

Full length Research Paper

Demineralized bone matrix gelatin as scaffold for tissue engineering

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The aim of the present study was to do a comparative light microscopical and histochemical analysis of bone regeneration processes in rabbits, with the use of demineralized bone matrix gelatin scaffolds. Our histological results showed that no gelatin scaffold was extruded, indicating that neither body fluid nor blood swept the gelatin scaffold from the implantation site, nor was the gelatin scaffold biodegraded. Additionally, no abscess or inflammation of the peripheral osseous tissues at the implantation site was observed, suggesting that the implantation of the gelatin scaffold in the vertebra bone defect did not cause histopathology or exhibit malbiocompatibility with the peripheral osseous tissues.

Key word: Demineralized bone matrix gelatin scaffolds, tissue engineering.

INTRODUCTION

Bone tissue engineering is an emerging interdisciplinary field that seeks to address the needs for bone reconstruction by applying the principles of biology and engineering to the development of viable substitutes that restore and maintain the function of human bone tissues. This form of therapy differs from standard drug therapy or permanent implants in that the engineered bone becomes integrated within the patient, affording a potentially permanent and specific cure of the disease state (Ohgushi and Caplan, 1999; Connolly et al., 1989).

The field of bone tissue engineering has been at the forefront of research and product development in the fields of stem cell biology, tissue engineering and regenerative medicine (Deckard, 1988). Osteoblasts are the building blocks of bone in appendicular, craniofacial and axial skeletons. The differentiation of osteoblasts from stem cells is one of the first cell lineages that were identified from mesenchymal, umbilical cord, blood and embryonic stem cells. In comparison with a number of other systems whereby the differentiation of stem cells or progenitor cells into tissue-forming cells is being

explored, osteogenic differentiation from several types of stem cells is a conventional practice in many laboratories (Berry et al., 1997; Ratanavaraporn et al., 2009; Ma et al., 2010).

The basic requirements for bone-tissue-engineering scaffolds are also valid for delivery systems of bone-regenerating factors, since they are basically both biomaterials (Yamamoto et al., 2000). Demineralized bone matrix (DBM) is human allograft bone that is processed in hydrochloric acid to remove its nonorganic materials, theoretically leaving its organic collagen matrix. Martin et al. (1999) and Urist et al. (1967) previously showed in an animal model that DBM does have osteo-inductive properties, although this remains controversial. The lack of nonorganic mineral in the preparation provides the theoretical advantage that the graft may mineralize directly without undergoing remodeling, as mineralized allograft might be in the process of creeping substitution (Frankenburg et al., 1998; Zhu et al., 2010; Madhumathi et al., 2009). Bone that lacks remodeling or remodels by creeping substitution, lacks structural strength, which is an important desired characteristic of a distal radius graft.

In this study, using our staining method, we investigated the surface morphology of the demineralized bone matrix gelatin scaffolds. In the report, we still demonstrated

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the biomechanical and immunity test of the fine structures of demineralized bone matrix gelatin graft in rabbits.

MATERIALS AND METHODS

Preparation of bone matrix gelatin (BMG) grafts

Demineralized bone matrix gelatin was prepared from segments of distal femur that had been harvested from freshly euthanized adult Japanese white rabbits. All soft tissues were removed and the bones were washed in sterile deionized water. The cleaned bones were extracted for 1.5 h in a 1:1 mixture of chloroform and methanol (30 ml/g of bone), and then subjected to the following steps: (1) Demineralization at 2°C with 0.6 M hydrochloric acid (60 mg/g) overnight; (2) Washed with sterile deionized water to pH 7.4; (3) Washed with 2 M CaCl₂ for 1 h at 2°C; (4) Washed with 0.5 M EDTA for 1 h; (5) Washed with 8 M LiCl for 1 h; (6) Washed with deionized water at 55°C. Between each step, the bone material was washed with sterile deionized water. The bone matrix gelatin was then incubated with Dulbecco's modified eagle's medium (DMEM) supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin for 1 h at 37°C. BMG was cut into 5 × 5 mm and stored at -70°C until used.

Histology

All the samples were processed for histological examination. The samples were first fixed in 10% formalin for 72 h and decalcified in 15% formic acid in 10% formalin for 2 – 6 weeks. Samples were then embedded in paraffin and 5 μm-thick tissue sections were obtained for H&E staining. In addition, the reconstructed intercostal tissue was also stained by H&E and the symmetrical costal tissue was examined as a normal control.

