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Human Ex Vivo Bone Cultures as a Model for Orthopedic Translational Research

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Abstract

Introduction: Human ex vivo bone tissue cultures may provide a translationally relevant research model while reducing reliance on animal experiments, particularly for Orthopedic infections remain. We aimed to establish human bone tissue cultures and evaluate their viability and metabolic activity over 21 days as a foundation for future orthopedic research.

Methods: Trabecular bone tissue from six patients undergoing elective hip prosthetic surgery was collected and cultured at 37°C. Samples were assessed at baseline and days 7, 14, and 21. Cell viability was evaluated using live/dead staining, metabolic activity using alkaline phosphatase (ALP) and lactate dehydrogenase assays, calcium content using Alizarin Red staining, and bone-related gene expression using quantitative PCR.

Results: The number of living cells remained stable throughout the 21-day period. Damaged cells decreased significantly between baseline and days 14 and 21 ($p=0.028$), while dead cells decreased significantly by day 21 ($p=0.028$). ALP levels decreased approximately fourfold after baseline ($p=0.028$). Calcium content and expression of the investigated bone-related genes remained stable, indicating preservation of selected tissue characteristics despite reduced ALP activity.

Conclusions: Human trabecular bone can be maintained as an ex vivo tissue culture for at least 21 days, with stable cell counts, calcium content, and bone-related gene expression. Although metabolic activity was not fully maintained, the findings support further development of this model for orthopedic infection research. Future studies should evaluate its suitability for investigating osteomyelitis and testing antibiotic-releasing biomaterials, potentially reducing the need for animal experiments.

Keywords: Organotypic human bone cultures, Ex-Vivo, Reduce-Replace-Refine, Orthopedic translational research

Mini abstract: Human trabecular bone can be maintained ex vivo for 21 days, preserving selected tissue characteristics, and may provide a platform for orthopedic infection research and reducing animal experimentation.

Introduction

Orthopedic translational research aims to improve the understanding and treatment of musculoskeletal disorders, including orthopedic infections such as periprosthetic joint infections and osteomyelitis. Achieving successful translation of experimental findings into clinical practice requires models that adequately reflect human tissue biology (Bellido and Delgado-Calle 2020). Although numerous cell culture systems are available, such as primary osteoblast cultures and osteogenic cell lines, models preserving the structural and cellular complexity of native human bone tissue remain limited. Conventional cell cultures are valuable for investigating specific cellular processes under controlled conditions but cannot fully reproduce the three-dimensional architecture, extracellular matrix, and heterogeneous cellular environment and cellular interactions of intact bone (Marino, Staines et al. 2016, Bellido and Delgado-Calle 2020). *Ex-vivo* tissue cultures offer an alternative by preserving aspects of native tissue structure and cell–matrix interactions. Although these remain *in-vitro* models, they may better approximate *in-vivo* conditions than conventional cell-based systems, potentially improving the clinical relevance of experimental findings (Bellido and Delgado-Calle 2020, Cramer, Ito et al. 2021).

Organotypic tissue cultures derived from various animal tissues have previously been established as reliable models for investigating specific biological processes and addressing targeted research questions, such as tissue regeneration, inflammatory responses, host–pathogen interactions, and the evaluation of novel therapeutic strategies (Stavridis, Dehghani et al. 2009, Schizas, Rojas et al. 2014). Human bone tissue obtained during routine orthopedic procedures, such as hip arthroplasty, represents a potential resource for developing such models. However, the ability of bone tissue to maintain viability and metabolic activity during prolonged *ex-vivo* culture must be established before the model can be used for more complex investigations. Developing human tissue-based models may also support the principles of the 3Rs (replacement, reduction, and refinement) by providing an alternative to some animal experiments (Tannenbaum and Bennett 2015).

The aim of this study was to establish an *ex-vivo* culture model using human femoral head bone tissue and evaluate tissue viability and selected indicators of metabolic activity over 21 days. We

hypothesized that human bone tissue cultures could maintain viability and metabolic activity over this period and thereby represent a reliable model for orthopedic translational research.

