Cuttlebone used as a bone xenograft in bone healing

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ABSTRACT: This study was conducted to examine the potential of cuttlebone xenograft in the healing of bone using radiography and histology for a period of 24 weeks. One hundred and five New Zealand male rabbits with radius defects in the metaphyseal region were divided into five groups treated with cuttlebone, demineralized bone matrix, bovine cancellous graft, and tricalcium phosphate. The control was no treatment. Clinical, radiological, biochemical and histological evaluations were made 1, 2, 3, 4, 6, 12, and 24 weeks after surgery. Physiological measurements (body temperature, heart rate, and respiratory rate) were not affected by the treatments. The radiological score was greatest in the demineralised bone matrix and tricalcium phosphate groups (score of 8), followed by the bovine cancellous graft (score of 6), cuttlebone (score of 6), and control groups (score of 5). The histological score was greatest in the tricalcium phosphate group (score of 55), followed by the cuttlebone (score of 50), bovine cancellous graft (score of 48), demineralized bone matrix (score of 44) and control groups (score of 42). Oxidative enzyme activities were not different across the treatments. The lack of reinfection and infection responses and faster bone union highlight the potential of cuttlebone xenograft in orthopaedic surgery.

Keywords: cuttlefish backbone; bone xenograft; bone healing; radius; rabbit

List of abbreviations

BCG = bovine cancellous graft, CB = cuttlebone, DBM = demineralized bone matrix, TCP = tricalcium phosphate

Although there have been great advancements in fixation methods used in orthopaedic surgery over the last 30 years, the available techniques have not completely resolved all problems associated with these procedures. Healing of fractures with loss of tissue, diaphyseal sectional fractures of long bones, various types of bone defects, osteomyelitis, non-union, and arthrodesis continue to be major problems in orthopaedic surgery (Oh et al. 2006). For instance, shortening of the limb and bone dysfunction due to tissue loss cannot be prevented in fractures of the limb bones (Miranda et al. 2005). The potential of filling bone defects with bone graft to maintain bone integrity has been investigated (Alexander 1987). After blood, bone grafts are the second most common transplants (Candas 1983). Autogenous bone grafts are the most popular among bone graft materials because they are bio-compatible, osteo-conductive, and cost-effective (Ghodasra et al. 2014). Autografts reduce the risk of infectious disease transmission, and they exhibit optimal osteo-conductive, osteoinductive, and osteogenic properties (Shafiei et al. 2009). However, grafting has disadvantages, such as the limited supply of bone for autografts, immune responses to allografts and alloimplants, potential transfer of infectious agents, technical difficulties with vascularised grafts, presence of pain, seroma, bleeding, and infection in the donor area and instability in the affected region (Altundal et al. 2005). As a result, scientists are searching for alternative bone fillers (Damien and Parsons 1991). Numerous experimental studies have been conducted on cuttlebone (CB) (Rocha et al. 2006; Yildirim et al. 2007; Ivankovic et al. 2009). Due to its aragonite structure, its feasibility as bone graft has been inves-

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tigated *in vitro* (Ivankovic et al. 2009). It can be obtained easily and inexpensively from all the seas of the world, it is cheap (Rocha et al. 2006), it can be shaped easily due to its morphology and mineral composition, it is compatible with other types of bone structure and it has high osteo-inductive capacity (Murugan et al. 2006). The pore diameter of bone grafts should be between 200 and 500 μm for bone tissue revascularisation and structuring (Wiesmann et al. 2004). The pore diameter of CB is between 200 and 600 μm (Rocha et al. 2006), suggesting that CB could be an ideal graft. Moreover, its porous structure can allow physical contact with the host tissue and facilitates mineral exchange and vascularisation (Birchall and Thomas 1983).