Scaffold characterizations

The surface morphology of the scaffolds was observed by SEM (Philips XL30 FEG). The scaffolds were coated with gold using a sputter coater (DeskII, Denton vacuum Inc.). During the process of gold coating, the gas pressure was kept at 50 mtorr and the current was 40 mA. The coating time was 200 s. Samples were analyzed at 10 kV.

The surface area was measured by N₂ adsorption experiments at liquid nitrogen temperature on a Belsorp- Mini (Bel Japan, Osaka, Japan), after evacuating samples at 25°C for 10 h (<7 × 10⁻³ Torr). Porosity was calculated as: $P = D_p/D_0$. Where D_p is the skeletal density of gelatin foam and D_0 is the density of gelatin. D_p was determined by: $D_p = 4m/d^2h$.

Swelling studies

The swelling studies were performed in PBS (pH 7.4) at 37°C. The dry weight of the scaffold was noted as W_i . Scaffolds were placed in PBS buffer solution (pH 7.4) at 37°C for 1 h and then removed. The surface adsorbed water was removed by filter paper and wet weight was recorded W_w . The ratio of swelling was determined using the Equation (1):

$$\text{Swelling ratio} = \frac{W_w - W_i}{W_i} \quad (1)$$

Swelling ratio was expressed as mean ± SD (n = 3).

In vitro degradation studies

The degradation of the scaffold was studied in PBS (pH 7.4) medium containing lysozyme at 37°C. Three scaffolds were immersed in lysozyme (10,000 U/ml) containing medium and incubated at 37°C for 7 days. Initial weight of the scaffold was noted as W_i . After 7 days, the scaffold was washed in deionised water to remove ions adsorbed on surface and were freeze dried. The dry weight was noted as W_t . The degradation of scaffold was calculated using Equation (2):

$$\text{Degradation (\%)} = \frac{W_i - W_t}{W_i} \times 100 \quad (2)$$

Degradation rate was recorded as mean ± SD (n = 3).

Mechanical test

The sixth and seventh ribs of all groups were harvested without cartilage at 24 weeks. The ribs of the left thoracic cages were deemed as normal intact controls. The length of the samples was uniformly processed as 7 cm, including the reconstructed part. Each sample was tested using a three bending point test. The parameters of the test were as follows: L (test span) = 70 mm, load rate = 0.5 N/mm, primary load = 1 N. The bending stress was calculated using the equation: $\sigma = 3PL/2bh^2$, where σ , P, L, b, and h represent the bending stress, the bending strength load, test span, the width and thickness of the specimen, respectively.

Immunity experiment

Eighteen healthy Japanese white rabbits of both sexes weighing 2.6 – 3.0 kg were used. The experiments followed the Principles of Laboratory Animal Care by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, and all protocols were approved by the Animal Care and Use Committee of our university. Rabbits were anesthetized with an intramuscular injection of 50 mg/kg ketamine hydrochloride and 5 mg/kg xylazine. The segmental defect model was as described in Liu et al. (2009). Briefly, unilateral 15 mm segmental bone defects were created in the middle of the radial diaphysis. All bone debris and interosseous membrane at the defect site was washed and wiped away. The defect was irrigated with sterile physiological saline solution and the gelatin scaffold was fitted into the defect. Muscles, fascia and skin were separately closed over the defect with use of 4-0 sutures. After operation, bone and tissues specimens were removed at 2, 4, 7, 14, 30 and 60 days, respectively. Specimens were placed in formalin, embedded in paraffin after decalcification, sectioned parallel to the diaphysis at a thickness of 4 μm and examined on Masson's trichrome and hematoxylin and eosin staining. Inflammation around scaffold was measured.