Methods

Experimental design: Bone tissue cultures were obtained from a total of six patients that underwent primary total hip replacement. All experiments were conducted under aseptic conditions using sterile instruments after approval from the Swedish Ethical Review Authority (dnr: 2020-04462). The removed femoral heads arrived at the orthopedic laboratory and immediately placed in 50mL tubes containing α-MEM (GE Healthcare) supplemented with 10 % FBS (Sigma-Aldrich), 50 U/mL penicillin and 50 mg/L streptomycin (PeSt), and 2 mmol/L L-glutamine (SVA, Uppsala, Sweden). By using a 3-mm bone biopsy punch three to four trabecular bone cylinders were obtained and subsequently chopped into 1-mm thick bone discs using a tissue chopper (McIlwain Tissue Chopper; Mickle Laboratory Engineering, Surrey, UK). The bone discs were then transferred into 24-culture well plates containing the above-described medium solution. A total of 32 bone discs was isolated from each patient to serve as bone tissue cultures (Figure 1). The samples were analyzed at the onset of the experiments, one, two and three weeks after bone culture preparation creating four time points (onset, one week, two weeks, three weeks). Each time point a total of 8 bone cultures was randomly chosen to be analyzed for cell viability by live/dead staining, metabolic activity using alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) assays, calcium content using Alizarin Red staining, and bone-related gene expression using quantitative PCR. Two cultures were used in each analysis.

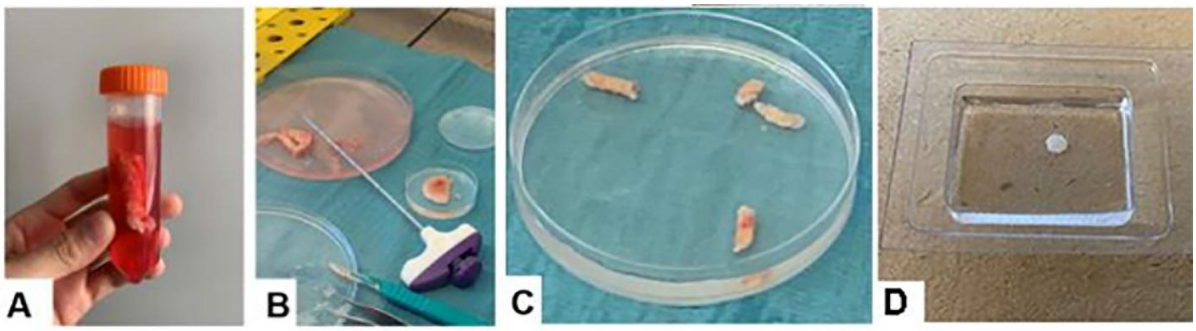

Figure 1 (A-D): *Human bone culture preparation. Bone sample arrived at the lab and transferred in culture medium (A). Bone cylinders are obtained with a biopsy punch (B, C) and sliced to bone discs (D).*

Live/dead staining: Live/dead staining was performed on two bone cultures at each time point using Calcein AM (4 µM) and propidium iodide (PI; 16 µg/mL). Bone pieces were initially incubated with 500 µL of 0.1% Triton X-100 in alpha-MEM for 15 min at 37°C to increase membrane permeability. Calcein AM and PI were separately diluted in alpha-MEM, and equal volumes of the two solutions

were subsequently mixed. A total of 500 µL of the mixed staining solution was added to each bone culture, followed by incubation for 90 min at 37°C. The samples were then washed six times with alpha-MEM, with 15-min incubation intervals between washes, followed by rinsing with Dulbecco's phosphate-buffered saline (DPBS). Bone cultures were fixed in 4% buffered formaldehyde for one hour at room temperature and rinsed again with DPBS. Finally, the samples were transferred to µ-Slide 18-well chambers, covered with PBS, and examined using confocal microscopy. Live cells were identified by green fluorescence, dead cells by red fluorescence, and damaged cells by yellow fluorescence resulting from the overlap of green and red signals, as illustrated in Figure 2. Cells in each category were manually counted using confocal microscopy.