An increase in oxygen free radicals due to stress during and after anaesthesia and operative intervention is common in experimental animals (Cornell and Lane 1992). After a fracture, enzymes released by neutrophils and polymorphonuclear leukocytes accumulate in the region of tissue damage and increase reactive oxygen metabolites (Ikeda et al. 1989). Oxygen free radicals not only lead to soft tissue degradation but also exert a negative effect on fracture healing (Durak et al. 1996). The increase in free radicals is particularly apparent two to three weeks after a fracture (Turgut et al. 1999). Therefore, bone grafts not only accelerate healing but decrease the occurrence of free radicals.

This study examined the feasibility of CB as a xenograft in bone healing based on clinical, radiographic, biochemical, and light microscopic results *in vivo* in the short, medium, and long term.

MATERIAL AND METHODS

Animals, experimental groups, and management. To take six samples from all sub-groups, one hundred and five one-year-old male New Zealand white rabbits were divided into five groups, each of which were further divided into seven subgroups that were sampled at 1, 2, 3, 4, 6, 12, and 24 weeks. The animals were anaesthetised with an injection of 8 mg/kg xylazine HCl and 30 mg/kg ketamine HCl. A 1–2 cm skin incision was made in the metaphyseal regions of the left and the right radius on all animals. *M. ext. dig. communis* and *m. ext. carpi radialis* were retracted. After removal of the periosteum using a periosteal elevator, a 5 mm diameter unicortical defect with a depth of 3 mm was created with a burr (Leppestr. 62, 51766, Germany)

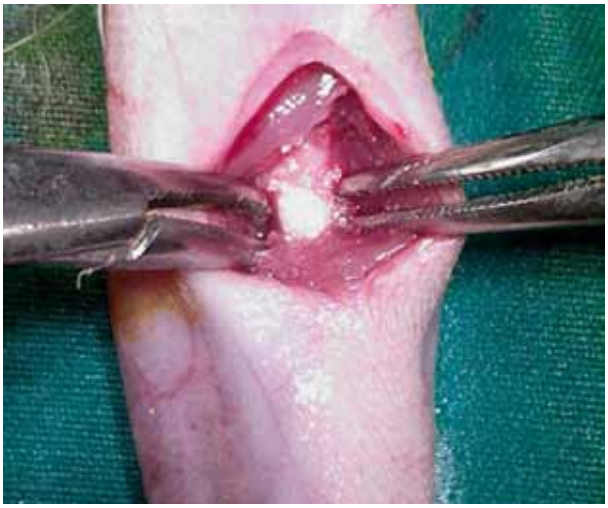

Figure 1. CB graft placement in a 5 mm defect

under saline irrigation. In groups I, II, III, and IV, the defects were filled with CB (n = 21, Figure 1) (Vitaking, Stone, Yalcin Aquarium, Ankara, Turkey), demineralised bone matrix (DBM, n = 21) (UltraFill DBM Putty, USA), bovine cancellous graft (BCG, n = 21) (LifeTEK, OrthoBiologics, USA), and tricalcium phosphate (TCP, n = 21) (Bi-Ostetic™, USA) after mixing with vancomycin powder (1 g Vancomycin HCL, Hospira, USA). Group V (n = 21) was not treated and served as a control. After administering 0.5 ml of a broad-spectrum antibiotic (Rifocin 125 mg amp., Sanofi-Aventis, Turkey) locally against possible contamination in the region of the defect, the operative wound was closed with muscle (4/0 coated vicryl, Ethicon, USA) and skin (2/0 mersilk, Ethicon, USA) sutures. A protective dressing was applied to the region. During the postoperative period, 20 mg/kg of cefazolin (1 g *i.m.*/*i.v.*) (Iespor®, Ulugay, Turkey) was administered for five days and 1.15 mg/kg of flunixin meglumine was administered *i.m.* (Vial Fluvil®, Vilsan, Turkey) for three days. The skin sutures were removed on the 7th postoperative day. During the experiment, the rabbits were fed *ad libitum*.

Cuttlebone graft. The CB was purchased from a pet shop where it is sold as a calcium supplement for birds and beak rasp (Vitaking, Stone, Yalcin Aquarium, Ankara, Turkey). The CB were crushed into small pieces with a scalpel blade and sterilised with ethylene oxide (55 °C, 4 h, 40% humidity, 12 h propagation time).

