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Evaluation of osteoinduction properties of the demineralized bovine foetal growth plate powder as a new xenogenic biomaterial in rat

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ABSTRACT

The aim of this study was evaluation of osteoinductive properties of demineralized bovine foetal growth plate in submuscular transplantation (ectopic osteoinduction) as a new xenogenic biomaterial in rat model. Demineralized bovine foetal growth plate was ectopically implanted in 18 male Sprague–Dawley rats. In 18 of the animals under aseptic conditions two submuscular pouches were created between external and internal oblique abdominal muscles in the two flanks: the right was left empty (sham) and the left was filled with 20 mg of demineralized bovine foetal growth plate powder. Radiographs were taken in 2, 4 and 6 weeks after the surgery, then six animals were pharmacologically euthanized after 2, 4 and 6 weeks for histopathological evaluation. Results showed: (1) osteoinductivity of xenogenic demineralized bovine foetal growth plate powder, and (2) earlier mineralization of ectopically implanted demineralized bovine foetal growth plate in the submuscular implanted area. Our results show that submuscular implantation of xenogenic demineralized bovine foetal growth plate has osteoinductive properties in a rat model.

1. Introduction

Demineralized bone matrix (DBM) has been used for several decades in human surgery for the treatment of nonunions, osteomyelitis, and large defects resulting from benign tumor removal (Jin, 1991). The process of demineralization with hydrochloric acid not only destroys and decreases antigenic stimulation, but also may enhance the release of bone morphogenic proteins (BMPs) (Riley et al., 1996). BMPs stimulate local undifferentiated mesenchymal cells to transform into osteoblasts (osteoinduction), and the collagenous framework of the DBM particles allows for migration of tissue into the site (osteoconduction). Extensive research continues to identify the different BMPs that might be osteoinductive, and these are being readied for clinical application (Reddi, 1995). Osteoinductive properties of allogenic and xenogenic demineralized bone matrix has been studied previously (Bigham et al., 2009).

TGF-β is an important and multifunctional autocrine regulator of bone formation (Noda and Camilliere, 1989). It has been demonstrated that TGF-β down regulates alkaline phosphatase, osteocalcin, osteopontin, collagen I and BMP-2 mRNA expression. This provides evidence that TGF-β1 acts as a powerful bone growth stimulant at the level of pre-osteoblasts (Harris et al., 1994). BMP-2 up-regulates the phenotypic expression of osteoblasts (Takuwa et al., 1991; Ohta et al., 1992; Hughes et al., 1995). BMPs 2, 4, 6, 7 and 9 increase osteocalcin expression and alkaline phosphatase expression in pre-osteoblasts, leading to mineralization (Phillips, 2005).

The presence of growth factor β (TGF-β) in growth plate (Rosier et al., 1998) and bone morphogenetic proteins 2 and 7 in human and rat foetal growth plate have been identified and reported previously (Anderson et al., 2000). These proteins promote the chondroblastic differentiation of mesenchymal cells followed by new bone synthesis by endochondral osteogenesis.

The growth plate has a discoid form and is surrounded by a perichondrial ring. Bordering the central part of the plate, limited by the bony epiphysis and metaphysis and adjacent to the perichondrium, the marginal region is a cartilaginous tissue, with no secondary ossification center at this level. Its reserve zone is contiguous with that of the central region. It is wider and has a greater number of cells in the proliferative zone; these cells are smaller compared to those in the central region (Miralles-Flores and Delgado-Baeza, 1990). The hypertrophic cells are similar in size and number and undergo osteogenesis similar to that of cells in the central part. In that respect, marginal regions represent pure

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chondroplasia. The function of this zone is not well specified but it is interesting that its expression of growth factors is higher than that observed at the central region, which corresponds to the faster growth in the marginal regions (Lazowski et al., 1994). Chondroblasts of the marginal region can differentiate into hypertrophic chondrocytes and then undergo apoptosis, or they may differentiate into osteoblasts and contribute to subperiosteal ossification (Hochberg, 2002). Bovine growth plate graft have been used previously as a xenogenic segmental bone graft has potential osteoinductive properties (Dehghani et al., 2008). There are some advantages for using bovine fetus growth plate as donor that includes availability of the calf fetus in the bovine slaughterhouses therefore preparation of the new osteoinductive biomaterial from the bovine fetus is more affordability in comparison to the preparation of the other biomaterials. The aim of this study was evaluation of osteoinductive properties of demineralized bovine foetal growth plate in submuscular transplantation (heterotopic osteoinduction).

2. Materials and methods

2.1. Animals

Animal selection and management, surgical protocol, and preparation followed the routines approved by the Institutional Animal Care and Use Committee that authorized by ethic committee of the University.

Eighteen male Sprague–Dawley rats (body weight 200–300 g, Razi Institute, Karaj, Iran) were used in this study. They were maintained in plastic cages in a room with a 12 h-day/night cycle and an ambient temperature of 21 °C, and were allowed ad libitum access to water and standard laboratory pellets (BK001P, B&K Universal Ltd., Grimstone Aldbrough, Hull, UK), consisting of 18% protein, 18% cellulose, 2% vitamin mix, 2% salt mix, and 60% maize starch.

