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Radiographic Survey of Effecte of the Inner Eggshell Membranes on the Tibia Bone Repair in Animal Model

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Abstract: The efficiency of chicken inner eggshell membranes combined with a minimally invasive small osteotomy procedure of the tibia to accomplish an efficient release of the tibia so that it can continue to grow in an unstressed manner was tested in dogs. All the experiments were carried out in accordance to the rules of Institutional Animal Care and Use Committee (IACUC) and the research protocol was approved by the Research Ethics Committee of Veterinary Medicine Collegue, Shahrekord Branch, Islamic Azad University. Inner Eggshell membranes were extracted from chicken eggs, rinsed, dried and sterilized with ethylene oxide for 24 h. Subsequently, 8 dogs were anaesthetized and a complete 8 mm wide osteotomy was performed in both the right and the left distal tibia. A piece of eggshell membranes was interposed in the osteotomy site of one tibia. The opposite osteotomized tibia was left as a negative control. After 30-60 days, tibia osteotomized regions were radiologycal examined. After radiological evaluation by densitometry and morphology, we demonstrate here for the first time that inner eggshell membranes as interpositional material in dogs osteotomized tibia experiments acted as an active against bone bridging. The degradation of the inner eggshell membrane due to host reaction, appeared sufficiently effected of inner eggshell membranes on bone repair was well done and useful.

Key words: Egg, inner eggshell membranes, bone, tibia, densitometry, biomatherial

INTRODUCTION

Much effort has gone into making artificial bone grafts work better and more efficiently, especially in highly comminuted fractures or those with a large amount of bone loss where the quality of the graft is often the determining factor in success (healing) or failure (non-union) of bone regeneration. Non-union current solutions include transfer of vascularized bone, distraction osteogenesis and the use of cancellous autografts, especially when defects are ≥4-5 cm (De Boer and Wood, 1989).

Forelimb growth deformities are commonly a result of an irreversible injury to the growth plate of the physis, resulting in premature physeal closure. The distal radial and ulnar physes are responsible for most of the bone length with the distal radial physis determining 60% of radial length and the distal ulnar physis determining 85% of ulnar length (Fox *et al*., 2006). The distal ulnar physis is most commonly injured causing excessive radial procurvatum with external rotation and carpal valgus, potentially resulting in osteoarthritis in the carpal and elbow joints (Fox *et al*., 2006).

In addition, the egg is the largest biological cell originating from one cell division. It is an important source of nutrients, containing all of the proteins, lipids, vitamins, minerals and growth factors required by the developing embryo as well as defense factors against bacterial and viral infection. Eggs consist of about 9.5% eggshell (including shell membrane) 63% albumen and 27.5% yolk. The main components are water (75%) proteins (12%) lipids (12%) as well as carbohydrates and minerals (Leslie and Clem, 1969; Mine, 2002).

Eggshell membrane is primarily composed of fibrous proteins such as collagen type I (Wong *et al*., 1984). However, eggshell membranes have also been shown to contain glycosaminoglycans such as dermatan sulfate and chondroitin sulfate (Baker and Balch, 1962) and sulfated glycoproteins including hexosamines such as glucosamine (Picard *et al*., 1973). Other components identified in eggshell membranes are hyaluronic acid (Long *et al*., 2005) sialic acid (Nakano *et al*., 2003) desmosine and isodesmosine (Starcher and King, 1980) ovotransferrin, lysyl oxidase, lysozyme and β-N-acetylglucosaminidase (Hincke *et al*., 2000).

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Diverse biological activities have now been attributed to egg components including antimicrobial and antiviral activity, protease inhibitory action, vitamin-binding properties, anti-cancer activity and immunomodulatory activity (Li-Chan and Nakai, 1989).

A recent study demonstrated that a 21,000 Da protein present in soluble eggshell matrix proteins may play an important role in increasing calcium transport across intestinal epithelial cells *in vitro* (Daengprok *et al*., 2003). Eggshell membrane is composed of collagen-like proteins which are largely located in the inner membrane (Wong *et al*., 1984). Eggshell membrane-derived peptides, prepared by alkaline treatment were shown to stimulate human skin fibroblasts *in vitro* (Suguro *et al*., 2000). Eggshell membranes also contain antimicrobial substances with lysozyme (Vadehra *et al*., 1972) and b-N-acetylglucosaminidase (Winn and Ball Jr., 1975) activity reported.

One method of assessing bone density using radiography is light processor. White light passing through the radiograph and absorption them by absorption sensor can be used to determine bone density in different areas (Arias *et al*., 1997).