Clinical evaluation. Daily body temperature, heart rate, and respiratory rate were measured in the preoperative and postoperative periods (seven days). The same measurements were taken from

all groups prior to sacrifice at 2, 3, 4, 6, 12, and 24 weeks. Body temperatures were measured using a clinical thermometer. Heart rate and respiratory rate were measured with patient monitoring (Bionet BM3 Vet, Korea). Dressings were opened every other day postoperatively to examine the suture line and the circumference, redness, oedema, exudation, and aperture of the wound edges.

Radiographic evaluation. The rabbits were sacrificed postoperatively using high doses of anaesthetic agent (60 mg/kg of xylazine HCl, 200 mg/kg of ketamine HCl, *i.v.*) at weeks 1, 2, 3, 4, 6, 12, and 24, and radiographs of the radial bone applied graft material were taken in the anteroposterior and mediolateral positions (52 mA, 0.6 mAs). The radiographs were evaluated according to the modified scoring system (bone formation, union, and remodelling) of Lane and Sandhu (1987). To correct the assessment, the radiographs were scanned with a computer, and callus formation was measured using the AutoCAD 2012 (CAD Software) program.

Biochemical evaluation. Approximately 5 g of muscle tissue just above the region where the graft was placed were taken postoperatively at weeks 1, 2, 3, 4, 6, 12, and 24. The muscle tissue samples were pulverized by grinding with liquid nitrogen in marble mortar, and the activities of antioxidant enzymes, including catalase (Aebi 1984), superoxide dismutase (Sun et al. 1988), myeloperoxidase (Bradley et al. 1982), glutathione reductase (Carlberg and Mannervik 1985), total glutathione levels (Sedlak and Lindsay 1968), and glutathione S-transferase (Habig and Jakoby 1981), were measured, in addition to the level of lipid peroxidation (Ohkawa et al. 1979). All the measurements were performed at room temperature.

Histological evaluation. The proximal metaphyseal portion of the radial bones was dissected and decalcified with 3% nitric acid for three weeks. Decalcified tissues were embedded in paraffin blocks. Subsequently, 5–7 μm thick sections were cut on a microtome and stained with haematoxylin-eosin. The samples were examined under a light microscope connected to a camera (Nikon Eclipse 50 I, Japan) and scored using a modified version of Heiple's (Heiple et al. 1987) and Lane and Sandhu's (1987) system (union, cancellous bone, cortical bone).

Statistical analysis. Continuous variables (respiratory rate, heart rate, body temperature, enzyme activity) were analysed using a two-way analysis of variance (group, time and group × time interaction). Histological and radiographic scores were analysed using the Kruskal-Wallis test (SAS 2002). The data are presented as the LS mean ± standard error. Group differences were detected using the LSD, with $P < 0.05$ considered statistically significant. The correlation between the radiographic and the histological scores was determined using Pearson's correlation test (SPSS 10.0, SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Clinical and radiographical parameters

There were no group differences in body temperature (38.8 °C), heart rate (296 beats/min), and respiratory rate (56/min).

The control group and the BCG group showed 75% callus formation and radiographic union in the postoperative 3rd week. In contrast, 100% callus formation and radiographic union were noted in the groups treated with CB (Figure 2), DBM, and TCP. Callus formation and radiographic union were clear in all the groups in the 4th postoperative week. By postoperative week 6, remodelling could be observed in the animals treated with CB, whereas remodelling was not evident until week 12 in the control group and the groups treated with TCP, DBM, and BCG. In terms of the radiographic

