defect whose length is greater than 2 or 2.5 times the diameter of the affected bone (Dimitriou *et al.*, 2011) Additionally, bone is constantly undergoing a cycle of resorption and renewal, with continuous chemical exchange and structural remodeling driven by internal factors and external mechanical demands. Due to its unique ability to regenerate without scarring, bone has been aptly described as the ultimate smart material. Bone is primarily composed of calcium crystals, hydroxyapatite (~70 wt%), embedded within a matrix of collagen and other mineralized extracellular matrices (ECM), along with various cellular components. The human skeletal system contains two types of mature bone, both structured around osteons: cortical and cancellous bone, also known as compact and spongy (or trabecular) bone, respectively (Lai *et al.*, 2015). Cortical bone is highly mineralized and dense, with typical void porosity of around 10%, ranging between 5% and 30%. This results in a higher elastic modulus (17 GPa), although at the cost of reduced toughness. The structure of cortical bone consists of compact cylinders that provide protection for the inner cancellous bone. In contrast, cancellous bones are highly porous (30-90%) and have a lower elastic modulus and tensile strength (<2 MPa). Its irregular, sponge-like structure helps absorb mechanical load while creating a microenvironment for biological activity. This structure is surrounded by organic components, such as marrow, blood vessels, and cellular elements, which make up less than 2% of the bone (Langer, 2000).

1.2. Bone Tissue engineering and Regeneration

Tissue engineering (TE) is an interdisciplinary field that integrates engineering, life sciences, and material science, with the goal of remodeling and restoring tissue function (Zhu *et al.*, 2019). It involves three main components: cells, scaffolds, and the bioreactor environment. One approach within tissue engineering is in situ TE, which aims to achieve functional tissue regeneration by implanting cell-free scaffolds designed to recruit cells and promote tissue reconstruction in vivo (Chen and Liu, 2016). In this context, biodegradable scaffolds play a crucial role. They serve not only as temporary templates for tissue regeneration and integration with the native tissue, gradually degrading while transferring mechanical load to the newly formed tissue but also interact with cells and bioactive molecules that regulate tissue remodeling. Therefore, it is essential for scaffolds to mimic the extracellular matrix (ECM), restore cell – ECM interactions, and deliver proregenerative signals to facilitate effective tissue remodeling (Chen and Liu, 2016). Autogenous bone transplantation remains the benchmark for clinical bone repair; however, it is associated with unavoidable complications, including secondary surgical trauma and significant donor site injuries (Alonzo *et al.*, 2021). In contrast, allogeneic bone serves as an alternative but carries risks such as immune rejection and potential disease transmission (Haugen *et al.*, 2019). Furthermore, its osteogenic potential and biological efficacy are often inferior to those of autologous bone (PérezGonzález *et al.*, 2020). Recent advancements in research have introduced a range of bone tissue engineering (BTE) strategies, particularly focusing on scaffold materials, which offer promising solutions for the treatment of bone defects.

