CASE REPORT

Successful treatment of refractory tibial nonunion using calcium sulphate and bone marrow stromal cell implantation

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Successful healing of a nine-year tibial nonunion resistant to six previous surgical procedures was achieved by tissue engineering. We used autologous bone marrow stromal cells (BMSCs) expanded to $5 \times 10^6$ cells after three weeks’ tissue culture. Calcium sulphate ($\text{CaSO}_4$) in pellet form was combined with these cells at operation. The nonunion was clinically and radiologically healed two months after implantation.

This is the description of on healing of a long-standing tibial nonunion by tissue engineering. The successful combination of BMSCs and $\text{CaSO}_4$ has not to our knowledge been reported in a clinical setting.

It is estimated that over 10% of fractures might be at risk of developing delayed or nonunion. Some cases are refractory to all existing forms of treatment, leading to numerous operations and recurrent infections. Bone tissue engineering has great potential in the management of refractory nonunion but its translation to clinical practice has been limited, with only a few reports. Vacanti et al. reported the successful use of a tissue-engineered distal phalanx to replace this bone in a patient who suffered partial avulsion of the thumb, and Wanner et al. reconstructed a mandible, using a titanium construct which incorporated bone morphogenetic protein (BMP), bone blocks and stromal cells. Quarto et al. used a graft of hydroxyapatite (HA) and stromal cells stabilised by external fixation in three patients to reconstruct 4 cm to 7 cm long bone defects, with satisfactory biomaterial incorporation and bone formation. Here, we report the successful use of cell-based tissue engineering to induce bone healing in a patient with a long-standing refractory nonunion of the tibia.

Case report

In November 1995, a 34-year-old man suffered a high-speed road traffic accident. He sustained a closed mid-shaft segmental left tibial fracture and a head injury. His tibial fracture was initially treated with intramedullary nailing.

In 1996, he was referred with disabling pain at the fracture site. This was warm and tender and inflammatory markers were raised. Radiographs showed nonunion, with sclerosis and hypertrophy. Because of suspected infection, antibiotic treatment was started. Between 1996 and 2004, several operations were performed, including the application of a monolateral external fixation, functional bracing, and two programmes of Ring circular external fixation with autologous bone grafting, all of which failed (Fig. 1). He agreed to have stem cell therapy with calcium sulphate ($\text{CaSO}_4$) pellets as a carrier with the approval of the local Ethics Committee and Research Panel.

Under general anaesthesia, approximately 10 ml of bone marrow was harvested from the posterior superior iliac spine by repositioning a Jamshidi needle into several areas. The aspirate was thoroughly mixed with 10 ml sterile phosphate-buffered saline (PBS; Invitrogen, Paisley, United Kingdom) in a Blue Max centrifuge tube (Becton Dickinson, Oxford, United Kingdom). Blood was sampled before anaesthesia and serum prepared to supplement the culture medium for cell growth. At the Good Manufacturing practice laboratory in our institution, the diluted aspirate was split into two equal volumes which were layered over pre-prepared 10 ml aliquots of Lymphrep (Axis Shield Diagnostics, Dundee, United Kingdom) in Blue Max centrifuge tubes. The tubes were centrifuged at 900 g for ten minutes, after which time a ‘buffy coat’ layer was seen in each. These layers were harvested and
added to 10 ml of culture medium (DMEM/F12, Invitrogen). This was centrifuged at 750 g for ten minutes. The resulting cell pellets were resuspended in 2 ml of DMEM/F12, as is laboratory procedure, and a cell count performed using a haemocytometer. A total of $7.5 \times 10^6$ mononuclear cells were isolated, which were plated out into T75 tissue culture flasks (Becton Dickinson) in 15 ml of a mixture of 85% DMEM/F12 and 15% autologous serum at a seeding density of 20 million cells per flask. The flakes were incubated for 24 hours at 37°C, 5% CO$_2$ and 90% humidity. After this, the tissue culture fluid and non-adherent cells were removed, with an aliquot from each flask being sent for microbiology. The adherent cells were washed twice with 10 ml PBS and fresh culture medium was added. The flakes were again incubated as described. Three days later, additional fresh medium was added and examination was performed with an inverted microscope, when fibroblast-like cells were noted. Further culture medium was added to the flakes every three to four days with an examination until the fibroblastic cells proliferated sufficiently to cover 70% of the flask base; at this stage, they were passaged. Passage one cells were seeded into T75 flasks at a concentration between 3000 and 4000 cells per cm$^2$ in 15 ml DMEM/F12/10% serum. Every three to four days, more culture medium was added to the flakes until the cells were harvested and returned to theatre suspended in 0.8 ml DMEM/F12 culture medium. The final yield was $5 \times 10^6$ cells (Fig. 2). At operation, the nonunion site was decorticated and a fibrous nonunion confirmed. Biopsy samples were sent for histology and microbiology. The results showed established fibrocartilaginous nonunion with little evidence of new bone formation and no signs of infection. The grafting material was prepared by adding autologous serum and the cells to 10 ml high-purity 4.8 mm and 3 mm calcium sulphate pellets (Stimulan, Biocomposites Ltd, Keele, United Kingdom). This material is synthetic and does not contain insoluble ‘earth’ impurities, which can be present in natural gypsum rock. This material was impacted by hand around and into the fracture site and periosteum sutured over it (Fig. 3). The patient was discharged home on the third post-operative day and allowed to bear weight as tolerated in a removable cast brace. By eight weeks, he was able to place full weight on the leg without assistance. His symptoms had resolved and examination showed clinical union. Radiographs showed abundant callus and the CaSO$_4$ pellets could not be identified (Fig. 4). There were no complications. Two years post-operatively, he was able to bear full weight unaided and had only minimal residual discomfort. Radiographs showed good alignment with union across all four cortices (Fig. 5). He has regained his pre-injury level of independent function and mobility.