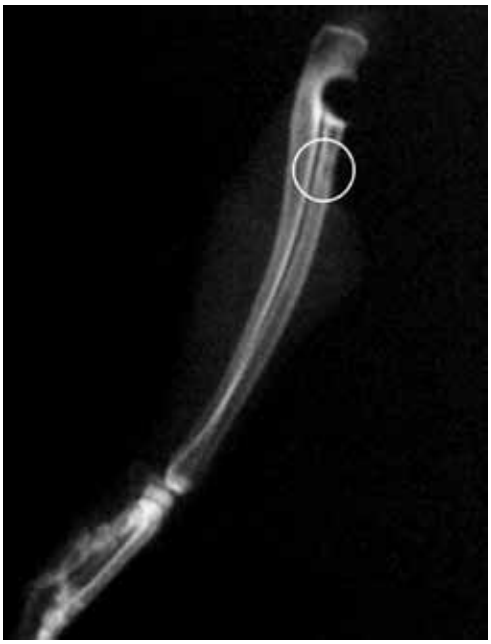

Figure 2. Radiographic image from the CB group; three weeks postoperatively

score (bone formation, union, and remodelling), DBM ranked first (score of 8), followed by TCP (score of 8), BCG (score of 5), CB (score of 6), and the control (score of 6).

Biochemical and histological parameters

The treatments affected the activity of oxidative system enzymes. Superoxide dismutase, glutathione S-transferase and glutathione reductase activities were increased from the first week to the 24th week in all groups, while catalase and lipid peroxidation activities were highest in the first week in all groups; this variable decreased until the 24th week. While myeloperoxidase activity was decreased in all other groups until the 24th week, it was increased only in the CB group. Glutathione S-transferase activity increased until the 24th week; it was lowest at the 12th and 24th weeks in the CB group and highest in the TCP group.

Fibrous union was initiated in the defects filled with the CB graft at postoperative week 1, and the rate of vascularisation was greater than that observed in the other groups. At postoperative week 2, there was a clear Havers' systems zone in the CB group, in addition to fibrous tissue and a high rate of vascularisation (Figure 3). At postoperative week 3, osteochondral union in the CB group resulted in increased cellular activity in terms of new bone formation (activation process). Fibrous tissue began to form in the DBM group at postoperative week 3, and cell activation led to the formation of cancellous bone. However, there was no sign of healing in the cortical region. When all the groups were compared, the TCP group recovered first and showed more rapid graft union. By postoperative week 4, osteochondral union had developed in the control and the CB groups. Moreover, the cancellous bone showed new bone formation, the cortical bone exhibited ossification, and bone marrow cavities were reconstructed. Signs of healing developed faster in the TCP group than in the control group, as reflected by the existence of cancellous bone. Osteochondral union had formed in the DBM group by week 4, and bone remodelling of the cancellous bone had begun. However, the recovery rate was slower than that in the other groups. Bone healing had progressed in the TCP group at week 4, bone clusters were organised, and restructuring was observed in the cancellous and cortical bones. The BCG group exhibited rapid recovery observed in the cancellous and cortical bones. In general, histologically advanced bone resorption was apparent in all the groups by the postoperative 4th week. The remodelling was fastest in the CB and TCP groups. At the 12th postoperative week, the restructuring process was completed in all groups. At the 24th postoperative week, the process of fracture healing and compact bone formation was completed in all the groups. Inflammatory cells and foreign body giant cells were not seen during the experiment in any of the groups. Based on their histological scores, the groups were ordered as follows: TCP (score of 55), CB (score of 50), BCG (score of 48), DBM (score of 44) and the control group (score of 42).

The highest correlation between the radiography and histology parameters was noted in the CB group, followed by the TCP, control, BCG, and DBM groups (Figure 4).

