Augmentation of bone healing of nonunion fracture using stem cell based tissue engineering in a dog: a case report

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ABSTRACT: A 4-year-old, intact male crossbreed dog, weighing 27 kg, was referred for the treatment of a nonunion fracture. The radiographs revealed displacement of the radius and ulna bone fracture fragment and a sclerotic fracture end of the radius. Autologous adipose derived stem cells (ADSCs) were isolated and expanded ex vivo in a culture. The ADSCs (3.2 × 107 cells) were seeded on a composition scaffold made from hydroxyapatite (HA) and chitosan (CH) fibers. The seeded scaffold with ADSCs was placed on the fracture site and the bone fracture was stabilized. A sample of seeded scaffold with ADSCs was taken to evaluate the extent of cell attachment and morphology on the scaffold using scanning electron microscopy (SEM). SEM showed that ADSCs adhered to the scaffold well and many bone nodules formed from the bone matrix secreted by ADSCs. Three months after surgery, the nonunion had successfully healed with no complications. The application of a composition scaffold of HA and CH containing ADSCs can be used to treat a nonunion fracture by augmenting bone healing and may decrease the risk of surgical failure of nonunion fractures.

Keywords: adipose derived stem cells; scaffold; nonunion fracture; tissue engineering

A nonunion bone fracture is a failure of the ends of fractured bones to unite. In a nonunion bone fracture, the progression of fracture healing has ceased and there is motion at the fracture site (Tomlinson, 1991). For the successful treatment of a nonunion bone fracture, the addition of cancellous bone autograft at the fracture site is recommended to stabilise the bone fracture (Sumner-Smith and Cawley, 1970; Hurov, 1984).

A cancellous bone autograft facilitates excellent bone formation, which can lead to bone union through its osteogenesis, osteoconduction and osteoinduction. It can be utilized to treat patients with nonunion, poor osteogenic potential, highly comminuted fractures and osteomyelitis (Martinez and Walker, 1999). Hence, a cancellous bone autograft is still considered the 'gold standard' for bone graft. However, there are several disadvantages with this approach. These include a high risk of fracture and infection and pain at the donor site (Martinez and Walker, 1999). In addition, the amount of cancellous bone is limited. Therefore, many recent studies have focused on bone tissue engineering to overcome these disadvantages (Yoshikawa and Myoui, 2005; Drosse et al., 2008).

Bone tissue engineering involves the use of a combination of scaffolds with osteoblasts or osteogenic potential cells to form bone tissue, which can lead to new bone formation at the affected area when implanted *in vivo*. This attractive property of bone tissue engineering has resulted in considerable developments in the field. However, the use

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of bone tissue engineering in clinical practice is still limited (Goessler et al., 2005; Cancedda et al., 2007). In human medicine, there are only a few reported clinical cases of bone tissue engineering being used to treat bone defects and nonunion fractures (Quarto et al., 2001; Vacanti et al., 2001).

In the present case, bone tissue engineering was applied to a dog with a nonunion fracture to augment bone healing. Adipose derived stem cells (ADSCs), as osteogenic potential cells, were seeded on a composition scaffold made from hydroxyapatite (HA) and chitosan (CH) fibers (fiber composite; 30% of HA, 70% of CH). The scaffold containing the ADSCs was placed on the fracture site. A sample of scaffold with the ADSCS was taken to evaluate the level of cell attachment and their morphology on the scaffold by scanning electron microscopy (SEM). To the best of our knowledge, there are no known case reports addressing the clinical applications of a scaffold of HA and CH containing ADSCs to augment bone healing of nonunion fractures in dogs.

Case presentation

A 4-year-old, intact male crossbreed dog, weighing 27 kg, was referred for the treatment of a nonunion fracture. The clinical history of the dog included a surgical correction of radius and ulna bone fracture on the right forelimb three months prior to the referral. The bone fractures were initially repaired using implants. After the lapse of two months, the implants had failed. A cast was placed from the paw to the elbow for one month after removing the implants. Complete blood count and serum biochemistry were performed, which revealed leukocytosis (18.9×10^3/µl; normal range $6–15 \times 10^3$/µl). Blood gas analysis and urinalysis were within the normal range. The radiographs revealed soft tissue swelling, displacement of the radius and ulna bone fracture fragment and a sclerotic fracture at the end of the radius. After removing the cast, an open purulent wound with skin necrosis was observed at the site of the previous surgical incision. While the dog was under sedation, sterile gauze was placed on the wound, and the area was shaved and scrubbed with a chlorhexidine solution. The necrotic skin was debrided, and pressure irrigation with lactated Ringer solution was performed. The debrided tissue was cultured and an antibiotic sensitivity test was carried out. The surgical correction was postponed due to wound contamination. Cefazolin (25 mg/kg *i.v.* every 8 h) and enrofloxacin (5 mg/kg *i.m.* every 12 h) were administered. A wound lavage was performed using chlorhexidine diacetate (0.05%) every 12 hours, and the wound was dressed with sterile gauze in a wet to dry bandage. A cranial and caudal splint was placed from the paw to the elbow.