2.2. Preparation of demineralized bovine foetal growth plate matrix

A 6-month old bovine fetus was collected from the local slaughter house. Metacarpal bones were dissected aseptically from foetal calf (Holstein Friesian) and all soft tissue was removed. Radiographs were taken to determine the growth plate's margins and limitations. With an oscillating osteotom, proximal and distal growth plates were cut and retrieved under aseptic conditions. The retrieved growth plates were cleaned from adjacent epiphyseal bone and primary Spongiosa and then were sliced. Demineralized materials were prepared as described by Reddi and Huggins (1972). The harvested growth plate cleaned of soft tissue and marrow, washed in sterile distilled water with continuous stirring, then washed three times in 95% ethanol for 15 min, rinsed in ether for 15 min, and finally air dried overnight. The cleaned and dried growth plate were then milled (Universal Mill A-20; Tekmer Co., Cincinnati, OH) to obtain 400–700 μm granules and then demineralized in 0.6 N HC1 three times for 1 h (50 ml HC1 per g of bone). The growth plate powder was rinsed with several changes of sterile distilled water to adjust the pH, three times in 95% ethanol and once in ether. The growth plate powder was air dried and stored in sterile plastic containers at 4 °C until being used for implantation. This entire process was performed under sterile conditions (except for the milling) and a sample was cultured to demonstrate that specimens contained no bacterial or fungal contamination.

All animals were anaesthetised by means of a subcutaneous injection of ketamine 100 mg/kg and xylazine 10 mg/kg (Flecknell, 2009) and the right and left flank areas were shaved and prepared aseptically with povidon iodine and draped with sterile drapes. Under aseptic conditions two submuscular pouches were created between external and internal oblique abdominal muscles in the two flanks: the right was left empty (sham) and the left was filled with 20 mg of demineralized growth plate powder.

Dorsal–ventral radiographs were taken in 2, 4 and 6 weeks using a step-wedge to calibrate radiodensity. The implanted area was radiographed using low energy X-ray (Faxitron, Hewlett Packard, Model 43855B, McMinnville, OR) with an exposure time of 30 s (15 kV). Radio-opacity of the implanted area was scored using range from 0 (minimally opaque) to 4 (most opaque) by an investigator blinded to treatment mode.

Every 2 weeks after the surgery (14th, 28th and 42nd postoperative days) six animals were pharmacologically euthanized (pentobarbital was injected intravenously 100 mg/kg) (Riviere and Papich, 2009) for histopathological evaluations. The implants were removed from each animal and paraffin wax was embedded following fixation in formalin and formic acid decalcification. The sections of 5–6 μm (Microrn HM 340 E microtome) were stained with Hematoxylin–Eosin (HE) and examined under a light microscope. All histopathological scores were evaluated using Emery's histopathological criteria (Emery et al., 1994) (Table 1).

The radiological and histopathological data were compared by Kruskal–Wallis, non-parametric ANOVA, when *P*-values were found to be less than 0.05, then pair wise group comparisons were performed by Mann–Whitney *U* test (SPSS 15.00).

3. Results

There was no intraoperative and postoperative death during the study. None of the rats sustained a wound infection or surgery complication. Radiographs showed significant differences ($P = 0.004$) between 14th and 42nd postoperative days. The radio-opacity of the implanted area showed gradually increasing at 14th, 28th and 42nd postoperative days (Table 2). An increased radio-opacity with discrete foci of densities and imparting a granular appearance to the images was observed after 42nd postoperative day (Fig. 1).

Staining of specimens with hemotoxylin and eosin revealed that submuscular implantation of the demineralized bovine foetal growth plate lead to fibrotic response with apparent bone formation without any inflammatory response. Histopathological evalu-

Table 1
Emery's histopathological bone formation criteria.

Score (points)	Tissue present
0	Empty
1	Fibrous tissue only
2	More fibrous tissue than fibrocartilage
3	More fibrocartilage than fibrous tissue
4	Fibrocartilage only
5	More fibrocartilage than bone
6	More bone than fibrocartilage
7	Bone only

Table 2
Radiological and histopathological findings for ectopic bone formation in rat over the various time intervals.

	Med (min–max)			P^a
	2nd week ($n = 6$)	4th week ($n = 6$)	6th week ($n = 6$)	
Radiological	1 (0–2)	2 (1–3)	3 (2–3)[b]	0.009
Histopathological	3 (2–4)	5 (3–6)[c]	6 (5–7)[d]	0.003

[a] Kruskal–Wallis non-parametric ANOVA.
[b] $P = 0.004$ (compared with 2nd week by Mann–Whitney *U* test).
[c] $P = 0.02$ (compared with 2nd week by Mann–Whitney *U* test).
[d] $P = 0.02$ (compared with 2nd week by Mann–Whitney *U* test).