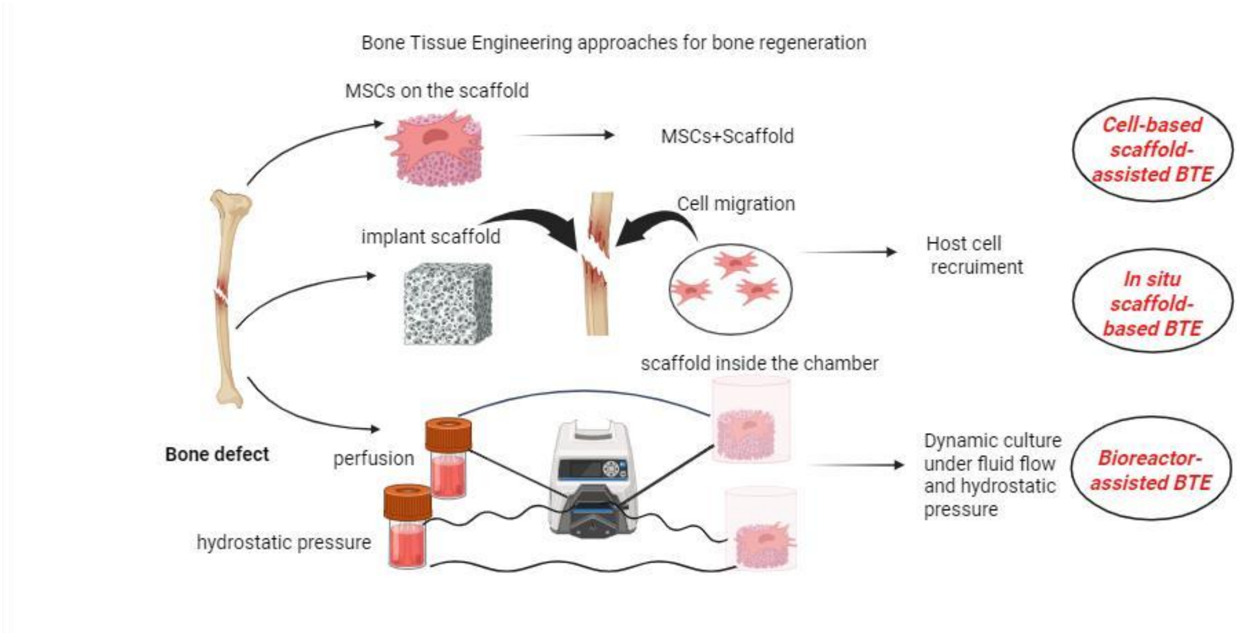

***Figure** 1 Conceptual overview of bone tissue engineering and regeneration strategies. The schematic illustrates the integration of mesenchymal stem cells (MSCs), biodegradable scaffolds, and mechanical stimulation within a bioreactor or in vivo environment to promote cell recruitment, osteogenic differentiation, extracellular matrix remodeling, and functional bone repair at the defect site.*

1.3. Mesenchymal Stem Cell in Bone Tissue Engineering

Mesenchymal stem cell (MSCs) is a crucial resource for cell-based therapies in regenerative medicine. They have demonstrated significant potential in repairing damaged tissues across a range of degenerative diseases, as evidenced by studies in both animal models and human clinical trials (Nakamura *et al.*, 2013; Kawai *et al.*, 2015; Oh *et al.*, 2018; Xu *et al.*, 2018). One of the key features of MSCs is their homing ability, which enables them to migrate to sites of injury, where they can differentiate into the local tissue components and secrete chemokines, cytokines, and growth factors that promote tissue regeneration (Zhang *et al.*, no date; Huang *et al.*, 2014; Folestad, Kunath and Wågsäter, 2018). Among MSCs, bone marrow-derived MSCs(BMSCs) stand out as non-hematopoietic stem cells found in the bone marrow, with the capacity for multipotent differentiation (Liu *et al.*, 2017).MSC transplantation can enhance wound healing through both cell differentiation and the release of paracrine factors (Chen *et al.*, 2004; Wu *et al.*, 2007; Jackson, Nesti and Tuan, 2012). However, the limited viability of MSCs at the transplantation site often reduces their therapeutic potential. Therefore, improving the survival of transplanted MSCs and enhancing their secretion of factors and biological functions in vivo is crucial for optimizing their effectiveness in tissue regeneration (Chen *et al.*, 2008). MSCs have garnered significant attention for their potential in engineering bone grafts, primarily due to their inherent ability to differentiate and contribute to bone formation during the natural developmental process. This potential has sparked extensive research into characterizing MSCs and identifying numerous sources for their isolation (Arvidson *et al.*, 2011). These cells can be isolated from a wide variety of adult tissues, including bone marrow, peripheral blood, umbilical cord blood, synovial membrane, dental pulp, adipose tissue, and others. Their isolation typically relies on a simple technique that capitalizes on their ability to adhere to plastic surfaces in culture (Miura *et al.*, no date; Kuznetsov *et al.*, 2001; Rosada *et al.*, 2003). Importantly, MSCs exhibit a high capacity for proliferation and can withstand freezing, allowing for large-scale in vitro expansion to obtain sufficient cell numbers for clinical applications (Kuznetsov *et al.*, 2001). In addition to adult sources, MSCs have also been derived from embryonic stem cells and induced pluripotent stem cells (iPSCs). These embryonic- and iPSC-derived MSCs share similar multipotent characteristics with their adult counterparts, but they show enhanced survival and expansion potential, due to higher telomerase activity. However, before they can be used in clinical settings, further studies are needed to rule out the risk of teratoma formation. Incorporating MSCs into biomaterials for BTE has been widely studied as a promising strategy for enhancing bone regeneration and osteointegration during bone repair. MSCs contribute to bone formation both directly, through osteogenic differentiation, and indirectly, by promoting the migration and differentiation of host osteoprogenitor cells. Predifferentiating MSCs into the osteogenic lineage before implantation has been shown to accelerate bone healing in preclinical models of large bone defects, craniofacial deformities, and spinal fusions.