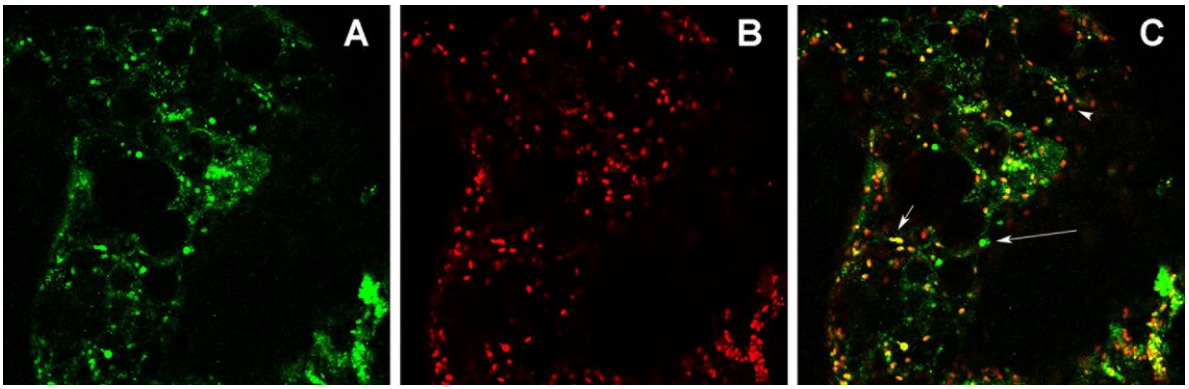

Figure 2 (A-C): *Micrographs of sections through bone cultures after a 7-day incubation using a confocal microscope. The samples were stained with calcein (A) and propidium iodide (B) to study live/dead staining. Live cells are shown as green objects (C, long arrows), dead cells are shown as red objects (C, arrowheads) and damages cells are shown as yellow objects (C, short arrows).*

ALP/LDH assay using ELISA: Bone cultures were immersed in liquid nitrogen for approximately 30 seconds and transferred to pre-cooled TissueLyser holders for homogenization at 30 Hz for 20 seconds. Subsequently, 400 µL of lysis buffer was added to each Eppendorf tube, which was shaken on a rocking platform for 15 minutes and centrifuged at 1,700 × g for 5 minutes; the resulting lysates were collected in a 48-well plate. ALP activity was measured using the Sigma ALP Assay Kit (P7998) by adding 100 µL of p-nitrophenyl phosphate substrate to each sample, followed by incubation at 37°C in 5% CO_2 for 60 minutes and absorbance measurement at 405 nm using a Multiskan Ascent ELISA reader (Thermo). LDH activity was assessed for normalization using a reaction mixture prepared by combining equal volumes of LDH Assay Substrate Solution (L2402), LDH Assay Cofactor Preparation (L2527), and LDH Assay Dye Solution (L2277). A volume of 100 µL of the LDH reaction mixture was added to each well, and the plate was incubated covered at room

temperature for 60 minutes. Absorbance was then measured at 490 and 690 nm using a Multiskan Ascent ELISA reader (Thermo).

Alizarin red staining (ARS): Bone mineralization was assessed using ARS to quantify calcium content in the bone cultures. Following fixation, the cultures were stained with ARS and rinsed with PBS. The bone pieces were subsequently disrupted using a tissue lyser, followed by decolorization with hexadecyl pyridinium chloride monohydrate (CPC) (Gregory, Gunn et al. 2004, Srinivasaiah, Musumeci et al. 2019). The samples were vortexed and centrifuged to obtain the dye-containing supernatant. Absorbance was measured using a spectrophotometer, and calcium concentration was determined in mM using a standard curve.

