Optimal Seeding Densities for In Vitro Chondrogenesis of Two and Three Dimensional-Isolated and Expanded Bone Marrow-Derived Mesenchymal Stromal Stem Cells within a Porous Collagen Scaffold

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Introduction: Bone marrow-derived mesenchymal stromal stem cells (BMSCs) are a promising cell source for treating articular cartilage defects. BMSCs have conventionally been isolated by plastic adherence and expanded in a two-dimensional (2D) environment within tissue-culture flasks [1]. Although this method has been shown to produce cells capable of chondrogenic differentiation, major drawbacks include loss of differentiation capacity, inability to produce extracellular matrix (ECM) proteins and cellular senescence [2-4]. Three-dimensional (3D) isolation and expansion of BMSCs has been proposed as a method of mimicking the natural bone marrow microenvironment and improving chondrogenic capacity [5]. Cell collections containing bone marrow-derived mononucleated cells (BMNCs) – some of which are BMSCs – are seeded within 3D biomaterials for isolation and expansion [5,6]. Cell seeding density is a transplantation variable that has not been evaluated in detail for expanded BMSCs. The optimal density for cell organization and chondrogenic differentiation is currently unknown. The objectives of this study were to perform in vitro chondrogenesis of BMSCs within a collagen I scaffold and to assess the impact of cell seeding density on chondrogenesis following 2D and 3D expansion. It was hypothesized that hyaline-like cartilage would be produced within collagen I scaffolds by 2D- and 3D-expanded BMSCs with an optimal seeding density of 10 × 10^6 cells/cm^3.

Methods: Bone marrow aspirates (BMAs) were obtained from the iliac crest of six sheep following ethical approval from the University of Alberta’s Animal Care and Use Committee. BMSCs were isolated in a 2D environment by plastic adherence, expanded to passage two (P2) in flasks containing expansion medium supplemented with 5% fetal bovine serum, and seeded within collagen I scaffolds (6-mm diameter; 3.5-mm thickness; 115 ± 20 μm pore size) at densities of 50, 10, 5, 1, and 0.5 × 10^6 BMSCs/cm^3. For 3D isolation and expansion, BMAs were seeded on collagen I scaffolds with 50, 10, 5, 1, and 0.5 × 10^6 BMNCs/cm^2 and cultured in expansion medium for an equivalent duration to 2D expansion. Constructs were differentiated in vitro in serum-free chondrogenic medium containing 10 ng/ml transforming growth factor-beta three and 100 nM dexamethasone for 21 days. Cell number was calculated from hemacytometer counting and DNA quantification using nucleic acid fluorescence. Gene (mRNA) expression was determined using RT-qPCR. ECM deposition was assessed with safranin O staining of proteoglycans, collagen immuno-fluorescence and glycosaminoglycan (GAG) quantification using dimethylmethylen blue absorbance. Statistical analyses were performed using a Kruskal-Wallis analysis of variance to assess differences in mRNA, GAG and DNA between seeding density groups. A Mann-Whitney U test evaluated differences between pre- and post-differentiation mRNA expressions.

Results: The duration of time from plating of BMNCs to 80% confluence at P2 during 2D expansion was 23.8 ± 0.8 days. 2D expansion yielded 27.6 ± 4.4 × 10^6 BMSCs per P0 flask, which equated to 34.2 ± 4.5% of the BMNC count during flask seeding. 3D expansion produced BMSC counts that were 5.8 ± 0.9%, 14.9 ± 6.2%, 24.2 ± 9.9%, 89.5 ± 59.2%, and 100.3 ± 92.2% of seeded BMNC counts for seeding densities of 50, 10, 5, 1, and 0.5 × 10^6 BMNCs/cm^2, respectively. 2D-expanded BMSCs seeded at all densities were capable of proteoglycan production and displayed increased expressions of aggrecan and collagen II mRNA relative to pre-differentiation controls (Figures 1A and 1B). Collagen II deposition was apparent in scaffolds seeded at 0.5-10 × 10^6 BMSCs/cm^2. Chondrogenesis of 2D-expanded BMSCs was most pronounced in scaffolds seeded at 5-10 × 10^6 BMSCs/cm^2 based on aggrecan and collagen II mRNA (Figures 1A and 1B), safranin O staining, total GAG and GAG/DNA (Figure 1C). For 3D-expanded BMSC-seeded scaffolds, increased aggrecan and collagen II mRNA expressions relative to controls were noted for all densities (Figures 1D and 1E). Proteoglycan deposition was present in scaffolds seeded at 0.5-50 × 10^6 BMNCs/cm^2, while collagen II deposition occurred in scaffolds seeded at 10-50 × 10^6 BMNCs/cm^2. The highest levels of aggrecan and collagen II mRNA (Figures 1D and 1E), total GAG, and GAG/DNA (Figure 1F) occurred in scaffolds seeding at 50 × 10^6 BMNCs/cm^2.

Discussion: Isolation and expansion of ovine BMSCs in both 2D and 3D environments yielded cells capable of producing hyaline-like cartilaginous tissue within a collagen I scaffold. The effect of cell seeding density on chondrogenic gene expression and ECM formation was demonstrated, and optimal seeding densities for chondrogenesis were 5-10 × 10^6 BMSCs/cm^2 for the 2D expansion protocol and 50 × 10^6 BMNCs/cm^2 for the 3D expansion protocol. Accordingly, these densities could be considered when seeding collagen I scaffolds with BMSCs in transplantation protocols related to articular cartilage regeneration.

Significance: This study provides evidence that both 2D- and 3D-expanded BMSCs are capable of producing hyaline-like engineered cartilage. Within a collagen I scaffold, optimal seeding densities are 5-10 × 10^6 BMSCs/cm^2 for a 2D expansion protocol and 50 × 10^6 BMNCs/cm^2 for a 3D expansion protocol.


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Figure 1. Aggrecan mRNA expression, collagen II mRNA expression and GAG deposition per DNA of 2D-expanded BMSCs (A, B, C) and 3D-expanded BMSCs (D, E, F) seeded within collagen I scaffolds. White bars represent pre-differentiation controls. Statistics: unlabeled, not significant; *, p<0.05; **, p<0.01; ***, p<0.001; #, pre-differentiation controls compared to post-differentiation constructs with significance of p<0.05.