1.4. Three-dimensional scaffolds for Bone Tissue Regeneration

Historically, scaffolds in tissue engineering have been employed as supportive structures for cell attachment, vascularization, tissue growth, and regeneration (Hutmacher, 2000; Liu and Ma, 2004). In BTE, scaffolds are designed to mimic the function of native bone tissue (Kashirina *et al.*, 2019), either acting as substitutes or encouraging tissue ingrowth from surrounding bone (Sharma *et al.*, 2021). The “ideal” BTE scaffold should also regulate cellular interactions, promote vascularization, and replicate the mechanical properties of the bone at the target site (Kantaros, Chatzidai and Karalekas, 2016). Furthermore, the preparation and sterilization processes for these scaffolds must adhere to industry and regulatory standards (Thayaparan *et al.*, 2021). BTE scaffolds need to possess a highly porous, three-dimensional structure that closely replicates the characteristics of native bone, including porosity, pore size, and interconnectivity. This design is crucial to support cellular attachment proliferation, and to provide space for new tissue growth and vascularization. While high porosity is essential for promoting these biological processes, it also presents challenges as it can significantly compromise the mechanical properties of the scaffold due to the increased void space. Therefore, for BTE applications, it is important to strike a balance by developing scaffolds that maintain high porosity while preserving adequate mechanical strength and structural integrity to ensure their effectiveness in supporting bone regeneration (Mohamad Yunos, Bretcanu and Boccaccini, 2008; Loh and Choong, 2013).

1.5. Bioreactors in Bone Tissue Engineering

The perfusion of culture medium through scaffolds plays a crucial role in ensuring an even distribution of nutrients and the efficient removal of waste products. To achieve this, various bioreactor systems have been developed, such as spinner flasks, rotating wall vessel bioreactors, and laminar flow bioreactors. Not only do these systems support cell growth, but fluid flow within them also influences cellular behavior, potentially guiding the mechanical regulation of progenitor cell growth and differentiation (Gaspar, Gomide and Monteiro, 2012). This approach has gained significant attention in bone tissue engineering due to the role of fluid micromovement in bone remodeling, which is naturally stimulated by the slight deformations in bone tissue caused by physical activity (Wittkowske *et al.*, 2016). However, mammalian cells are sensitive to fluid shear forces, and excessive shear stress can lead to cell death (Tanzeglock *et al.*, 2009). This risk is heightened in systems like spinner flasks and rotating wall vessels, where turbulent flow is more likely to occur, exacerbating shear-induced damage (Tanzeglock *et al.*, 2009; Brindley *et al.*, 2011). In contrast, laminar flow bioreactors offer a more controlled and predictable environment, with fluid flow patterns that reduce the likelihood of cell damage (Brindley *et al.*, 2011; Tsai *et al.*, 2019). These bioreactors ensure an even distribution of gases and nutrients across the scaffold at lower shear rates, making them a favorable choice for influencing progenitor cell fate through fluidic stimuli (Bergemann *et al.*, 2015). By providing a less harmful and more controlled mechanical environment, laminar flow bioreactors present clear advantages for bone tissue engineering applications (Song *et al.*, 2020).