Tissue extraction and quantitative PCR: Bone discs were shock-frozen in liquid nitrogen and mechanically homogenized using a TissueLyser II (Qiagen), and total RNA was isolated from each sample using TRIzol Reagent (Invitrogen), according to the manufacturer's instructions. RNA concentration and purity were assessed using a NanoDrop spectrophotometer (ND-1000). Equal amounts of total RNA from each sample were used for reverse transcription to synthesize complementary DNA (cDNA) using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems). Gene expression was subsequently quantified by real-time quantitative polymerase chain reaction (RT-qPCR) using a 7500 Fast Real-Time PCR System (Applied Biosystems). The expression of five bone-related genes, ALPL, SPP1, COL1A1, COL1A2 and RUNX2 (Taqman, Table 1) was analyzed, with β-actin (ACTB) serving as the housekeeping gene for normalization. Gene expression was calculated using the comparative delta-delta threshold cycle ($2^{-\Delta\Delta Ct}$) method. For each patient and time point, the mean cycle threshold (Ct) values from two bone culture samples were calculated for each target gene and the housekeeping gene. The ΔCt value was determined by subtracting the mean Ct of β-actin from the mean Ct of the respective target gene. The ΔΔCt value was then calculated by subtracting the ΔCt at baseline (day 0) from the ΔCt at the respective follow-up time point (days 7, 14, or 21). Relative gene expression was expressed as fold change using the formula $2^{-\Delta\Delta Ct}$, with baseline expression serving as the reference.

Table 1: Taqman probes used for gene expression quantification

Acronym	Name	Taqman assay number ID
ALPL	Alkaline phosphatase	Hs01029144_m1
SPP1	Sectreted phosphoprotein 1	Hs00959010_m1
COL1A1	Collagen type I alpha 1	Hs00164004_m1
COL1A2	Collagen type I alpha 2	Hs01028970_m1
RUNX2	Runt related transcription factor 2	Hs00231692_m1

Statistical analysis: Statistical analysis was conducted using the SPSS software. Non-parametric Wilcoxon test for dependent matched pairs was performed to identify differences between different timepoints. The level of significance was set to $p \leq 0.05$ and is indicated with * throughout the analysis. The results are presented as means ± standard error of the mean (SEM).

Results

The number of cells after live/dead staining and assessment are shown in Figure 3. The number of live cells did not differ significantly between onset and any of the subsequent time points (day 7: $p = 0.25$, day 14: $p = 0.60$, and day 21: $p = 0.67$). The number of damaged cells decreased significantly from onset to day 14 ($p = 0.028$) and day 21 ($p = 0.028$), but not to day 7 ($p = 0.34$). Similarly, the number of dead cells decreased significantly between onset and day 21 ($p = 0.028$), whereas no significant differences were observed between onset and day 7 ($p = 0.35$) or day 14 ($p = 0.17$).