After consulting the owner regarding the treatment options for a nonunion fracture, the owner agreed to the use of bone tissue engineering to promote bone healing. One day after admission, approximately 20 g of fat was harvested from the subcutaneous over the cutaneous trunci fascia. The fat was washed extensively with a phosphate buffered saline (PBS) solution (Invitrogen, USA) before being minced with a surgical blade and digested in an equal volume of a filtered (0.2 µm) PBS solution containing 0.1% of collagenase (Sigma, USA) with constant shaking at 37°C for 50 minutes. The resulting solution was centrifuged at 300 × g for 5 min and the supernatant was discarded. The resulting cell pellet was plated into a T1 50 cm^2 tissue culture flask (Becton Dickinson, USA) in 35 ml of a mixture of 80% DMEM (Invitrogen, USA), 15% autologous serum and 1% antibiotic/antimycotic solution (Invitrogen, USA). The flask was incubated at 37°C in an atmosphere containing 5% CO_2. Four days later, the adherent cells population reached ~ 80–90% confluence (Figure 1). The adherent cell layer was washed twice with PBS and the cells were detached from the flask using 0.025% trypsin-1mM EDTA (Sigma, USA). The number of cells was calculated from hemocytometer counts. A total of 3.2×10^7 cells were harvested. The composi-

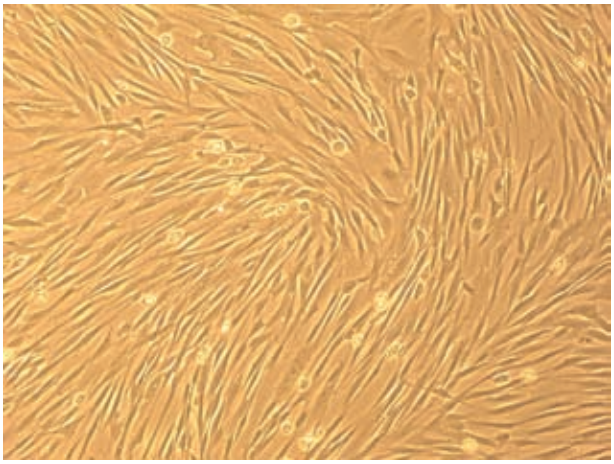

Figure 1. Cell culture image of a population of ADSCs (× 10 magnification) after four days in culture

tion scaffold was obtained from the Laboratory of Textile Engineering, College of Engineering, Chonbuk National University, Korea. This scaffold was made from hydroxyapatite (HA) and chitosan (CH) fibers (fiber composite; 30% of HA, 70% of CH) as described in a previous report (Chung, 2002). The scaffold (4 × 4 × 0.2 cm) was sterilized with ethylene oxide and submerged in the culture medium containing 90 % DMEM and 10 % autologous serum. Two hours later, the pre-wetted scaffold was placed into a petri dish (Becton Dickinson, USA), and 800 µl of the culture medium containing 3.2×10^7 cells was pipetted onto the top surface of the scaffold. The scaffold was incubated at 37°C in an atmosphere containing 5% CO_2 for 3 h. Before giving a prepared scaffold to the surgeon, a small piece (0.5 × 0.5 × 0.2 cm) was taken and placed into osteogenic medium [DMEM, 10% FBS, 1% antibiotic/antimycotic solution, 10 mM β-glycerophosphate (Sigma, USA), 20nM dexamethasone (Sigma, USA), and 50 mg/ml of sodium 2-phosphate ascorbate (Sigma, USA)]. The sample was cultured in an osteogenic medium for 21 days , which was changed every three days. After 21 days, the scaffold was fixed in a 2.5wt.% gluteraldehyde solution with a sodium cacodylate buffer for 2 h. After rinsing with the buffer, the scaffold was submerged in 1wt.% osmium tetroxide in 0.1M sodium cacodylate for 90 min. After a buffer rinse, the scaffold was dehydrated using a graded series of ethanol. Finally, the scaffold was placed in hexamethyldisilazane for 45 min and left under a fume hood until it was completely dry. The scaffold sample was mounted and sputter- coated with gold-palladium prior to examination by scanning electron microscopy (SEM) at 5kV. SEM revealed clusters of ADSCs attached to the scaffold (Figure 2). However, most

