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British Journal of Oral and Maxillofacial Surgery 54 (2016) 316–321
BRITISH Journal of Oral and Maxillofacial Surgery
www.bjoms.com

# Sheet of osteoblastic cells combined with platelet-rich fibrin improves the formation of bone in critical-size calvarial defects in rabbits

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Accepted 17 December 2015
Available online 15 January 2016

**Abstract**

Techniques that use sheets of cells have been successfully used in various types of tissue regeneration, and platelet-rich fibrin (PRF) can be used as a source of growth factors to promote angiogenesis. We have investigated the effects of the combination of PRF and sheets of mesenchymal stem cells (MSC) from bone marrow on the restoration of bone in critical-size calvarial defects in rabbits to find out whether the combination promotes bony healing. Sheets of MSC and PRF were prepared from the same donor. We then implanted the combined MSC and PRF in critical-size calvarial defects in rabbits and assessed bony restoration by microcomputed tomography (microCT) and histological analysis. The results showed that PRF significantly increased bony regeneration at 8 weeks after implantation of sheets of MSC and PRF compared with sheets of MSC alone ($p = 0.0048$). Our results indicate that the combination of sheets of MSC and PRF increases bone regeneration in critical-size calvarial defects in rabbits, and provides a new way to improve skeletal healing.
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*Keywords:* Mesenchymal stem cells; Cell sheet; Platelet-rich fibrin; Bone regeneration; Rabbit calvarial critical-size defect

## Introduction

The technology of engineering bone tissue remains the most promising treatment for the reconstruction of bony defects associated with microvascular flaps from the fibula, scapula, and iliac crest that are required for the treatment of trauma, congenital malformation, and after resection of a tumour.[1,2] However, there are still some problems that we need to resolve about tissue-engineered bone grafts.[3] For example, the lack of a good vascular supply seriously affects the formation of large areas of engineered bone as it relies on diffusion to supply nutrition as well as to remove any metabolic waste

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http://dx.doi.org/10.1016/j.bjoms.2015.12.015
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products. Diffusion can sustain cell viability only within a maximum of 200 μm into the matrix,[3–5] so vascularisation is essential for successful engineering of bone.

In recent years, many techniques have been introduced to try and solve the problem of vascularisation including microsurgery, the design and modification of the structure of scaffolds, coculture systems, and incorporation of growth factors.[6] Prefabrication of arteriovenous loops and flaps have been the most common microsurgical methods that have been used.[7,8] One of the main purposes of the current design of scaffolds is the promotion of vascularisation by modification and sustained release of growth factors.[9,10] The coculture of endothelial progenitor cells with mesenchymal stem cells (MSC) has also been reported.[3,11] However, these techniques have their own shortcomings and still cannot produce sufficient vascularisation for adequate tissue engineering.[12]

It is inevitable that growth factors are vital to neovascularisation and the formation of bone so many growth factors have been used in regeneration, including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor-beta 1 (TGF-β1), insulin-like growth factor-1 (IGF-1), fibroblast growth factor-2 (FGF-2), and endothelial cell growth factor (ECGF).[13] Fortunately, platelet-rich fibrin (PRF), which is rich in many autologous growth factors such as these, and is capable of releasing them continuously and slowly, offers an appropriate solution for delivery.[14] Engineered scaffold-free bone based on sheets of osteogenic MSC alone, without exogenous scaffolds, has also been made successfully.[15,16] In a previous study we showed that PRF can considerably increase the osteogenic capacity of sheets of MSC in vitro and at an ectopic site in mice with severe combined immunodeficiency (SCID).[17]

Based on our previous findings, we investigated the effects of the combination of PRF and sheets of MSC on the restoration of bone in critical-size calvarial defects in rabbits to find out whether such a combination would promote bone healing.

## Material and methods

### *Cell isolation and culture*

New Zealand rabbits were obtained from the animal holding unit of the Fourth Military Medical University (FMMU), and the protocol was approved by the Institutional Animal Care and Use Committee of FMMU. Briefly, MSC were isolated from the aspirated marrow of the iliums of adult rabbits and cultured in low-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% penicillin/streptomycin (Gibco, Carlsbad, CA, USA), and0.272 g/l L-glutamine (Sigma-Aldrich, St. Louis, MO, USA). Sheets of MSC were then cultured in osteogenic medium for two weeks as reported previously.[17] The medium was changed every 2 days. The microstructure was observed by scanning electron microscopy (EM) (S-4800, Hitachi, Japan).

### *Preparation of PRF*

PRF was prepared as reported previously.[17] Briefly, blood 10 ml was drawn from each rabbit and centrifuged immediately for 10 minutes at 3000 rpm in a laboratory centrifuge (XIANGYI, Hunan, China). The clot was then harvested with tweezers and gently pressed into a membrane between two sterile pieces of gauze. PRF membranes were also prepared for histological examination, scanning EM, and transmission EM (JEM-1230, JEOL, Japan).

### *Protocol*

A total of 15 New Zealand rabbits (3 months old, weighing about 2.5 kg) were used in the study. The rabbits were randomly divided into 3 groups: in the first the defect was repaired with combined sheets of MSC together with PRF (n=6), in the second (n = 6) with sheets of MSC alone, and in the third (control, n = 3) the defect was left untreated. After anaesthesia a tongue-shaped incision was made to expose the cranium. A full-thickness defect 15 mm in diameter was carefully prepared and treated as described. Native periosteum was removed to exclude any influence on bony regeneration. The scalp was closed with sutures. After 8 weeks rabbits were killed humanely with an overdose of barbiturate (200 mg/kg) given intravenously.

### *MicroCT analysis*

Each specimen was scanned with a MicroCT system (Inveon, Siemens, Germany; 80 kV, 500 mA, 1200 milliseconds integration time). The scans used a 360° radiographic projection (total scan time 30 minutes). Scanning images were switched into 3-dimensional volumes (21 μm resolution) using Cobra software (Siemens reconstruction software). The volume of new bone in the defect, and bone volume/total volume (BV/TV), were also calculated.

### *Histological analysis of bone*

After CT the specimens were divided into two parts, one half of which was prepared and stained with haematoxylin and eosin and the other with Masson's trichrome, and they were viewed under light microscopy. Sections were selected from each specimen for histomorphometrical examination as reported previously.[17] Three high-resolution, low-magnification, micrographs were randomly selected from each section and analysed twice by two unbiased examiners (who were unaware of the experimental conditions) using computer-based image analysis techniques (Leicas Qwin Pro-image Analysis System). The cross-sectional area of mineralised bone and cartilage (blue staining) was measured and expressed as the relative percentage of the total cross-sectional area.

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