A radiographic survey of eggshell powder effect on tibial bone defect repair tested in dog

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Abstract. Objective: Nowadays, skeletal system injuries are of major importance. In addition, it is recommended to use materials for hard tissue repair in open or closed fractures. It is important to use complex minerals with a beneficial effect on hard tissue repair, stimulating cell growth in the bone. Materials that could help avoid bone fracture inflammatory reaction and speed up bone fracture repair are of utmost importance in the treatment of bone fractures.Material and Method: Similar to minerals, the inner eggshell membrane consists of carbohydrates, lipids, proteins with high pH, high calcium absorptive capacity and with faster bone fracture repair ability. In the present radiographic survey, eggshell-derived bone graft substitutes were used for bone defect repair in 8 dog tibia, measuring bone density on the day of implant placement and 30 and 60 days after placement. Results: In fact, the result of this study shows the difference in bone growth and misshapen bones between treatment and control sites. Conclusion: Cell growth was adequate in treatment sites and misshapen bones were less frequent here than in control sites.

Key Words: radiography, eggshell powder, bone repair.

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Introduction

A complication-free and fail-safe healing process in defect sites is of vital significance when considering the reconstruction of cranial, craniofacial and oral defects associated with congenital malformation, surgical oncology and traumatic injury (Lew et al 1997). For this purpose, allogeneic and alloplastic bone substitutes, as well as autogenic bone grafts, have been used for a very long time. Moreover, in the last decade, the concept of guided bone regeneration (GBR) has become widely accepted and different membranes have been experimented in combination with various powdered materials used as space fillers. In cranial bone defects, space-filling materials have been intensively assessed in order to enhance bone formation in combination with GBR (Dupoirieux et al 2000). It has been suggested that demineralized bone matrix (DBM) grafts accelerate bone healing due to the possible osteoinductive effects of bone morphogenetic proteins (BMPs) in the graft (Bonder 1996). Although vascularized and cancellous autografts show optimum skeletal incorporation, host morbidity limits autograft availability (Bauer&Muschler 2000). Synthetic bone substitutes have also been introduced into clinical use, but they are rather expensive. Diverse biological activities have been attributed to egg components, including antimicrobial and antiviral activity, protease inhibitory action, vitamin-binding properties, anti-cancer activity and immunomodulatory activity (Li-Chan&Nakai 1998). In the last decade, chicken eggshell powder has been suggested as a possible solution (Dupoirieux et al 2000; Dupoirieux et al 1995). The mineral and organic matrix of the chicken eggshell, specifically ostrich eggshell, has recently been introduced as a bone substitute candidate in reconstructive surgery (Dupoirieux et al 2001; Dupoirieux 1999) and in jaw cyst repair (Baliga et al 1998), as a result of its thickness, suitable for preparing particulate materials of different size and of its inorganic phase, mainly consisting of calcite crystals (Feng et al 2001). It closely resembles the mineralized bone matrix and it is mainly composed of calcium carbonate (97.4%), magnesium phosphate (1.9%) and tricalcium phosphate (0.7%). Moreover, the calcified eggshell contains an organic matrix, accounting for 2% of the total eggshell weight. This organic matrix contains several proteins and proteoglycans, such as ovocleidin-116, ovotransferrin, ovalbumin, ovocalyxin-32, ovocleidin-17, osteopontin (OPN), and lysozyme, some of them being able to modify the morphology of calcite crystals and the precipitation of calcium carbonate (Pines et al 1995; Panheleux et al 1999). OPN plays an important role in calcification, as it increases osteoblast adhesion onto the matrix and binds to hydroxyapatite (Pines et al 1995; Li-Chan&Nakai 1998). Understanding the biological effects and resorption kinetics of autograft, allograft and synthetic bone substitute materials is necessary for their optimum use, since they play important roles in reconstructive surgery. In experimental studies, the calvarial defect model has been regarded as the most selective experimental model for bone regeneration due to the poor blood supply and the membrane structure precluding spontaneous healing (Dupoirieux et al 2001). In the present study, the process of bone healing was assessed in the experimental dog model of tibial bone defect without filling, on the one hand, and filled with either same size eggshell granules or commercially available DBM, on the other hand, using clinical, radiological and densitometric methods.