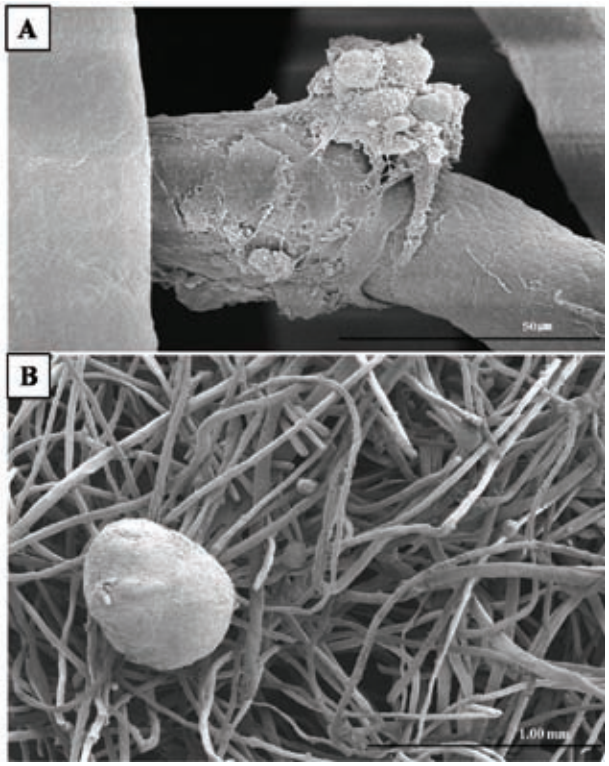

Figure 2. SEM image of the scaffold seeded with ADSCs after 21 days of culturing in an osteogenic medium. The ADSCs adhered to the scaffold and were surrounded by extracellular matrix (A). Many bone nodules made by the secreting bone matrix of the ADSCs also were shown (B)

of the cells were surrounded by an extracellular matrix. Many bone nodules had formed from the bone matrix secreted by the ADSCs.

Surgical correction of the bone fracture was performed five days after admission. During the operation, the sclerotic fracture ends of the radius were drilled to expose the medullary cavity and the callus was removed to allow contouring of a compression

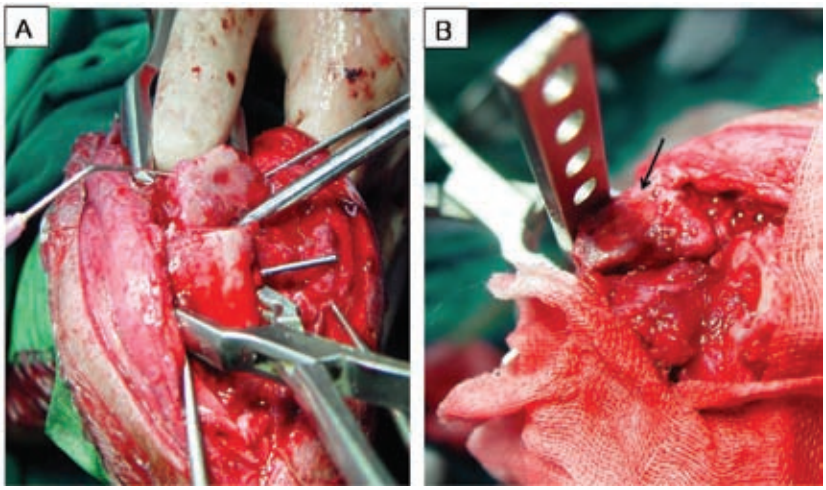

Figure 3. Photograph showing the sclerotic fracture ends of the radius (A), and the scaffold seeded with ADSCs (B; black arrow) that placed on medullary cavity after exposing the medullary cavity by drilling