MATERIALS AND METHODS

Preparation of animal models: In this experimental study, 8 mature male 3-4 years old native dog breeds weighting 20-30 kg were selected. The animals were kept in a restricted access room under controlled temperature (22°C) and light/dark cycles (12/12 h) and with free access to food and water. All the animals were randomly divided into 2 groups. The research protocol was approved by the Research Ethics Committee of Veterinary Medicine Collegue, Shahrekord Branch, Islamic Azad University. All the experiments were carried out in accordance to the rules of Institutional Animal Care and Use Committee (IACUC). The study design and parameters for tissue reaction evaluation were in accordance with the ISO 10993-6 (1994) Standard.

Eggshell Membrane (ESM) collection and preparation: Eggshell membranes of white leghorn hen eggs were obtained by opening the eggshell at the opposite pole to the air chamber, then emptying the albumen and yolk and thoroughly washed with distilled water several times, then the membranes were manually extracted, rinsed and dried at 25°C for 24 h and cut in sheets of 1 cm^2. The sheets were then individually packed and sterilized with ethylene oxide for 24 h at room temperature.

Rabbit *in vivo* ostectomy and implantation of eggshell membrane: In order to assess the effect of eggshell membranes as a surgical implant to effect bone healing (ASTM F981-04, 2004), 10 adult male New Zealand White rabbits, 2-3 months old and 3-3.5 kg in weight, divided into five groups of two individuals were used. The animals were then anaesthetized via isoflurane inhalation and operated on under sterile conditions.

But in this study, the right tibia were approached through 1-1.5 cm incisions in a caudo-medial manner over the distal one-third of the tibia and the medial digital extensor muscle separated and expose the distal tibia. After elevating the surrounding musculature to isolate the tibia just proximal to the physis, a complete 8 mm wide osteotomy was performed using an oscillating saw. Then, a 1 cm^2 piece of ESM was fold in two and interposed into the osteotomy site. The fixation of the implant was accomplished by two simple discontinuous sutures (2-0 nylon) of the ESM against the periosteum of the cranial and caudal face of the proximal fragment. The opposite tibia was only osteotomized and up of this part for natural healing as a negative control.

Wound closure was accomplished by a simple discontinuous subcutaneous pattern (2-0 polyglactin 910) with a simple discontinuous skin pattern (2-0 nylon) and ketoprofen (1 mg kg^{-1} every 24 h for 3 days) was administrated as analgesic/anti-inflamatory treatment.

The animals were euthanized at 30, 60 days and X-rays were taken. Implanted and negative part of tibia were retrieved with the surrounding soft tissues and cut into two saggital halves. One half was fixed in a 10% formaldehyde solution, decalcified with 10% formic acid and embedded in paraffin. Serial sagittal studies (5 μm) were made through the implant and were stained with H&E and Mallory stains to be examined by light microscopy for histological evaluation of inflammation, fibrosis and osteogenesis in other study. The radiographs of this study send to evaluation denditometry for survey of bone healing from on the radiograph.

RESULTS

Morphology evaluation from radiographic results: Morphology evaluation from radiographic results in dogs tibia are between the 1st and 60th day, the control and implanted inner eggshell membrane shown in first radiograph that is befor surgery who tell us about healthy bone without any fracture in cortex and other site of tibia's bone (Fig. 1a and Table 1).

Group I (eggshell): In the 1st day of implantation there is not any reaction in the cortex and new bone formation. This phase we can see diffrent density measurement in the site diffects (Fig. 1b and Table 2).

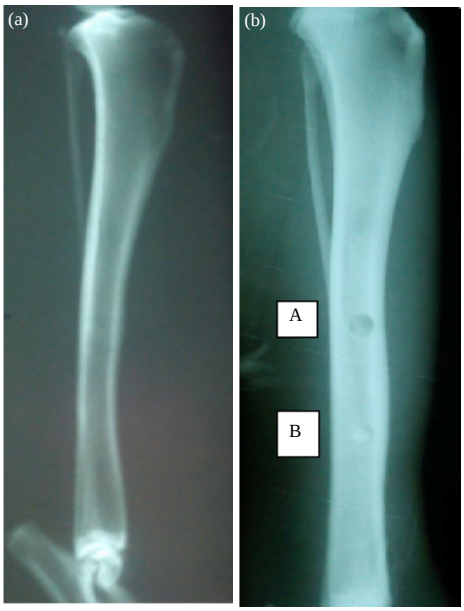

Fig. 1: a) This radiograph is before surgery and implantation of the biomatherial and b) tibia's radiograph after surgery and implantation. Site A: negative control; Site B: biomatherial site