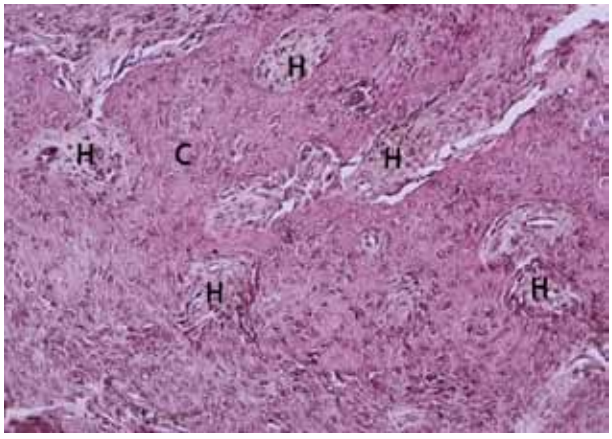

Figure 3. Histological image from the CB group (HE × 40); two weeks postoperatively; C = cartilage formation, H = Havers system formation

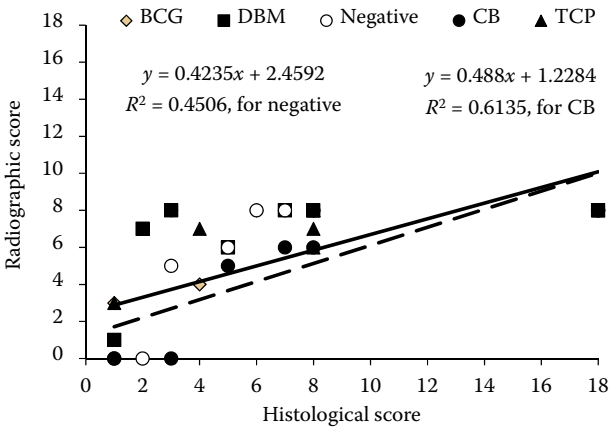

Figure 4. Correlation between radiographic scores and histological scores

A number of research studies have focused on the healing of bone defects with remodelling (Bruder et al. 1998; Khan 2000; Pinheiro et al. 2014; Thormann et al. 2014). In recent years, many commercial bone graft materials have been introduced. However, none of these have been entirely successful (Tomin et al. 2002). Thus, DBM, BCG, and TCP, all of which are currently used in human orthopaedic surgery and CB, were tested as bone xenografts in a 24-week experimental study. Bone defects may take from four weeks to six months to heal after surgical interventions. After the application of a xenograft, an excessive rise in body temperature, irregular heart rate rhythm, and respiratory disorders, manifesting in a rejection, can be seen (Halling et al. 2004); these were not noted in this experiment. The mean body temperature, heart rate, and respiratory rate were within reference values (38.5–39.5 °C body temperature, heart rate 300 beats/min, respiratory rate 60/min) as provided by Havenaar et al. (2003). Given that the measured parameters were within normal limits and that lymphocytes, neutrophils, macrophages, plasma cell infiltration, and foreign body giant cells were not seen histologically, infection appears not to have been present.

Ossification in the fracture zone is first expressed as endosteal and periosteal growth. This ossification later expands, and a fibro-cartilaginous callus forms, which begins to spread after 20 days (Aslanbey 2002). In the present study, callus formation was detected radiographically at postoperative day 21. However, the histological findings showed that bone healing was faster in the CB and TCP groups than in the other groups. Moreover, bone healing in the CB group was faster than in all the other groups 28 days postoperatively.

In the CB group, the degree of postoperative vascularisation at postoperative day 14 and the degree of bone-graft union and the ossification rate at postoperative day 42 was faster than in all the other groups. According to these results, grafting with cuttlebone induces the process of osteogenesis. Owing to the high degree of vascularisation in the CB group compared to the other xenograft group and the acceleration of osteogenesis and onsteointegration, CB was considered the optimal graft in the current study.

According to the results of the biochemical analysis, there was no statistically significant difference between the experimental groups. Compared with the other grafts, CB is associated with decreased formation of free radicals in soft tissue, and it allows bone healing without causing oxidative stress. The lack of difference in the oxidative enzymes in the CB group compared to those of the other xenografts, all of which are currently on the market, suggests that CB can be processed for bone recovery.

We can conclude, based on the clinical and histological data that the CB graft was not rejected, and did not cause infection. Thus, CB appears to be a compatible material, which can establish bone union earlier than other xenografts, without leading to excessive oxidative damage. The data suggest that CB can be processed and employed in orthopaedic surgery.

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