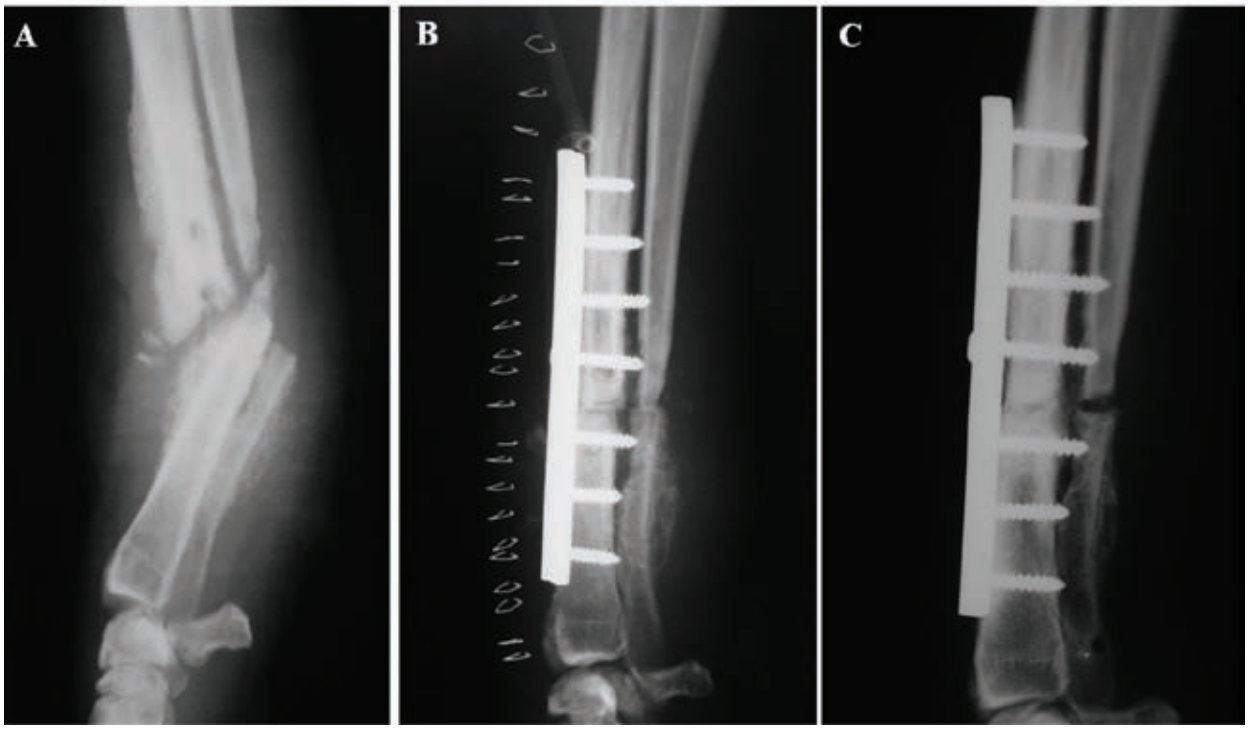

Figure 4. Preoperative cranial caudal radiograph showing the displacement of the bone fracture fragment and sclerotic fracture end of the radius (A). Immediate post-operative lateral radiograph showing good alignment and apposition of bone fracture (B). Follow-up radiograph, which obtained seven weeks after the surgical repair, showing callus bridged the radius bone fracture line and disappearance of the fracture line (C)

plate on the cranial radius bone surface. The compression plate was positioned with a screw on the proximal radius fracture bone. The prepared scaffold containing ADSCs was folded and then placed on the medullary cavity of the proximal radius bone fracture (Figure 3). After reducing the bone fractures, the remaining screws were placed on the digital radius bone fracture for stabilization. At that time, swabs from the bone fracture were taken for culture and for an antibiotic sensitivity test. The wound was then irrigated, a closed suction drain was placed into the fracture site, and subcutaneous tissue and skin were closed in a routine manner. Postoperatively, enrofloxacin (5 mg/kg *i.m.* every 12 h) was administered for one week as a result of the culture and sensitivity test of the open wound. However, the culture and sensitivity test of bone fracture revealed no infection. The drain was removed after two days. The surgical site healed without complications. A Robert Jones bandage was placed from the paw to the elbow for four weeks and no further external support was provided. Seven weeks after surgery, radiographs showed good alignment and apposition of fracture fragment, and a callus bridged the fracture gap (Figure 4). Ten months after surgery, the implants were removed in a local animal hospital and the dog had good use of its limb according to a telephone follow-up.