2.Objective of the study

Therefore, the main objective of this research is to explore how different biophysical cues affect the behavior and differentiation of bone marrow-derived mesenchymal stem cells (MSCs) within 3D, bioengineered microenvironment that mimic the architecture of trabecular bone found in long bones. Specifically, this study investigates two key physical stimuli: fluid flow-induced shear stress and hydrostatic pressure generated using a pinch valve system. By examining how these forces influence MSC fate decisions, the study aims to enhance our understanding of the mechanobiological mechanisms that guide stem cell differentiation. These insights could inform the development of more effective strategies for bone repair and regeneration.

3.Materials and methods

3.1. Design and Fabrication of 3D-Printed Biomimetic PLA Scaffolds

Biomimetic 3D-printed scaffolds made from polylactic acid (PLA), here referred to as P3S3, were developed to closely replicate the structural features of human trabecular bone under physiological conditions. The scaffold design was inspired by the internal architecture of bones such as the ulna, tibia, and femur, incorporating a trabecular spacing of approximately 284 μm and a trabecular thickness of 258 μm, resulting in an overall porosity of 44.7%. A custom script was used to generate a trabecular-like structure within a cylindrical volume (10 mm diameter, 5 mm height).
The resulting computer-aided design (CAD) files were imported into idea Maker software (Raise3D Inc., Irvine, CA, USA) and sliced along the Z-axis at 0.25 mm intervals. The scaffolds were fabricated using a Raise3D N2 3D printer (Raise3D Inc., Irvine, CA, USA) and PLA filament (FormFutura BV, The Netherlands). The nozzle temperature was set to 205 °C, the build plate to 60 °C, and a 0.4 mm nozzle was used for extrusion. The P3S3 scaffolds were designed according to a previously validated computational protocol and fabricated by external research partners using standardized 3D-printing prosedures, were designed to balance structural integrity and porosity, making them suitable for bone tissue engineering applications. Their architecture was optimized to mimic native trabecular bone morphology. Characterization of bioactivity and permeability is currently in progress and follows procedures previously reported in related studies (Ledda *et al.*, 2022; Gabetti *et al*, 2024).

3.2. Bioreactor

A custom-designed, parallelized bioreactor platform was developed to deliver controlled mechanical stimulation to 3D tissue constructs (Gabetti *et al,* 2022). The parallelized, automated perfusion bioreactor consists of three independent 3D-printed culture chambers (CCs), connected to three independent closed-loop hydraulic circuits controlled by a custom control unit, designed for housing and culturing 3D bone-like tissues. This system enables the application of both fluid flow-induced shear stress and cyclic hydrostatic pressure, two key mechanical cues known to influence cell behavior and tissue development (Bancroft *et al.*, no date; Wittkowske *et al.*, 2016). Each CC (Dental SG Resin, Formlabs, United States) is composed of two screwable parts that allow housing constructs of different sizes, which are in turn press-fit in a tailored polydimethylsiloxane (PDMS) holder (SYLGARD 184, Dow Corning, United States). Each closed-loop hydraulic circuit is composed of oxygen-permeable tubing (Darwin Microfluidics, France) and a culture medium reservoir, and is connected to a multichannel peristaltic pump (G100–1 J, Longer Precision Pump, China) suitable to be incubated at 37 °C. The pump is controlled by a custom control unit equipped with a microcontroller board (Arduino Micro, Arduino, Italy), that allows setting the pump's parameters and guarantees the same D conditions in each CC. The system operates in D (The condition designated as D refers to constructs maintained under continuous direct perfusion at a constant flow rate of 0.3 mL/min.) mode with adjustable flow rates ranging from 0.006 to 24 mL/min, ensuring continuous medium circulation through the constructs and enhancing mass transport and exposure to shear stress. In parallel, RP1 is applied by means of a solenoid-actuated pinch valve, which intermittently closes with programmable periods (T) ranging from 0.25 to 30 seconds and duty cycles (DC) between 0 to 100%, enabling precise modulation of pressure dynamics. This versatile setup allows simulation of various physiological conditions, making it an effective tool for studying mechanobiological responses in engineered bone-like tissues.