**Discussion**

Recent advances in cell biology and biomaterials science have been amalgamated in the new discipline of tissue engineering. However, the translation of this rapidly expanding discipline to clinical practice is so far limited, and this is the first report of healing of a long-standing tibial nonunion using the method. The successful combination
of BMSCs and CaSO₄ as a carrier has not to our knowledge been reported in the clinical setting.

Stromal cell therapy introduces new applications for fracture healing, including nonunion.⁶,⁷ These are ideal for this purpose, resulting from their ease of manipulation and expansion \textit{in vitro}. They are multipotent progenitor cells, capable of differentiating into various cell types, including osteoblasts.⁸,⁹ Methods have been developed for the isolation and expansion of stem cells from bone marrow stroma and there is some experimental and clinical evidence that supports the efficacy of BMSCs in enhancing osteogenesis.⁴,¹⁰-¹² At a site of nonunion, osteoprogenitor cells may be insufficient in quantity or unable to recognise cellular cues. Thus, a local boost with a large number of active and viable cells can be achieved through implantation of culture-expanded BMSCs. They are thought to work not only by local osteogenesis but also by releasing signalling molecules, thereby causing autocrine and paracrine effects by recruiting and activating endogenous stromal stem cells to undergo differentiation and osteoproduction. This leads to reactivation and augmentation of the cellular fracture healing mechanism.

Percutaneously-injected autologous bone marrow without tissue culture has been tested clinically for the treatment of tibial nonunion, with mixed results.¹³-¹⁵ The efficacy appears to be related to the number of progenitor cells in the graft.¹⁶ However, the quantity of stromal cells in bone marrow aspirates is only < 0.01%.¹⁷ Also, by percutaneous injection, a substantial number of BMSCs might be lost by apoptosis because of inadequate cellular attachment. Loading the BMSCs on a scaffold is more effective, as it allows immediate functional cellular attachment to an osteoconductive carrier. This leads to cellular proliferation, differentiation, and extracellular matrix production in the required area. Petite et al¹⁸ showed in an animal model that tissue-engineered bone using a coral scaffold and culture-expanded BMSCs performed better than bone marrow and scaffold alone. Whereas culture expansion might have the disadvantage of losing possible ‘supporting cells’ in the bone marrow aspirate, its use allows a large number of known progenitors to be introduced into the site of nonunion.

The extent of bone healing depends on the source of cells. Filling a defect with scaffold alone allows osteogenesis to occur in the medullary areas. This could be caused by bone marrow cells migrating from the adjacent marrow cavity onto the osteoconductive scaffold. There is experimental evidence that implantation of stromal cells to various scaffolds leads to the formation of abundant, biomechanically stable bone \textit{in vitro} and \textit{in vivo}.¹⁰,¹⁹-²¹
Attempts to combine stromal cells seeded on to porous ceramic scaffolding have given suboptimal results as a result of the slow resorption of the HA-based ceramics, producing bone ingrowth into the porous surface rather than true bone regeneration.\(^{22,23}\) We used a combination of CaSO_4 and BMSCs and found a progressive, complete resorption of the scaffold, leaving relatively abundant calcium phosphate-containing ceramics. High phosphate concentrations in tissue medium slow down differentiation and mineralisation\(^{23}\), and induce osteoblast apoptosis.\(^{24}\)

Increasing calcium levels may also accentuate this effect.\(^{25}\) It is not known whether the combination of decortication, BMSCs and CaSO_4, or one of these factors alone, was responsible for healing in the present case. Therefore, this treatment method should be further validated in a double-blinded randomised controlled trial.

In conclusion, CaSO_4 can be combined with BMSCs for successful bone-tissue engineering. Such advances in regenerative medicine can be safely applied in the treatment of long-standing nonunion of the tibia.

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References