Materials and methods

Preparation of animal models

This experimental study was conducted on adult male mixed breed dogs (n=8) aged 3 to 4 years and weighing 20 to 30 kilograms. The animals were kept in a restricted access room (at 22°C and on a 12:12 light: dark cycle), with free access to food and water. Animals were randomly divided into 2 groups. The research protocol was approved by the Research Ethics Committee of the College of Veterinary Medicine, Shahrekord Branch, Islamic Azad University. All the experiments were carried out in accordance with the rules of the Institutional Animal Care and Use Committee (IACUC). The study design and the parameters for tissue reaction evaluation were in accordance with the ISO 10993-6 (1994) standard.

Eggshell powder (ESP) collection and preparation

Eggshell powder was obtained from White Leghorn hen eggs by breaking the eggshell opposite the air chamber, discarding the albumen and yolk and washing them thoroughly with distilled water several times. The outer and inner shell membranes were carefully removed with a forceps. Then, the eggshell was manually extracted, rinsed and dried at 25°C for 24 h. After being crushed, the sheets were individually packed and sterilized with ethylene oxide for 1 hour at room temperature. (AX-400, Axis, Izmir, Turkey) It has been suggested that the methods used for the harvesting and sterilization of graft materials had a strong impact on their osteogenic capacity (Dupoirieux et al 1994).

Experimental Animals and Surgical Technique

In order to assess the effect of eggshell powder-derived graft substitutes on bone healing (Adam 2004), the present study was conducted on adult male mixed breed dogs (n=8, 2-3 years, 23-23.5 kg), divided into 2 groups. The isoflurane anaesthetized dogs underwent surgery under sterile conditions. In this study, the caudomedial approach to the medial part of the right tibia was performed via 1–1.5 cm incisions. After removing the surrounding musculature to isolate the tibia, an 8-mm wide osteotomy was performed using an oscillating. Further, 1 mg of eggshell powder was implanted to the site of osteotomy. The fixation of the implant was accomplished using two simple discontinuous periosteal sutures (2-0 nylon) of the cranial and caudal face of the proximal fragment. The other site of the tibia was only subjected to osteotomy and served as negative control for natural healing. Densitometry (manufacture by MedicalExpo) was performed using the LC NDT FV-2009 Viewer.

Wound closure was accomplished by the apposition of the subcutaneous tissue in a simple continuous pattern (2-0 polyglactin 910) and by a simple discontinuous skin pattern (2-0 nylon), Postoperative care an analgesic/anti-inflammatory drugs were used.

The animals radiographs were obtained after, 0 and 30 and 60 days, (Trophy CCX Digital, Croissy-Beaubourg, France), at an exposure of 50 kV, 7 mA for 0.5 seconds. The radiographs obtained in this study were used in the laboratory for densitometric analysis of bone healing. Figure 1a shows part A (implant site) and part B (control site) on the X-ray images acquired after surgery. Figure 1b shows the X-ray image of the tibia 30 days after surgery in the first group and Figure 1c shows the X-ray image of the tibia 60 days after surgery in the second group. All radiographs consisted of both lateral and craniocaudal views.

The results of radiography and densitometry and the p values in this study are shown in Table 1.

The data were analyzed using SPSS software (version 17. SPSS Inc., USA) and P value was calculated using Chi-square and fisher's exact tests to fine any significant relationship. P value less than 0.05 was considered statistically significant.

Results

Clinical Results

All animals healed without any complications and gained body weight. Physical examination detected eggshell particle remnants under the skin, with no major displacement in bone defect and relatively smaller than the initial size of the particles.

Radiological Results

Radiological examination detected minimal bone regeneration at the periphery of the bone defect site in the control group, 1 month after surgery. Bone formation displayed centripetal progression from the entire circumference of the defect. In the experimental groups, small particles were absorbed more excessively than the larger ones. Bone regeneration was considerable in both groups when compared to the first group in the first month of the experiment. Bone regeneration significantly increased within the implanted site in the second group, in the second month of the experiment, when compared to the control group.