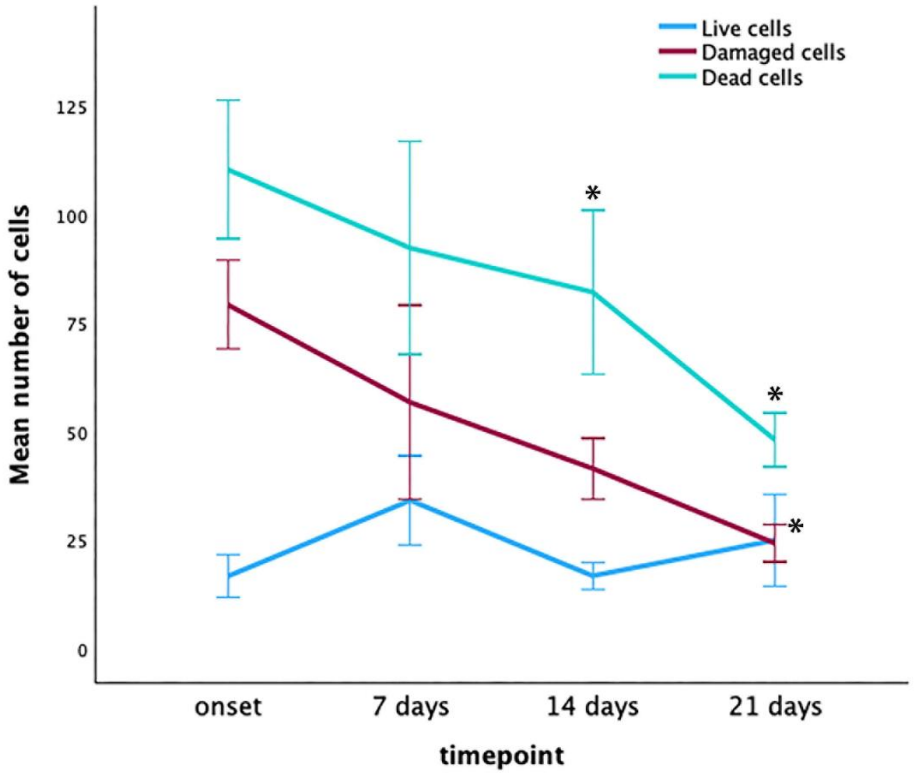

Figure 3: *Quantitative analysis of live, dead and damaged cells at each timepoint. Dead and damaged cells decreased significantly compared to onset after 21 days while live cells remained unaltered throughout the experiment (* $p<0.05$ compared to onset).*

Metabolic osteoblast activity by measuring ALP/LDH showed a nearly fourfold reduction at onset to the other time points (p= 0,028 between onset to timepoints 7, 14 and 21 days) as shown in Figure 4. No differences were noted from timepoint 7 up to timepoint 21.

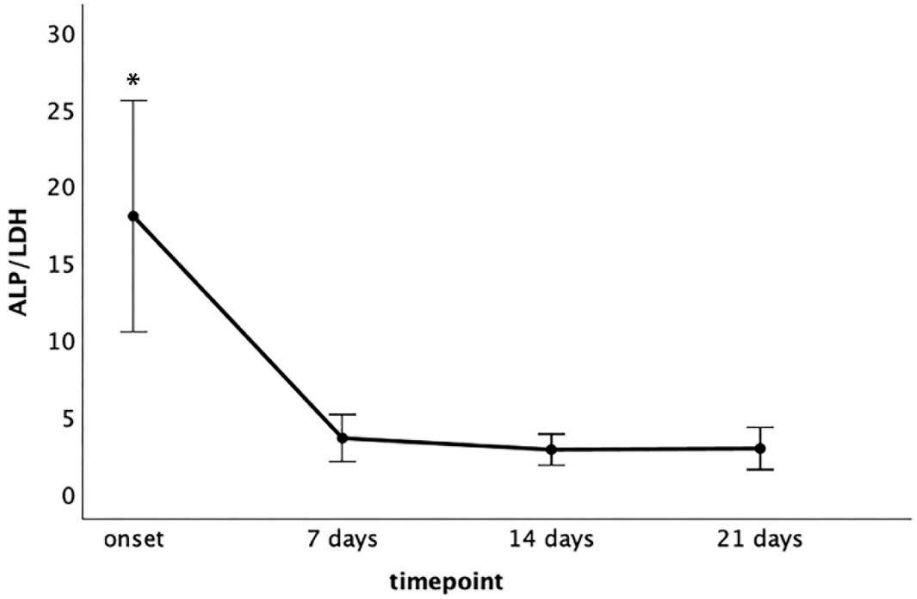

Figure 4: *Metabolic osteoblast activity decreased significantly during the first seven days of the incubation period and remained unaltered throughout the experiment (* $p<0.05$ compared to each timepoint).*

The calcium content using ARS remained unaltered from the onset up to timepoint 21(Figure 5).

