Initial Cell Seeding Density Determines the Heterogeneous Architecture and Nutrient Gradients in Human Chondrocyte Spheroids

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INTRODUCTION: Cartilage is an avascular tissue in which chondrocytes rely on diffusion of oxygen and nutrients for survival. Chondrocyte spheroids, as 3D *in vitro* models, more accurately mimic the cartilage microenvironment than 2D cultures and are increasingly used in basic research and preclinical drug screening for osteoarthritis (OA). However, the physiological state of spheroids undergoes substantial structural and functional alterations in response to varying culture conditions, such as initial seeding density, culture duration, and exposure to different inductive factors. This variability reshapes internal oxygen/glucose and other nutrient gradients, as well as cell viability. Such physiological variability can alter responses to the same drug across spheroid status, undermining the accuracy and reproducibility of *in vitro* screening. Thus, the spheroid status should be systematically characterized and standardized to minimize confounding. This standardization improves interpretability and reproducibility, supporting more precise preclinical *in vitro* assessment. This study focuses on elucidating the effects of initial seeding density and culture duration on the development of chondrocyte spheroids. By integrating experimental data and computational growth model, we aim to quantitatively investigate the structural evolution and internal nutrient distribution of spheroids under varying conditions. Defining and standardizing seeding density and culture duration aims to improve the reproducibility and interpretability of spheroid-based mechanistic studies and preclinical drug screening.

METHODS: Human chondrocyte cell line C28/I2 was used to generate spheroids with initial seeding densities of 500, 1000, 4000, and 8000 cells/spheroid and cultured for up to 9 days (D9). Bright-field imaging tracked morphology daily from D1 to D9. Spheroid diameter and volume were quantified on D1, D3, D5, D7, and D9 ($n \geq 8$) Apoptosis was quantified by TUNEL on D1, D5, and D9 to assess viability ($n \geq 8$). Histological staining, including Hematoxylin and Eosin (H&E) and Picrosirius Red, was performed to evaluate the general morphology and collagen content of the spheroids. A finite element model was developed in COMSOL Multiphysics to simulate oxygen, glucose, and lactate distributions and cell viability within chondrocyte spheroids of varying initial sizes, incorporating energy metabolism, nutrient diffusion on D1, D5, and D9. Two-way ANOVA tests were performed to determine statistical significance.

RESULTS: Spheroid growth depended strongly on initial seeding density (**Fig. 1A**). For 500 cells/spheroid and 1000 cells/spheroid, they showed rapid growth, with radii increasing from 75 to 210 μm and from 104 to 237 μm, respectively. In contrast, 4000 cells/spheroid and 8000 cells/spheroid exhibited limited size increase, with radii increasing from 158 to 235 μm and from 220 to 254 μm, respectively (**Fig. 1B, 1C**). High seeding density reduced viability and promoted internal zonation (**Fig. 1D**). At 500/1000 cells per spheroid, viability remained high from D1 to D9 with only a few TUNEL-positive cells. By contrast, 4000/8000 cells per spheroid accumulated TUNEL-positive cells in the core, with a marked increase by D9 (**Fig. 1E, 1G**). H&E staining revealed architectural heterogeneity among spheroids formed at different seeding densities, whereas Picrosirius Red staining demonstrated differences in collagen deposition, with spheroids generated from 8000 cells exhibiting greater collagen deposition (**Fig. 1F**). At 8000 cells per spheroid, a central necrotic zone was evident at D9. To better understand the underlying causes of this phenomenon, a finite element spheroid growth model was developed to characterize the spatial distribution of oxygen, glucose, and lactate within spheroids of varying sizes (**Fig. 2A, 2B**). For 500/1000 cells per spheroid, oxygen and glucose concentrations remained relatively high at D1 (≈close to media values) and only showed minor declines in the core by D9; lactate accumulation remained limited, consistent with sustained cell viability (**Fig. 2C, 2D**). In contrast, 4000 cells/spheroid and 8000 cells/spheroid displayed significantly nutrient gradients. Oxygen and glucose concentrations dropped sharply toward the spheroid center, particularly at D5 and D9. This was accompanied by increased lactate accumulation in the core, consistent with the development of a central necrotic region observed experimentally. The modeling results suggest that the combined effects of high cell density and limited nutrient diffusion lead to metabolic stress and cell death at the spheroid core (**Fig. 2E, 2F**).

DISCUSSION: Spheroid-based *in vitro* models are increasingly used to investigate osteoarthritis-related mechanisms and to evaluate pharmacological interventions. However, variations in seeding density can markedly alter spheroid physiology, compromising reproducibility and predictive accuracy unless careful standardization is applied. In this context, integrating computational modeling with experimental spheroid culture provides a powerful strategy to predict and modulate internal microenvironmental conditions, thereby improving the reliability of *in vitro* drug testing platforms. Overall, this study identifies the critical role of initial seeding density in shaping spheroid structure and function and highlights the value of combining experimental and modeling approaches to develop robust *in vitro* models for cartilage research and therapeutic evaluation.

SIGNIFICANCE/CLINICAL RELEVANCE: Combining computational modeling with experimental approaches enables the development of robust spheroid-based platforms for osteoarthritis mechanism studies and drug evaluation.

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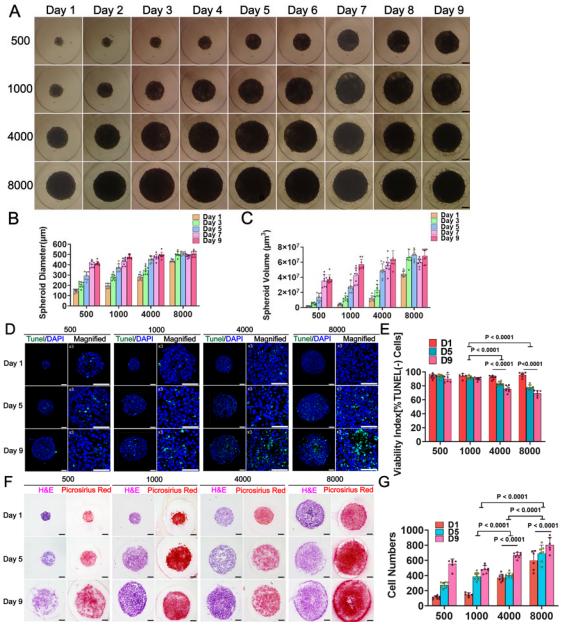

Fig 1. (A) Representative bright-field images of human chondrocyte spheroids. (B) Diameter and (C) volume were quantified on D1, D3, D5, D7, and D9. (D) Representative confocal images of spheroids stained for TUNEL (green) and DAPI (blue). (E) Quantification of cell viability index (%) based on TUNEL staining. (F) Representative H&E and Picrosirius Red images of spheroids. (G) Total cell number. (D–G) All measurements were performed at D1, D5, and D9. Statistical comparisons were performed using two-way ANOVA with Tukey's post hoc test. $P < 0.05$ is considered significant.

Fig 2. (A) Mesh representation of the 3D spheroid model. (B) Schematic representation of nutrient profile within the spheroid. (C–F) Radial distributions of glucose concentration (left) and cell viability (right) at D1 (blue), D5 (green), and D9 (red) for spheroids initialized with (C) 500, (D) 1000, (E) 4000, and (F) 8000 cells. (colors in legend).