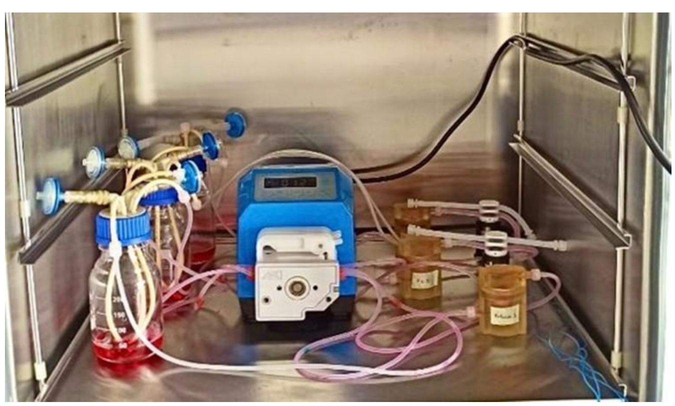

Figure 2. *Bioreactor setup with hydrostatic pump for controlled D and RP1 culture. (The condition designated as D refers to constructs maintained under continuous direct perfusion at a constant flow rate of 0.3 mL/min. The condition designated as RP1 represents constructs cultured under the same perfusion parameters as in D, with the additional application of hydrostatic pressure.)*

3.3. Cell Culture Conditions

Human telomerase reverse transcriptase (hTERT)-immortalized bone marrow-derived mesenchymal stem cells (BM-MSCs) from the clonal line Y201, originally developed by James et al (James *et al.*, 2015). were cultured under standard conditions. The cells were maintained in a basal medium composed of low-glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Thermo Fisher Scientific, Inc.), supplemented with 15% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco, Thermo Fisher Scientific, Inc.). Cultures were incubated at 37 °C in a humidified environment containing 5% CO_2. Once the cells reached 80–90% confluency, they were passaged using trypsin/ethylenediaminetetraacetic acid (trypsin/EDTA; Gibco, Thermo Fisher Scientific, Inc.) prior to being used in any subsequent assays.

3.4. Cell seeding and culturing under dynamic and mechanical load conditions

Immortalized BM-MSCs of the clonal line Y201 were directly seeded on the top of P3S3 scaffolds inserted into the holders of an automated bioreactor, which was placed in a 6-well cell culture plate. Seeding was performed using a defined cell density of 2×10^6 cells, resuspended in 40 µL of basal medium (BM) that was low-glucose and supplemented with 15% fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin), with a total volume of 150 µL, including 110 µL of Pure Col EZ (Merck, Italy). The samples were incubated for 4 h to allow cell infiltration and adhesion, after which BM was added. The samples were then incubated overnight to ensure proper cell attachment. Following overnight incubation, the samples were cultured under two different conditions for 14 days. In the first condition, constructions were cultured under continuous direct perfusion at a flow rate of 0.3 mL/min, referred to as the dynamic culture condition (D). In the second condition, termed rehabilitation program 1 (RP1), samples were exposed to a combination of D and RP1. The pressure, set at 15 kPa, was applied for 2 hours per day using pinch valves operating with a cycle time of 10 seconds (T = 10 s) and a duty cycle of 70% (DC = 70%) (n=3).

3.5. Metabolic Assay

The resazurin reduction assay was used to assess cellular metabolic activity, serving as an indicator of cell viability and proliferation. To perform this test, a 1% resazurin solution was prepared using resazurin sodium salt (Merck, Darmstadt, Germany) dissolved in 1X phosphate-buffered saline (PBS). On days 0 and 14 of cell culture, 15 µL of this 1% resazurin solution was added to each well containing 1 mL of culture medium, resulting in a final concentration of 0.015% (v/v). After incubating the samples for 3 to 4 hours, 100 µL aliquots were taken to measure fluorescence. Readings were obtained using a microplate reader (Tecan GENios Microplate Reader, Spark, Tecan Trading AG, Männedorf, Switzerland) with excitation and emission wavelengths set at 530 nm and 590 nm, respectively. All measurements were performed in triplicate (n = 3) and averaged for analysisClick or tap here to enter text.(Matic *et al.*, 2024).