DISCUSSION

The rate of nonunion fracture is reported to be 3.4% of the total bone fractures in dogs (Millis and Jackson, 2003). In nonunion fractures, the regeneration of bone is a major difficulty because the progression of fracture healing has ceased. Bone tissue engineering has been introduced as an alternative solution to induce bone regeneration instead of bone graft. However, there are few reports of the application of bone tissue engineering to clinical practice (Quarto et al., 2001; Cancedda et al., 2007). This is the first clinical case report to use bone tissue engineering (a composition scaffold of HA and CH containing ADSCs) to augment the bone healing of a nonunion fracture in a dog.

For the application bone tissue engineering, adipose derived stem cells (ADSCs) were used as

osteogenic potential cells. ADSCs are multipotent progenitor cells, capable of differentiating into a number of mesodermal lineages including osteoblasts (Xu et al., 2005; Moseley et al., 2006). A number of experiments have demonstrated the efficacy of ADSCs in enhancing osteogenesis (Xu et al., 2005; Bunnell et al., 2008). Furthermore, stem cells secrete bioactive factors that suppress apoptosis, enhance angiogenesis, and stimulate mitosis and differentiation of tissue intrinsic reparative cascades (Caplan and Dennis, 2006). These effects may lead to reactivation and augmentation of the fracture healing mechanism (Xu et al., 2005). Another reason for choosing ADSCs was that a high number of stem cells were needed. In human clinical case reports, the injection of bone marrow derived stem cells (BDSCs) in atrophic tibia nonunion fractures have elicited successful bone healing, and it has been suggested that the efficacy of bone healing appears to be related to the number of osteoprogenitor cells injected (Hernigou et al., 2006). Adipose tissue was chosen as the stem cell source because it has a large number of stem cells compared with bone marrow. One study indicated a 500 times higher number of stem cells derived from an equivalent amount of adipose tissue than from bone marrow (Fraser et al., 2006). In addition, increasing the amount of bone marrow does not coincide with a high number of stem cells whereas increasing the amount of adipose tissue does (Schaffler and Buchler, 2007). The stem cells were expanded *ex vivo* to increase their number. However, surgeons should consider that time is required to expand cells. Hence, the expansion of cells is limited to patients not requiring emergency treatment. In this case, surgical correction of the nonunion fracture could be delayed because a nonunion fracture does not require emergency surgical treatment. Furthermore, the treatment of the open wound contamination required some time.

Although ADSCs were obtained before implantation, there was some concern about how to place a large number of ADSCs on the fracture site so as not to lose cells. A direct injection of ADSCs at the fracture site did not appear to be a good method. Hence, stem cells were attached to a scaffold and this was placed precisely on the fracture site. It is believed that using a scaffold to attach stem cells promotes better bone healing than an injection of stem cells. This is supported by a previous study which reported that scaffolds containing stem cells elicit better bone formation than the scaffold and stem cells alone (Petite et al., 2000). In this case, a composition scaffold made from hydroxyapatite (HA) and chitosan (CH) fibers was used for seeding the ADSCs. HA is one of the major constituents of the inorganic component in bone and is used widely as a bone substitute in bone repair (Yoshikawa and Myoui, 2005). These advantages of HA makes it suitable as a scaffold for tissue engineering. However, there are some limitations to its usage due to its brittleness, which makes it difficult to shape. To solve this problem, composites of hydroxyapatite and chitosan have recently been reported (Xiao et al., 2008). Chitosan (CH) is a natural polymer and polysaccharide, and a deacetylated form of chitin. It exhibits biocompatible, biodegradable and osteoconductive properties (Kim et al., 2008). Many studies have demonstrated the effect of chitosan on the bone healing and formation process (Di Martino et al., 2005; Kim et al., 2008). From these results, a composite of HA and CH material is expected to be a suitable scaffold for bone tissue engineering. In this case, SEM confirmed that the ADSCs had adhered to the scaffold well and many bone nodules had formed from the bone matrix secreted by the ADSCs. This result is in agreement with a previous report (Xiao et al., 2008).

Although successful bone healing of a nonunion fracture was achieved by bone tissue engineering, it is unclear how many of the stem cells differentiated into osteoblasts once implanted, and how many stem cells are required to induce bone formation *in vivo*. Therefore, future studies will be needed to use bone tissue engineering widely in clinical practice.

In conclusion, a nonunion fracture was healed successfully using internal fixation and a composition scaffold of HA and CH containing ADSCs. *In vivo* analysis confirmed that the composition scaffold of HA and CH containing ADSCs induced new bone formation. The application of a composition scaffold of HA and CH with ADSCs can be used to treat a nonunion fracture by augmenting bone healing, and may decrease the risk of surgical failure of a nonunion fracture.

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