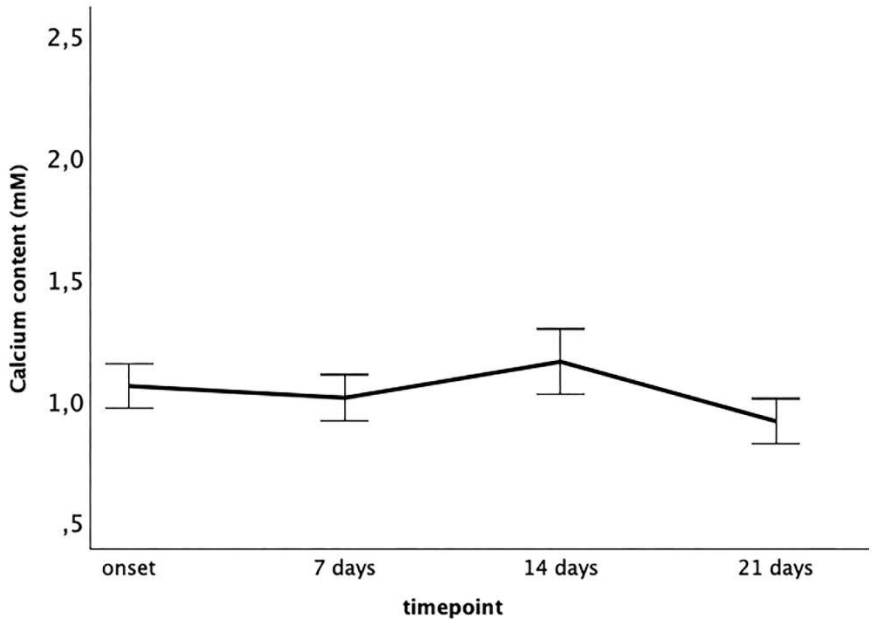

Figure 5: *The calcium content remained unaltered throughout the experiment*

To study the dynamic expression of the ALPL, SPP1, COL1A1, COL1A2, and RUNX2 genes a fold change between the expression of a specific timepoint in relation to onset was generated. Comparisons were made between fold changes at each timepoint (7, 14 and 21 days) in relation to the onset. As shown in Figure 6, no significant differences in relative gene expression were observed between the subsequent time points and baseline (onset) for any of the five genes analyzed. These findings indicate that the expression of genes associated with bone metabolism and formation remained stable throughout the 21-day culture period, with no significant changes detected after the first week in culture.

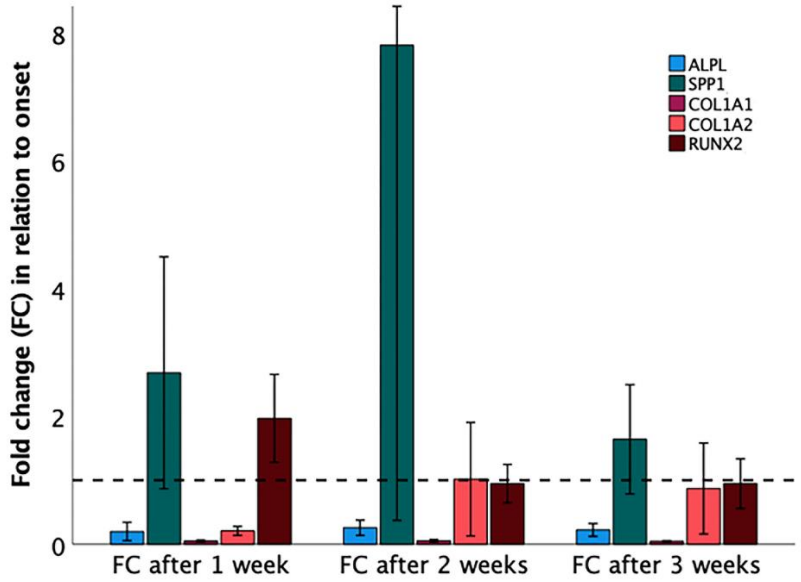

Figure 6: *No significant differences in fold changes were observed between timepoints in relation to onset regarding gene expression. That means that the gene expression was stable after the first week in culture.*

Discussion

The present study demonstrates that human femoral head bone tissue can be maintained in an ex vivo culture system for up to 21 days while preserving measurable cell viability, metabolic activity, calcium content, and expression of genes associated with bone metabolism and formation as well as tissue cytoarchitecture. Although ALP activity declined during the initial culture period, several key parameters remained stable thereafter. These findings support the potential use of human bone tissue cultures as a model for orthopedic translational research and provide a foundation for future studies investigating tissue responses to therapeutic interventions.