Table 1: Measurement of bone density in site A (negative control) and site B (implantation diffect) by bone denditometry test. The unit of donsitometry is Cd/m^2

Control site (Cd/m^2)	Befor surgery density degree	After surgery density degree	1st month density degree	2nd month density degree
Group I				
1st dog	3.90	3.90	3.90	-
2nd dog	2.50	3.90	3.90	-
3rd dog	3.00	3.80	3.80	-
4th dog	3.11	3.85	3.85	-
Group II				
5th dog	3.10	3.90	3.90	3.70
6th dog	2.50	3.90	3.90	3.70
7th dog	3.00	3.80	3.80	3.60
4th dog	3.11	3.85	3.85	3.70

Table 2: Measurement of bone density in site B (implantation diffect) by bone denditometry test. The unit of donsitometry is Cd/m^2

Biomaterial site (Cd/m^2)	Befor surgery density degree	After surgery density degree	1st month density degree	2nd month density degree
Group I				
1st dog	2.10	3.00	1.40	-
2nd dog	1.90	3.10	1.50	-
3rd dog	1.80	2.90	1.50	-
4th dog	1.85	2.90	1.50	-
Group II				
5th dog	3.10	3.00	2.50	2
6th dog	1.90	3.00	2.40	2
7th dog	1.80	2.80	2.50	2
4th dog	1.85	2.85	2.40	2

Group I (Inner eggshell membrane): After 30 days, there was some new bone formation between in negative control and implantation diffect that shown make new bone and little repair near the control site and implantion site. This couse of reaction of osteoblasto cells that made bridge in the bone cortex actually in the implantaion diffect there is quite as more repair as negative control site (Fig. 2a).

Fig. 2: a) Create some new bone formation after 30 days and b) group II after 60 days

Goup II (Inner eggshell membrane): After 60 days this group have best repair as last group and may be almost as more repair as last group that was in 30th day in addition when we see to this group, we can fine that made remodeling around bone cortex and there is not any new bone formation. Also, complet the tibia's bone cortex and make calcification in the bone cortex (Fig. 2b).

DISCUSSION

In this study, chicken eggshell membrane was shown to be a good interpositional material to be used in dog tibia experimental osteotomy. Eggshell Membranes (ESM), like fat grafts, discouraged vascularization, thus repairing osteoblast infiltration and proliferation which are necessary characteristics for good osteoconduction. We do not know whether the eggshell membrane effect is due to the described mineralization repairing effect of the type collagen and other component that there are in inner eggshell membrane that they contain or whether the inflammatory reaction associated with the eggshell membrane material may also contribute to bone healing. In fact, we have previously shown that experimental culture

of osteoblasts on eggshell membrane does not mineralize it unless the terminal ends of type collagens are removed (Arias *et al*., 1997).

Eggshell membranes are primarily composed of fibrous proteins such as collagen type I (Wong *et al*., 1984). However, eggshell membranes have also been shown to contain Glycosaminoglycans (GAGs) such as dermatan sulfate and chondroitin sulfate (Baker and Balch, 1962), hexosamines such as glucosamine as well as hexoses and fucose (Picard *et al*., 1973). More recently, significant amounts of hyaluronic acid have been detected in eggshell membrane (Long *et al*., 2005). Other components identified in eggshell membrane include sialic acid (Nagai *et al*., 2003), desmosine and isodesmosine (Starcher and King, 1980), ovotransferrin (Gautron *et al*., 2001), lysyl oxidase (Akagawa *et al*., 1999) and lysozyme (Hincke *et al*., 2000). There are a kind of process between these components that help to repair of tibia bone in the cortex of it. Whatever, the mechanism was this study shows that instead of a partial tibia osteotomy and complete periostum removal, minimally invasive surgery with a small osteotomy and ESM interposition is a plausible way to accomplish non-union at least for 30 and 60 days in dogs tibia.

In addition, a secondary surgery site is not needed to obtain ESM as it is for harvesting fat grafts. Moreover, ESM can be obtained in large quantities and is inexpensive, sterilizable with ethylene oxide and easily stored after freeze-dry liophilization. It is also important to note that splint protection was not needed in order to keep the implant in place between the two bone ends.

CONCLUSION

Finally, there have been many attempts to develop bone atrophic or traumatic non-union animal models for studying bone healing (Kokubu *et al*., 2003). In this regard, the interposition of eggshell membranes between the two ends of a sectioned bone would be interesting not only for the treatment of premature physeal closure but also because it could be an experimental procedure for developing a non-union model in dogs. Aftet that the inner shell membranes could usefull for structuer of it and measurement of flexibility when in surgery use them.

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