3.6. Gene Expression Analysis

To assess osteogenic differentiation, reverse transcription-quantitative PCR (RT-qPCR) was carried out. Total RNA was first extracted from the samples using TRIzol™ Reagent (Invitrogen Life Technologies) following the manufacturer's protocol. The concentration and purity of the RNA were then measured using a NanoDrop One spectrophotometer (Thermo Fisher Scientific).Next, 1 µg of RNA was treated with DNase I to eliminate any residual genomic DNA, and complementary DNA (cDNA) was synthesized using the iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad Laboratories) with a Mastercycler X50s thermal cycler (Eppendorf).Quantitative PCR was performed on the resulting cDNA (diluted to 1 ng/µL) using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories) and gene-specific primers at a final concentration of 2.5 µM. Reactions were run and monitored using the CFX96™ Real-Time PCR System (Bio-Rad Laboratories) in accordance with standard protocols. Gene expression levels were normalized to the housekeeping gene YWHAZ (Tyrosine 3monooxygenase/tryptophan 5-monooxygenase), and relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (Livak method)(Livak and Schmittgen, 2001; James *et al.*, 2015), with comparison to the control group. The osteogenic markers analyzed included OPN (osteopontin), OCN (osteocalcin), ALP (alkaline phosphatase), MMP3(matrix metalloproteinases-3), MMP9 (matrix metalloproteinases 9), and RUNX2 (runt-related transcription factor 2). Primers and probes are reported in Table 1.

Table 1. Description of the designed primers

Gene	**Forward Primer**	**Reverse Primer**
Runt-related transcription factor 2 (RUNX2)	5'- GAA TGC CTC TGC TGT TAT GA -3'	5'- GAA GAC GGT TAT GGT CAA GG -3'
Alkaline phosphatase (ALP)	5'- GAG TAT GAG AGT GAC GAG AAA G -3'	5'- GAA GTG GGA GTG CTT GTA TC -3'
Osteopontin (OPN)	5'- CCC ATC TCA GAA GCA GAA TC -3'	5'- TGG CTT TCG TTG GAC TTA C -3'
Osteocalcin (OCN)	5'- AGG CGC TAC CTG TAT CAA -3'	5'- CTC CTG AAA GCC GAT GTG -3'
Matrix metalloproteinase 3 (MMP3)	5'- TTT GAT GGA CCT GGA AAT GT -3'	5'- GGT CCC TGT TGT ATC CTT TG -3'
Matrix metalloproteinase 9 (MMP9)	5'- TTG ACA GCG ACA AGA AGT G -3'	5'- GGC ACT GAG GAA TGA TCT AAG -3'
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase (YWHAZ)	5'- GGA GGG TCG TCT CAA GTA T -3'	5'- ATC TCT TAG CTC CGT CTC AA -3'

4 Results

[1] 4.1. Cells' metabolic activity

The resazurin reduction assay was performed to evaluate the metabolic activity of cells cultured under two different conditions: D and RP1, (The condition designated as D refers to constructs maintained under continuous direct perfusion at a constant flow rate of 0.3 mL/min. The condition designated as RP1 represents constructs cultured under the same perfusion parameters as in D, with the additional application of hydrostatic pressure.) over a 14-day period. The assay measures the reduction of resazurin to resorufin as an indicator of cellular viability and proliferation.

As illustrated in Figure 2, cells cultured under the RP1 condition exhibited a higher metabolic activity compared to those in the D group after 14 days. The RP1 group demonstrated a mean fluorescence intensity of approximately 14,000 relative fluorescence units (RFU), whereas the D group exhibited a mean intensity of around 12,000 AU. Statistical analysis revealed that this difference was not statistically significant ($p > 0.05$), as indicated by the overlapping standard deviation bars ($n = 3$).