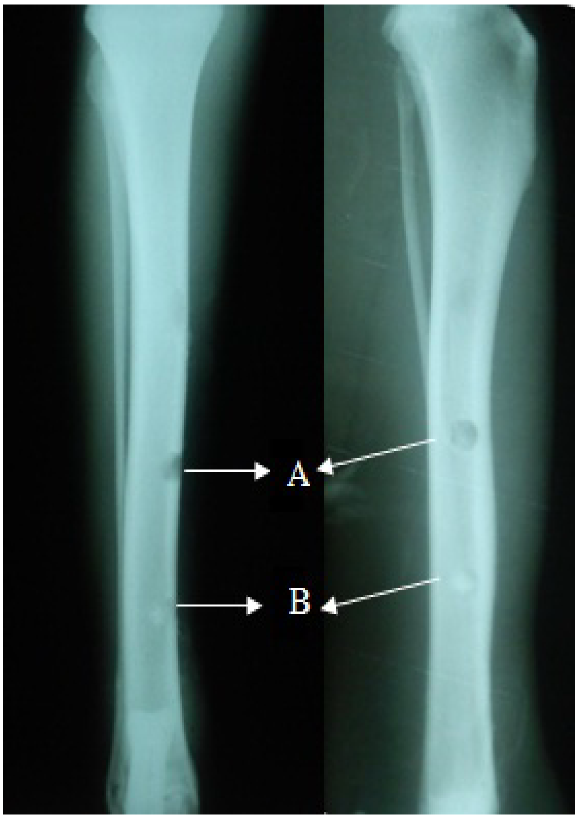

Figure 1a. Postoperative radiograph of the canine tibia on the day of the surgery

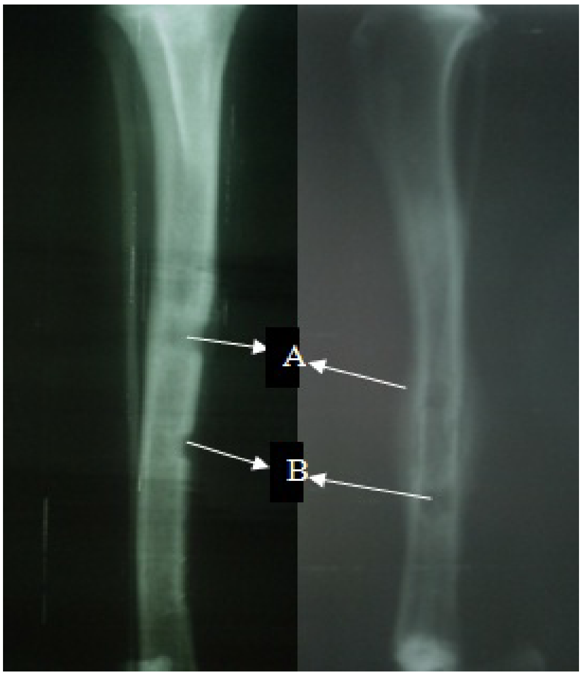

Figure 1b. Postoperative radiograph of the canine tibia 30 days after treatment

Figure 1c. Postoperative radiograph of the canine tibia 60 days after treatment

Table 1. Densitometry results and P values. ***Effective ($p<0.05$),*Non-Effective ($p<0.01$)

Defects	Positive controls				Treatment using biomaterial (eggshell powder)			
Days	**0**	**30**	**60**	**p value**	**0**	**30**	**60**	**p value**
Group 1	3.9	3.9	3.7	**0.01***	3.9	3.2	2.5	**0.001*****
Group 1	3.9	3.9	3.7	**0.01***	3.9	3.2	2.5	**0.001*****
Group 1	3.8	3.8	3.6	**0.01***	3.8	3.1	2.5	**0.001*****
Group 1	3.85	3.35	3.7	**0.01***	3.85	3	2.5	**0.001*****
Group 2	3.9	3.9	3.7	**0.01***	3.9	3	2.5	**0.001*****
Group 2	2.9	3.9	3.7	**0.01***	2.9	3.1	2.5	**0.001*****
Group 2	3.8	3.8	3.6	**0.01***	3.8	3	2.5	**0.001*****
Group 2	3.85	3.85	3.7	**0.01***	3.85	3.11	2.5	**0.001*****