The live/dead staining results demonstrated that the number of living cells remained stable throughout the culture period, with no significant differences between baseline and subsequent time points. This finding suggests that the culture conditions were sufficient to maintain the population of viable cells within the bone tissue over 21 days. In contrast, the numbers of damaged and dead cells decreased over time. One possible explanation is the gradual loss of damaged or dead cells and cellular debris

from the tissue during prolonged culture and repeated washing procedures. However, changes in cell integrity and the retention or detectability of stained cells may also have contributed to these observations (Stoddart, Furlong et al. 2006). Importantly, the decrease in damaged and dead cells should not necessarily be interpreted as evidence of cellular recovery, as the study did not directly investigate the mechanisms underlying these changes. Nevertheless, the stable number of live cells provides encouraging evidence that the tissue maintained its cellular viability during the experimental period.

ALP activity, used as an indicator of metabolic activity associated with bone tissue, decreased approximately fourfold during the initial culture period. This decline may reflect the physiological response of tissue to removal from its native environment, including changes in nutrient availability, cellular signaling, and interactions with surrounding tissues. The absence of normal vascular supply and systemic regulation may also contribute to gradual metabolic deterioration during ex vivo culture. Such changes are expected limitations of tissue culture models and emphasize the importance of evaluating tissue function over time (Bellido and Delgado-Calle 2020, Cramer, Ito et al. 2021). Despite the initial decline, ALP activity remained measurable during the subsequent culture period. This is an important finding because it suggests that the model retains sufficient biochemical activity to enable comparisons between experimental conditions. Stable or consistently measurable activity after the initial adaptation period may allow future investigations to assess whether interventions, such as antibiotic exposure or treatment with bioactive biomaterials, influence bone tissue metabolism beyond the changes associated with culture itself.

Calcium content remained stable throughout the 21-day culture period, suggesting that the mineral component of the bone tissue was largely preserved under the applied culture conditions. Calcium is a major constituent of the bone mineral matrix and contributes to its structural and mechanical properties (Boskey 2013) since it possesses a crucial role with -PO_4 in hydroxyapatite formation (Carino, Ludwig et al. 2018, Tourkova, Nelson et al. 2025). The maintenance of calcium content indicates that no substantial net change in the measured mineral content occurred during the observation period. However, stable calcium levels do not necessarily demonstrate the absence of

ongoing mineral turnover or local tissue degradation, as these processes may occur without a measurable change in total calcium content (Kenkre and Bassett 2018). Taken together with the viability and gene expression findings, the results support the preservation of important structural and biological characteristics of the bone cultures over time.

Gene expression analysis provided further insight into the stability of the cultured tissue. The five genes investigated—ALPL, SPP1, COL1A1, COL1A2, and RUNX2—are associated with bone mineralization, extracellular matrix composition, and osteoblast differentiation. No significant differences in relative expression were observed between baseline and subsequent time points, indicating that the expression of these genes remained stable during the culture period.

ALPL encodes tissue-nonspecific alkaline phosphatase, an enzyme involved in bone mineralization and phosphate metabolism (Millan and Whyte 2016). In addition to its role in promoting mineral deposition, alkaline phosphatase contributes to the regulation of phosphate availability within the bone microenvironment (Millan and Whyte 2016). The stable expression of ALPL, despite the decline in measured ALP activity, suggests that transcriptional stability does not necessarily correspond to stable enzyme activity. This discrepancy may reflect post-transcriptional regulation, changes in protein abundance or activity, or differences in the biological processes captured by the two measurements.

SPP1 encodes osteopontin, a multifunctional extracellular matrix protein involved in bone remodeling and mineralization. Osteopontin contributes to cell adhesion and interactions between cells and the mineralized matrix and can influence osteoblast and osteoclast activity (Sodek, Ganss et al. 2000). Its expression may therefore reflect processes involved in maintaining bone tissue structure and regulating remodeling. The stable expression of SPP1 suggests that the culture conditions did not induce a significant change in the transcription of this gene over the observation period.

COL1A1 and COL1A2 encode the alpha-1 and alpha-2 chains, respectively, of type I collagen, the principal collagen component of the bone organic matrix. Type I collagen provides structural support and forms a scaffold for mineral deposition (Selvaraj, Sekaran et al. 2024). Maintaining the expression of both genes is therefore relevant to the preservation of the bone extracellular matrix. Although stable expression does not directly establish that collagen synthesis or matrix integrity remained unchanged,

it indicates that no significant transcriptional alterations in these major collagen components were detected during culture.