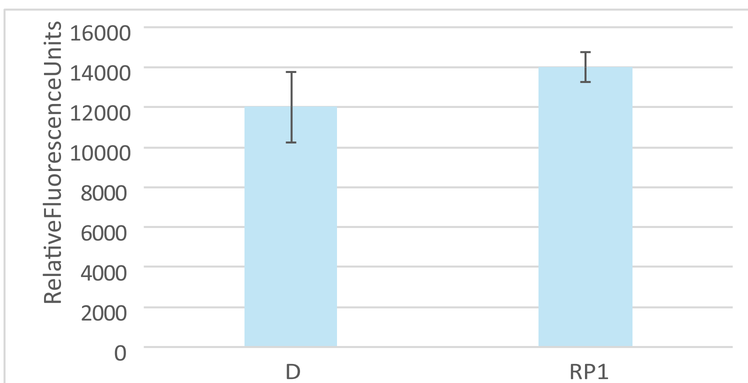

Figure 3. *Metabolic activity off cells under D and RP1 conditions on day 14, assessed by resazurin reduction assay. Values are mean ± SD (n = 3) difference not significant (p > 0.05). (The condition designated as constructs maintained under continuous direct perfusion at a constant flow rate of 0.3 mL/min. The condition designated as RP1 represents constructs cultured under the same perfusion parameters as in D, with the additional application of hydrostatic pressure.)*

These results suggest that both culture conditions support cell viability and proliferation over the 14-day period. Although the RP1 condition showed a trend toward higher metabolic activity, the lack of statistical significance indicates that further investigation with a larger sample size may be needed to confirm this observation

4.2. Gene Expression Analysis

Reverse transcription-quantitative PCR (RT-qPCR) was performed to assess the expression of osteogenic marker genes in the samples. The relative mRNA levels of RUNX2, ALP, OPN, OCN, MMP3, and MMP9 were normalized to the housekeeping gene YWHAZ and analyzed using the $2^{-\Delta\Delta Cq}$ method.

As shown in Figure 3, the osteogenic markers exhibited varied expression profiles. Among the genes analyzed, ALP and MMP3 showed the highest upregulation, with fold-change values exceeding 2.0, suggesting enhanced osteogenic activity in these groups. RUNX2 also displayed increased expression (~1.5 fold-change), consistent with its role as an early transcriptional regulator of osteogenesis. OPN, OCN, and MMP9 were expressed at lower levels, with RQ values below 1.0, indicating a modest or delayed response in these markers. Although the observed upregulation of RUNX2, ALP, OPN, OCN, and MMP9 was modest, it nevertheless indicates a potential stimulation of osteoblast differentiation and bone matrix mineralization. This outcome suggests that, while the applied hydrostatic pressure settings are capable of eliciting cellular responses, the degree of stem cell activation may be contingent upon both the intensity and the duration of the applied stimulus.

