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ORIGINAL PAPER

Decellularised Bone Scaffold Effectively Support The Adherence and Proliferation of Human Osteosarcoma (HOS) after Recellularisation

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ABSTRACT

Decellularised bone has been proposed in bone tissue engineering studies as a donor scaffold for bone implantation into patients. In this study, the success of a bovine bone decellularisation using 10 mM Tris, 1 mM EDTA, 0.1 % (v/v) Triton X-100 and 0.5 % (w/v) Trypsin has been established through analytical DNA assays. Further to establish the recellularisation protocol, reseeding using HOS cells was allowed to progress for up to 5 weeks. The adherence and proliferation within the scaffolds were then confirmed by the SEM after 21 days post reseeding. Overall, the findings presented that the decellularised bovine bone scaffolds are capable of supporting cell adherence and proliferation. Taken together, the studies carried out in this project show that this bovine donor bone scaffold could be used to repair bone defects in recipient patients.

INTRODUCTION

Bone defects caused by trauma and pathological and physiological bone resorption denote a major challenge and have become a global health problem[1]. Regenerative medicine strategies involving decellularised extracellular matrix scaffolds are developing fast[2] and, in particular, decellularized bone has been proposed for bone tissue engineering as both an allograft and autograft scaffold. Decellularisation is a method to develop biological scaffolds (templates) which minimise possible adverse immune responses and support host cell diffusion into the graft[3]. Basically, this approach segregates the extracellular matrix (ECM) components by extracting cells from tissues or organs. The process of tissue decellularisation preserves the natural ultrastructure (collagen, elastin etc.) and biochemical content of ECM[4,5]. The effectiveness of donor cell removal from a tissue is influenced by the origin of the tissue and is achieved by the use of specific physical, chemical, and enzymatic methods. Decellularisation should efficiently eliminate all donor cellular material while minimising any adverse effect on the composition, mechanical integrity, and biological activity of the remaining extracellular matrix (ECM). This study aimed to establish decellularisation and recellularisation protocols of the decellularised trabecular bone samples.

MATERIALS AND METHOD

Decellularisation

Twelve bovine cancellous proximal femur samples were decellularised by six cycles of overnight incubation at 37°C using 10mM Tris, 1mM EDTA, 0.1% v/v Triton X-100; and 0.5% w/v trypsin. Decellularisation was confirmed by the absence of DNA staining with 4',6-diamidino-2-phenylindole (DAPI) both by detecting any DNA remaining on the bone matrix spectrofluorometrically, and by microscopic examination.

Recellularisation

Recellularisation using human osteosarcoma (HOS) cells (seeded at $5x10^5$ cells per cm^2 bone) was allowed to progress for up to 3 weeks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-ascorbic acid, β-glycerophosphate, dexamethasone, foetal calf serum (FCS), penicillin/ streptomycin (PEST) and non-essential amino acids (NEAA). Reseeded bone samples were stained for DNA content in 0.6 nM DAPI and observed under the Zeiss microscope.

Scanning Electron Microscope (SEM)

After 21 days of reseeding, bone samples (n = 6) were washed with phosphate-buffered saline (PBS). Then, they were fixed in 2.5% glutaraldehyde for 2 hours. Tissue constructs were then washed in 0.1M sodium cacodylate buffer. Next, tissue constructs were put in tubes and 1ml of 0.1M sodium cacodylate buffer added. Finally, pieces of bone samples of around 1 cm^3 were freeze-dried in liquid isopentane immersed into liquid nitrogen and dried in a sublimation chamber at 5kPa. All the bone samples were placed in a freeze dryer (ScanVac CoolSafe Freeze Drying, LaboGene) overnight. The samples were then sealed and packed under vacuum. Before imaging, freeze-dried bone samples were fixed (glued) to the metal surface for coating with gold. The process of coating took approximately 20 minutes using the S150 Sputter Coater (Edwards, High Vacuum Co. International, USA). Finally, the inner and outer regions of coated tissue constructs were imaged under the Scanning Electron Microscope (Hitachi SU 6600 SEM, Japan).

