Bone & Joint Research

Supplementary Material

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METHODS

Fabrication of multi-layered scaffolds

The multi-layered scaffold was fabricated as reported previously.[1] Briefly, 3% (w/v) chitosan-gelatin (CG; 1:1) polymer solution was prepared using 3% acetic acid. This was poured into plastic moulds up to approximately 3 mm to 4 mm. Moulds containing CG solution were allowed to gel at 4˚C for 30 minutes before unidirectional freezing. The latter was performed keeping the moulds in direct contact with a metal plate, which in turn was in direct contact with ultra-low temperature liquid nitrogen. The unidirectional frozen layer obtained represented the superficial zone of the scaffolds. In a second step, preheated CG solution was directly poured on the frozen CG layer and kept in direct contact with a cold metal plate in the vertical position, to form the transitional as well as deep zones of the scaffolds. The frozen scaffolds were then freeze-dried to obtain macroporous multi-layered CG scaffolds. CG scaffolds with random pore architecture were used as controls and synthesized as reported previously.[1]

Multi-lineage differentiation

Chondrogenic differentiation

Third passaged cells were seeded in a 96-well plate at a density of 1.5×10^5 cells/well and then centrifuged at 500 g for five minutes to pellet down the cells. After 24 hours culture with mesenchymal stem cell (MSC) culture media, cells were differentiated into chondrogenic lineage by using Dulbecco's Modified Eagle Medium (DMEM)-high glucose medium supplemented with dexamethasone (100 μM), ascorbate-2-phosphate (40 μg/ml), 1 × insulin-transferrin-sodium selenite + 1 media supplement, L-proline (40 μg/ml), and sodium pyruvate (1 mM). The differentiation media was replenished two times per week. After three weeks of differentiation, the cartilage pellet was stained with Safranin O dye to confirm the glycosaminoglycan (GAG) deposition.

Osteogenic differentiation

Third passaged cells were cultured in a 24-well plate at a density of 1.5×10^4 cells/well and cultured with MSC cultured media. After 48 hours, the osteogenic differentiation media (Minimum Essential Medium Eagle - Alpha Modification supplemented with 10% fetal bovine serum (FBS), 10 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μg/ml ascorbate-2-phosphate) was added to the culture to induce osteogenesis. After three weeks of differentiation, cells were fixed with 70% ethanol for 15 minutes and then stained with Alizarin red staining to visualize the calcium deposition.

Adipogenic differentiation

Third passaged cells were seeded in a 24-well plate at a density of 1.5×10^4 cells/well and cultured with MSC cultured media. After 48 hours, the cells were differentiated into adipogenic lineage using DMEM-high glucose medium supplemented with 10% FBS, 1 μM dexamethasone, 10 μM 3-isobutyl-1-methylxanthine (IBMX), 10 μg/ml insulin, and 100 μM indomethacin. After three weeks of differentiation, cells were fixed with 4% paraformaldehyde for 15 minutes and then stained with Oil O Red staining to confirm the lipid droplet formation.

REFERENCE

1. **Arora A, Kothari A, Katti DS.** Pore orientation mediated control of mechanical behavior of scaffolds and its application in cartilage-mimetic scaffold design. *J Mech Behav Biomed Mater*. 2015;51:169-183.