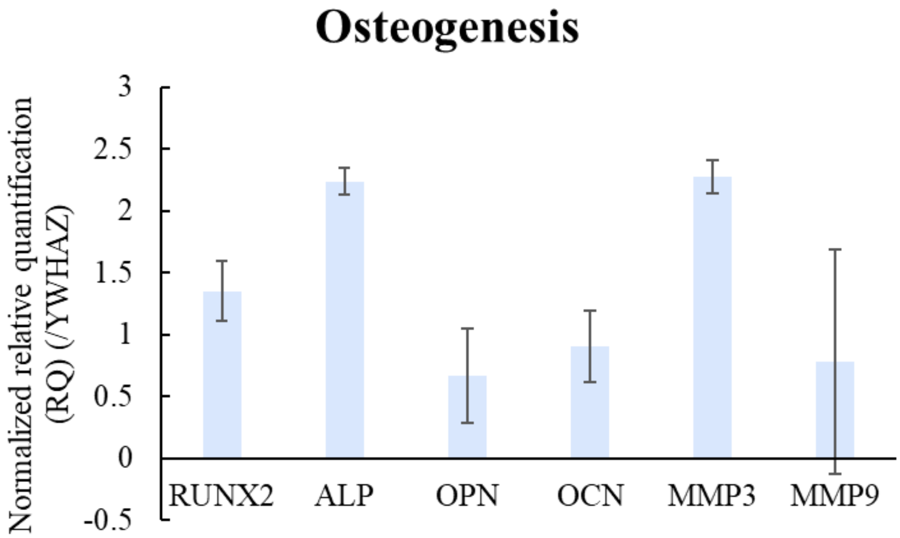

Figure 4. *RT-qPCR analysis of osteogenic markers normalized to YWHAZ. ALP and MMP3 were upregulated (mean ± SD, n = 3). The relative mRNA expression profiles of BM-MSCs exposed to fluid flow-induced shear stress in combination with a hydrostatic pressure of 15 kPa, simulating physiological loading for 2 hours per day (RP1), are shown in comparison with those of BM-MSCs subjected exclusively to fluid flow-induced shear stress (D).*

5. Discussion

The resazurin assay did not reveal any significant differences in metabolic activity between the D and RP1 conditions, indicating that the mechanical stimulation applied did not compromise cell viability. These findings suggest that the combination of hydrostatic pressure and perfusion was well tolerated by BM-MSCs and did not elicit cytotoxic effects. Consequently, the increased expression of osteogenic genes observed under the RP1 condition is likely driven by mechanotransducive signalling rather than by alterations in cell survival or metabolic function. The gene expression analysis conducted through RT-qPCR provides critical insights into the osteogenic differentiation of BM-MSCs cultured within 3D-printed P3S3 scaffolds under different mechanical stimuli. The experimental setup involved a custom-designed bioreactor system that applied two distinct culture regimens: continuous direct perfusion, referred to as the dynamic culture condition (D), which was maintained at a flow rate of 0.3 mL/min. A rehabilitation program (RP1), which combined perfusion with cyclic hydrostatic pressure. The RP1 was set at 15 kPa and was applied for 2 hours daily using pinch valves with a 10-second cycle time and a 70% duty cycle. The bioreactor platform used in this study was a custom-designed, parallelized system that delivers controlled mechanical stimulation to 3D tissue constructs. This system is composed of three independent 3D-printed culture chambers connected to three separate closed-loop hydraulic circuits, allowing for the application of both fluid flow-induced shear stress and cyclic hydrostatic pressure. This setup enables the simulation of various physiological conditions, making it a valuable tool for studying mechanobiological responses in engineered bone-like tissues. Notably, the expression levels of ALP and MMP3 were significantly upregulated in the RP1 group compared to the D condition, indicating a robust osteogenic response to the combined mechanical cues of D and RP1. ALP, an early osteogenic marker associated with matrix mineralization, exhibited the highest fold-change, suggesting that the applied stimuli favour the initiation of osteogenic pathways. The significant increase in MMP3 expression is also noteworthy, as matrix metalloproteinases are essential for extracellular matrix remodelling during bone formation. The combination of D and hydrostatic pressure in the RP1 regimen appears to create a favourable microenvironment for osteogenesis. This finding is consistent with previous studies (Ghasemzadeh-Hasankolaei *et al.*, 2023) that have shown the importance of fluid micromovement in bone remodeling, which is naturally stimulated by physical activity. Other research (Elashry *et al.*, 2021) has also demonstrated that dynamic mechanical forces can promote ALP activity and the osteogenic differentiation of MSCs. The application of RP1 likely simulates physiological loading conditions experienced by bone in vivo, thereby enhancing mechanotransduction pathways in MSCs. In fact, some studies, such as one on fluid shear stress and its effects on MC3T3-E1 cells, have shown that fluid flow can induce the expression of RUNX2, a key regulator of osteogenesis. In contrast, the expression levels of late-stage osteogenic markers such as OPN and OCN remained low and showed no significant changes. This may suggest that the differentiation process was still in the early to mid-stages at day 14 of culture, with further maturation requiring extended culture periods. Taken together, these results highlight the importance of dynamic mechanical stimulation in regulating gene expression patterns relevant to osteogenesis. This finding supports the hypothesis that integrating multiple biophysical cues within engineered microenvironments can synergistically promote osteogenic differentiation.

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