RESULTS AND DISCUSSION

The existence of DNA in the specimen can therefore be detected by staining with DAPI [2, 6]. Fluorescence is generated when the DAPI attaches to the AT clusters in the minor grove of DNA. DAPI staining therefore provides a sensitive, specific and reproducible quantification

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and detection method for DNA on the bone samples after decellularisation, and later after re-seeding cells on these scaffolds. DAPI staining was an effective method to detect the elimination of cells and it revealed that protocol used in this study removed all measurable DNA from the bone samples. There was no further detectable (spectrofluorimetrically measured) DAPI staining could be eluted from the bone matrix following detergent treatment, and microscopically the bone surface was free of cells. These studies have proved that combination of treatments such as chemical, biological, and physical could produce a successful decellularisation protocols.

In ***Figure 1***, the cells started to grow on the bone samples after day 3 and also tended to outgrow from the bone samples and stick to the 24-well plate. Cells spread over a large area of the bone samples after 10 days and formed subconfluent cell layers. Cell growth could be seen on both sides of the bone under the microscope.

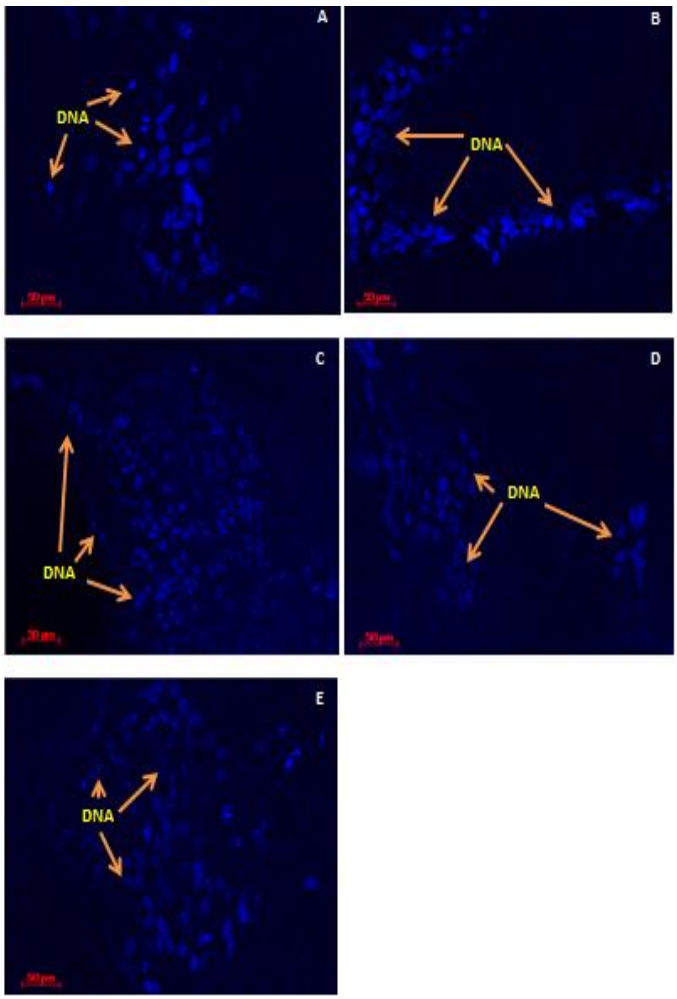

Fig 1*:* HOS cells were reseeded (at 5 x 10^5 cells per cm^2) onto bone. Bone samples were stained in 400µl of 0.6nM DAPI after **A)** 3 days, **B)** 7 days, **C)** 10 days, **D)** 15 days and **E)** 21 days. Cells seeded in plates.