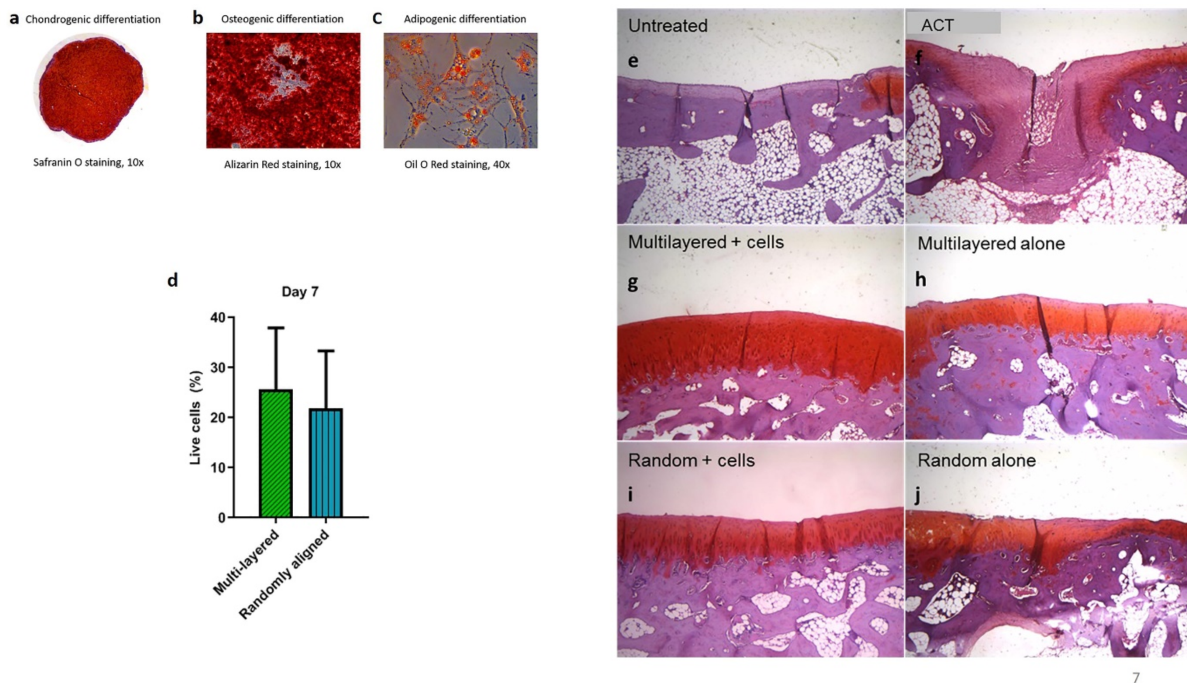

Fig a. Multi-lineage potential of rabbit bone marrow mesenchymal stem cells (MSCs) after 21 days of differentiation. a) Safranin O staining of glycosaminoglycan (GAG) deposited in cartilage pellet confirmed the chondrogenic differentiation. b) Alizarin Red staining of calcium deposits confirmed the osteogenic differentiation. c) Staining of lipid droplet with Oil O Red dye confirmed the adipogenic differentiation. d) Percentage of live cells on cell-seeded scaffolds on day 7 of culture. e) to j) Representative Safranin O histological images at 10× magnification showing the defect margins of: e) untreated control; f) allogeneic chondrocyte transplantation (ACT); g) multi-layered + cells; h) multi-layered without cells; i) random + cells; j) random without cells.

Table i. The O'Driscoll scoring system for quantification of articular cartilage repair.

Nature of predominant tissue		
Cell morphology	**Score**	**Maximum score**
Hyaline articular cartilage	4	4
Incompletely differentiated mesenchyme	2	
Fibrous tissue or bone	0	
Safranin O staining of matrix		
Normal or nearly normal	3	3
Moderate	2	
Slight	1	
None	0	
Structural characteristics and Surface regularity		
Smooth and intact	3	3
Superficial horizontal lamination	2	
Fissures 25% to 100% of the thickness	1	
Severe disruption, including fibrillation	0	
Structural integrity		
Normal	2	2
Slight disruption including cysts	1	
Severe disintegration	0	
Thickness		
100% of normal adjacent cartilage	2	2
50% –100% of normal cartilage	1	
0% –50% of normal cartilage	0	
Bonding to the adjacent cartilage		
Bonded at both ends of graft	2	2
Bonded at one end or partially at both ends	1	
Not bonded	0	
Hypocellularity		
Normal cellularity	3	3
Slight hypocellularity	2	
Moderate hypocellularity	1	
Severe hypocellularity	0	
Chondrocyte clustering		
No clusters	2	2
< 25% of the cells	1	
25% –100% of the cells	0	
Freedom from degenerative changes in adjacent cartilage		
Normal cellularity, no clusters, normal staining	3	3
Normal cellularity, mild clusters, moderate staining	2	
Moderate cellularity, mild clusters, moderate staining	1	
Severe hypocellularity, poor or no staining	0	
Total score		**24**