Finally, RUNX2 encodes a transcription factor that is essential for osteoblast differentiation and skeletal development. RUNX2 regulates the expression of several genes involved in bone formation and osteoblast function (Komori 2010). Its stable expression, together with the maintenance of ALPL, SPP1, COL1A1, and COL1A2 expression, supports the observation that key transcriptional markers associated with bone biology were preserved throughout the culture period.

Relative gene expression was evaluated using fold changes in relation to baseline. This approach facilitates standardized comparisons between time points and illustrates the dynamic changes in gene expression relative to the initial state of each culture. Normalization to a housekeeping gene helps account for differences in the amount of starting material and other sources of technical variation. Nevertheless, fold-change analysis describes relative rather than absolute transcript abundance, and its reliability depends on appropriate normalization and the assumptions of the comparative Ct method. In the present study, the absence of significant changes in fold expression supports the stability of the examined transcriptional markers during culture, although it does not exclude changes in other genes or biological pathways.

The development of human tissue-based models also aligns with the principles of the 3Rs: replacement, reduction, and refinement of animal use in research (Tannenbaum and Bennett 2015). By providing a human-derived experimental platform, ex vivo bone cultures may support the replacement of some animal experiments, reduce reliance on animal models during early-stage investigations, and contribute to the refinement of preclinical research strategies. Such models may be particularly valuable in orthopedic translational research involving tissue regeneration, biomaterial evaluation, and the development of therapeutic approaches for conditions such as periprosthetic joint infections and osteomyelitis (Srinivasaiah, Musumeci et al. 2019). For example, a viable human bone culture model could provide a platform for future studies investigating host tissue responses to bacterial challenge or evaluating the performance of antibiotic-releasing biomaterials. Although bacterial contamination and infection have been investigated in animal models (Mariano, Grenho et al. 2023), comparable studies

using human bone tissue cultures remain limited. Previously described human bone models have addressed specific research applications but have relied on relatively large tissue specimens and perfusion chambers (Swarup, Weidner et al. 2018, Kuehling, Schilling et al. 2022), which may require specialized equipment and be less readily implemented in routine laboratory settings. These approaches have been developed for specific applications and involved relatively large bone explants and/or dedicated perfusion systems, which may limit their accessibility and ease of implementation in standard laboratory settings.

Some limitations should be considered when interpreting our findings. The study focused on selected indicators of cell viability, metabolic activity, mineral content, and gene expression; these measurements do not fully characterize the functional integrity of the tissue. In addition, stable cell counts, calcium content, and gene expression cannot exclude changes in other cellular populations, tissue architecture, or biological pathways. Further studies should evaluate the reproducibility of the model across donors and investigate its responses to defined experimental interventions. In particular, bacterial challenge and exposure to antibiotic-releasing biomaterials would help establish its utility for research involving orthopedic infections.

In conclusion, human femoral head bone tissue could be maintained ex vivo for 21 days while preserving live cell numbers, calcium content, and the expression of selected genes associated with bone biology. Although ALP activity declined during the initial culture period, it remained measurable during subsequent culture. These findings support the potential of human bone tissue cultures as a model for orthopedic translational research. Further validation is required to establish their reproducibility and suitability for evaluating therapeutic interventions, including novel approaches to orthopedic infections.

Acknowledgments

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Ethics

The study was approved by the Swedish Ethical Review Authoriy (dnr: 2020-04462) and performed in accordance with the ethical standards laid down in the 1964 declaration of Helsinki and all subsequent revisions. According to Swedish legislation no need for informed consent is required if the tissue is anonymized and planned to be discarded within nine months.

Author Contributions

D.A: Manuscript preparation, data acquisition, statistical analysis and interpretation. I.G: Manuscript preparation, manuscript revision and drafting. N.H: Manuscript revision, study funding. N.S: Manuscript preparation and revision, drafting, data acquisition and interpretation, study funding.

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