Discussion

In the present study, 8-mm critical size defects were created in the tibial bone of dogs, as bone lesions above this critical size become scarred rather than regenerated, leading to the emergence of a cavity (Bauer&Muschler 2000; Lindholm&GAO 1993). Such defects have been traditionally treated with bone graft substitutes. When using a bone graft, the wound is expected to heal and new bone to form (Bodner 1996), further replacing the bone defect. Graft materials obtained from other sources have been introduced as a possible solution. Chicken eggshell has been recently introduced as a candidate for bone substitution in maxillofacial surgery (Dupoirieux et al 2000; Dupoirieux 1999). The chicken eggshell has a high mineral content (over 97%) and its mineralization is known as being the most rapid and with the largest amount of calcium deposition (Lavelin et al 1999). Previous researchers (Dupoirieux et al 2000; Dupoirieux et al 2001; Dupoirieux 1999; Durmus et al 2003) investigated the feasibility of the use of eggshell powder from various chicken breeds as bone graft material. In this study, the use of chicken eggshell was due to the size, strength, curvature, thickness and fine assembly, thus having excellent mechanical properties (Feng et al 2001). In addition, the egg is the largest biological cell originating from one-cell division (Mine 2002). Previously, chicken eggshell fragments were used as bone substitutes in facial reconstructive surgery in experimental animal models (Dupoirieux et al 2000; Dupoirieux et al 1995; Dupoirieux 1999; Durmus et al 2003). The researchers (Dupoirieux et al 2000; Dupoirieux et al 1995; Dupoirieux 1999; Durmus et al 2003) have focused on the material's biological behavior, biocompatibility, binding ability and resorption kinetics. However, previous studies have mostly taken into consideration the possible roles of the eggshell inorganic matrix as a tissue scaffold. Bone formation and bone remodelling are controlled by non-collagenous proteins of the bone matrix. These low-molecular weight polypeptides determine the crystal structure and mechanical strength of the material by modulating crystal nucleation in the bone and other extra cellular mineralized tissues. (Addadi and Weiner 1985) Osteocalcin (OCN), osteopontin (OPN) and bone sialoprotein (BSP) were found in the non-collagenous proteins of the bone matrix. Since OPN increases osteoblast adhesion onto the matrix and binds to hydroxyapatite, it plays important roles in bone formation and bone remodeling (Dominguez et al 2000; Lavelin et al 1999; Glimcher

1998; Gautron et al 1996). A recent study demonstrated that a 21 000 Da protein present in soluble eggshell matrix proteins may play an important role in the increase in calcium transport across intestinal epithelial cells in vitro (Daengprok et al 2003). Eggshell organic matrix proteins are also bioactive molecules that modify calcite crystal morphology and precipitation during the formation of chicken eggshell (Gautron et al 1997; Nakano et al 2001; Pines et al 1995; Panheleux et al 1999). Moreover, chicken OPN, which is an essential eggshell matrix protein, has 35% protein sequence homology and in the nucleotide level, has 65% sequence homology with mammalian OPN (Castagrola et al 1991; Pines et al 1995; Panheleux et al 1999). Transforming growth factor b1 (TGF-b1) and progesterone have also been isolated from the chicken eggshell powder (Schaafsma et al 2004). Recently, Mann and Siedler (Mann Siedler 2004) have shown two different C-type lectin-like proteins as major components of the matrix of the chicken eggshell calcified layer. Calbindin, a calcium binding protein is synthesized by the chicken uterus in a circadian fashion during the eggshell production (Lavelin et al 1999). Previous researchers (Dupoirieux et al 2001; Dupoirieux et al 2000; Dupoirieux et al 2001; Dupoirieux 1999) have concluded that the eggshell is a resorbable implant. In a previous study, 50 μm chicken eggshell particles were not detected by radiology after one month in any of the animals (Dupoirieux et al 2001). The 75 μm particles were completely resorbed after two months, whereas 150–300 μm particles were progressively resorbed during the fourth month. Some authors (Dupoirieux 1999), have suggested that chicken eggshell was a resorbable material, but with size-dependent degradation kinetics. In a similar study, assessed the potential beneficial effect of ostrich eggshell powder in combination with either inner or outer shell membrane treated with 2.5% glutaraldehyde or 0.05% trypsin (Durmus et al 2003). There was no encapsulation, inflammation or foreign body reaction three months after treatment, with larger eggshell particles partially resorbed and smaller particles moderately resorbed. In the present study, the density of the sites of implantation was higher than that of the control sites. Resorption was much slower in control sites. However, none of them has been completely degraded at the end of the experiment. Interestingly, bone formation was lower in control sites, whereas the sites of implantation had relatively higher bone density rates, with a greater osteogenic effect (Table 1). There might be a strong relationship between particle size and osteoinduction that needs further experiments in order to be determined (Dupoirieux 1999; Dupoirieux and et al 2001). have suggested that the reaction to eggshell particles was a non-immunogenic reaction without any detrimental effects. The use of eggshell powder in repair bone defect can be effectible.

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