Validation of a novel organoid model of osteonecrosis for high-throughput drug screening

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INTRODUCTION: Osteonecrosis (ON) is the death of bone marrow caused by lack of nutrients. Because of the precarious blood supply of the femoral head (FH) and its role as part of a major weight-bearing joint, ON of the FH (i.e., ONFH) is the most frequent site. The most common etiology of ONFH is due to corticosteroid use, and the pathogenesis is thought to be multi-factorial.[1] Current in vivo and in vitro models for ONFH have limitations such as inter-species differences in cell physiology, as well as the inability to effectively replicate cell-cell interactions and the 3D environment of the native joint. Organoid models are self-organizing 3D tissue models comprised of more than one cell type. Organoids have increasingly been used to minimize the use of animals, for personalized medicine, and for high-throughput drug screening (HTS).[2] To date, no studies have utilized organoids to study ONFH. We report the generation and validation of a novel organoid model that mimics the key features and pathophysiology of early-stage ONFH.

METHODS: Primary cells were isolated from cartilage and subchondral bone specimens collected during total joint replacement surgery performed for late-stage ONFH. Following tissue digestion, we harvested primary chondrocytes and mesenchymal stem cells (MSCs) for organoid generation. Our ONFH organoid model consisted of a dual-layer architecture: chondrocytes forming the outer layer to mimic articular cartilage, with an underlying MSC layer (osteoblast lineage cells) representing the subchondral bone compartment (Figure 1A through 1B). We plated 5000 MSCs and 5000 chondrocytes separately in individual wells using Corning® ultra-low attachment plates, promoting spontaneous spheroid formation over 24 and 48 hours, respectively. Following verification with an inverted microscope, chondrocyte spheroids were individually transferred to the wells containing MSC spheroids using a 200µL pipette tip and co-cultured with MSC spheroids to enable fusion. To facilitate tracking of cellular distribution and migration, selected MSCs were pre-labeled with Invitrogen® DeepRed cell tracker dye prior to plating. For imaging analysis, organoids were stained with DAPI and examined using confocal microscopy (Leica® SP8). Selected cultures were exposed to prednisolone at 3 ng/mL and 30 ng/mL to mimic glucocorticoid-induced ON. Cell survival was evaluated using the Invitrogen® Live/Dead viability assay. Confocal Z-stack images were acquired, mean fluorescence intensity was calculated across the Z-stack, and the ratio of live and dead cells as a proportion of total cells was quantified. Welch's t-test was used for group comparisons. Organoid width and diameter were also monitored daily. This study received approval from Stanford University's Institutional Review Board (Protocol #51386). Statistical analysis was performed using RStudio (Posit, Boston, MA, USA).

RESULTS: With regards to cell distribution and migration, on Day 1, the organoids showed a strong degree of separation between the inner MSC core (stained purple due to fluorescent overlap between the nonspecific blue DAPI staining and the red DeepRed staining of the MSCs) and the outer core of chondrocytes (solely stained blue with DAPI). While the thickness of the outer chondrocyte layer decreased between days 3 through 9, a distinct separation between the inner MSC core and the chondrocyte shell persisted (Figure 1). The average height and width of the organoids (N=3, daily) started at about 400 µm x 550µm and decreased over time, with both dimensions stabilizing to around 300µm to 350µm between days 4 through 9 (Figure 2). For cell viability (N=3 in each group, on all days studied), the proportion of dead cells to total cells was near zero in both control and 3 ng/mL prednisolone groups and was 0.12 in the 30 ng/mL prednisolone group, although the difference between groups was not significant. By day 3, both 3 ng/mL and 30 ng/mL treatment groups had a higher average proportion of dead cells to total cells relative to the control group, although the difference between groups was still not significant. By day 6, both 3 ng/mL and 30 ng/mL treatment groups had a significantly higher average proportion of dead cells/total cells relative to the control group ($p<0.01$ and $p=0.01$, respectively) but were not significantly different from each other (Figure 3).

DISCUSSION: Our organoid model of ONFH successfully replicated critical architectural elements of the bone-cartilage interface in the FH and exhibited progressive decreases in cell survival. The maintenance of a stratified cellular organization and quantifiable responses validates its use for future studies of the pathogenesis of ONFH and exploration of potential disease-modifying therapies.

SIGNIFICANCE/CLINICAL RELEVANCE: This organoid platform for ONFH simulates the biology of ONFH with potential future applications in personalized therapeutic approaches and large-scale drug discovery.

REFERENCES: 1. Baig SA, Baig MN. Osteonecrosis of the Femoral Head: Etiology, Investigations, and Management. *Cureus*. 2018;10(8):e3171. Published 2018 Aug 21. **2.** Zhao Z, Chen X, Dowbaj AM, et al. Organoids. *Nat Rev Methods Primers*. 2022;2:94.

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IMAGES AND TABLES:

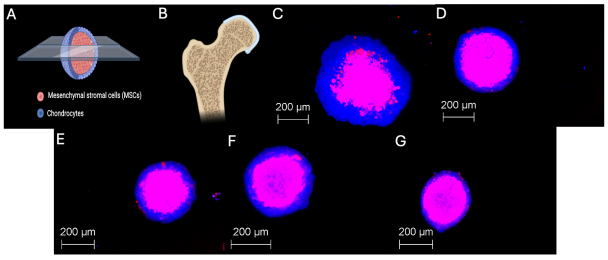

Figure 1: A) "Ideal" ONFH organoid and depiction of cross-section taken using confocal microscope. All images are viewed from above the organoid, looking downwards. MSCs are stained purple (i.e. both red and blue), and chondrocytes are stained blue. B) B) Normal femoral head, serving as an anatomical model for our ON organoid. Created using BioRender. D) through F): Cell distribution on Days 1, 3, 5, 7, and 9, respectively.

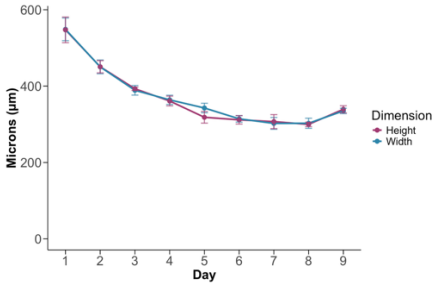

Figure 2: Average height and width of ONFH organoids in micrometers, by day

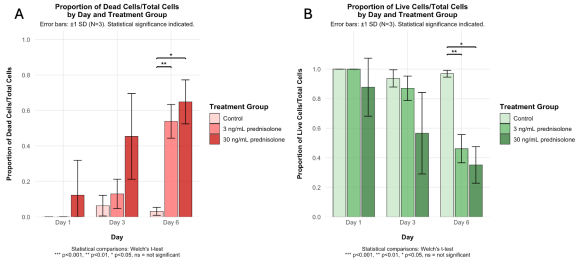

Figure 3: A) Average Proportion of Dead Cells/Total Cells by Day and Treatment with 3 ng/mL and 30 ng/mL prednisolone B) Average Proportion of Live Cells/Total Cells by Day and Treatment with 3 ng/mL and 30 ng/mL prednisolone