The methods for SEM used in this study were adapted from several studies [7, 8, 9, 10] that employed gold and palladium to coat the samples before imaging. The bone samples were only coated with gold in this study. Gold coating has a larger particle size which is visible at very high magnification above ~300k or 3 nm resolution. It was dried in a sublimation chamber at 5 kPa in order to preserve the microstructural of bone samples.

To be used for reseeding process, an ideal pore structure needs to be considered in which there should be interconnections in order for cell migration, nutrient movement and growth to occur freely. Representative images of scaffold structures after 21 days of reseeding were shown in ***Figure 2***. The images taken by SEM indicated that reseeded bone samples cultured on surfaces have a porous structure comprising of an interconnected network with ideal pore sizes. Additionally, the cells have migrated to the different areas in the scaffold and had colonised some sites of the scaffold. All in all, the SEM results also revealed that the bone samples after being treated with chemicals during decellularization process still have the capability to facilitate cell adhesion and proliferation.

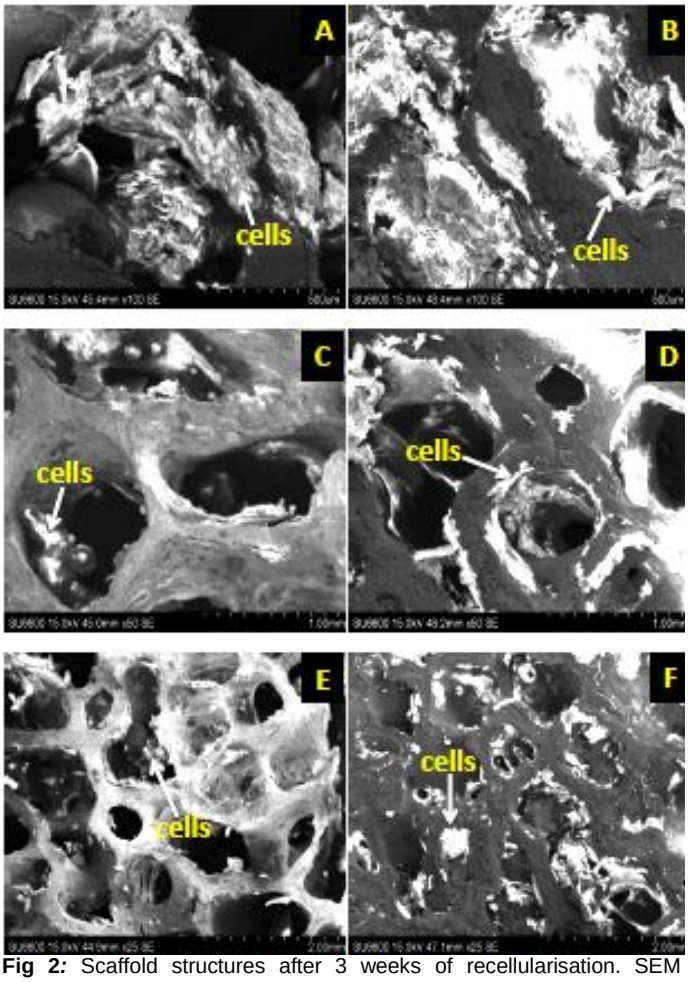

Fig 2*:* Scaffold structures after 3 weeks of recellularisation. SEM images. **A** and **B**, x100, scale bar=500µm, **C** and **D**, x50, scale bar=1.00mm, **E** and **F**, x25, scale bar=2.00mm.

CONCLUSION

In conclusion, the studies carried out have proved some important elements in determining the key success of recellularisation processes. The efficiency of cells adherence and proliferation within the scaffolds after recellularisation was successfully confirmed by DAPI staining and SEM. Furthermore, the attained findings indicated that the decellularized bone scaffolds are capable of supporting the cell adherence and proliferation. All in all, this bovine donor bone scaffold could be used to repair bone defects in recipient patients.

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