RESULTS AND DISCUSSION

Using an appropriate matrix, it may be possible to construct a single-unit that has the capability to repair both the chondral and subchondral aspects of the defect (Li et al., 2006). Theoretically, this could improve the initial

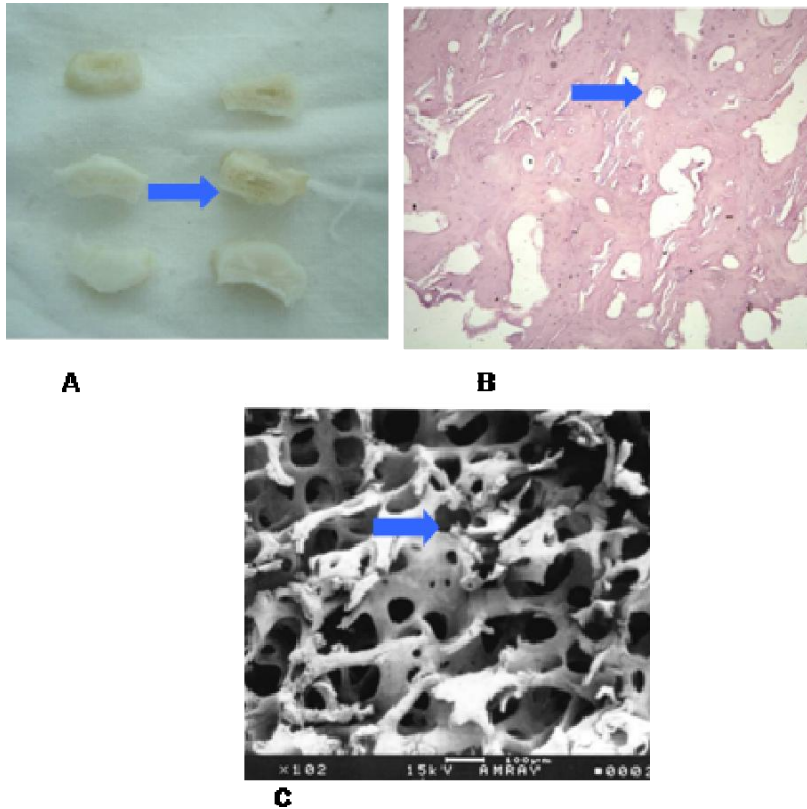


Figure 1. A, Observation of scaffold; B, H&E staining of scaffold ($\times 200$); C, SEM observation of scaffold ($\times 10^2$).

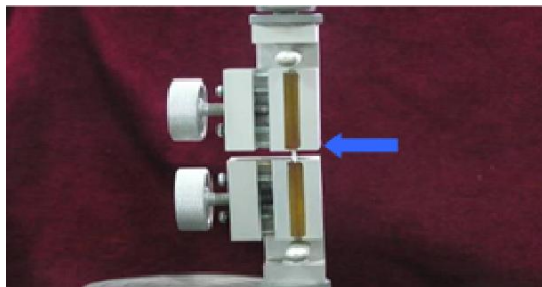


Figure 2. Biomechanics test of the scaffolds.

attachment and integration of the implanted construct into the joint.

In this study, the tissue samples treated with gelatin scaffold still exhibited a 3-D porous structure and a large amount of fibrin fibers attached around the original pores, and the pore size and porosity was reduced only a little as compared with the gelatin scaffold. Pore size did not differ between the two kinds of scaffolds (Figure 1). Schaefer et al. (2000) used separate biodegradable scaffolds to form bone and chondral tissue before combining the scaffolds into a composite. Integration between cartilage and bone was observed, but was

dependent upon the maturity of the neo-cartilage tissue. Frenkel et al. (1997) employed a two-layered collagen matrix consisting of a lower density collagen layer, as subchondral support and a porous upper matrix to support seeded chondrocytes. This combination showed promising results by the 24-week time point in a rabbit model.

Histological staining of the tissue-engineered constructs revealed chondrocytes located in lacunate, separated from each other by interterritorial extracellular matrix. Though the cartilaginous layer did not show normal stratification, different zonal-like layers (tangential layer, transitional layer and radial layer) were formed (Figure 1A, B and C). The prepared gelatin scaffold exhibited a homogeneously interconnected-3-D porous structure. The interconnected pores were detected in gelatin scaffold, with a pore size of $246 \pm 113 \mu\text{m}$ and mean porosity of $77.8 \pm 2.8\%$.

Swelling behavior and structural stability of scaffolds are critical for their practical use in tissue engineering. Most natural polymers, including chitosan, swell readily in biological fluids. *In vitro* culture studies (Li et al., 2005) indicated that initial swelling is desirable and the resultant increase in pore size facilitates cell attachment and growth in a three-dimensional fashion. However, continuous swelling would lead to loss of mechanical

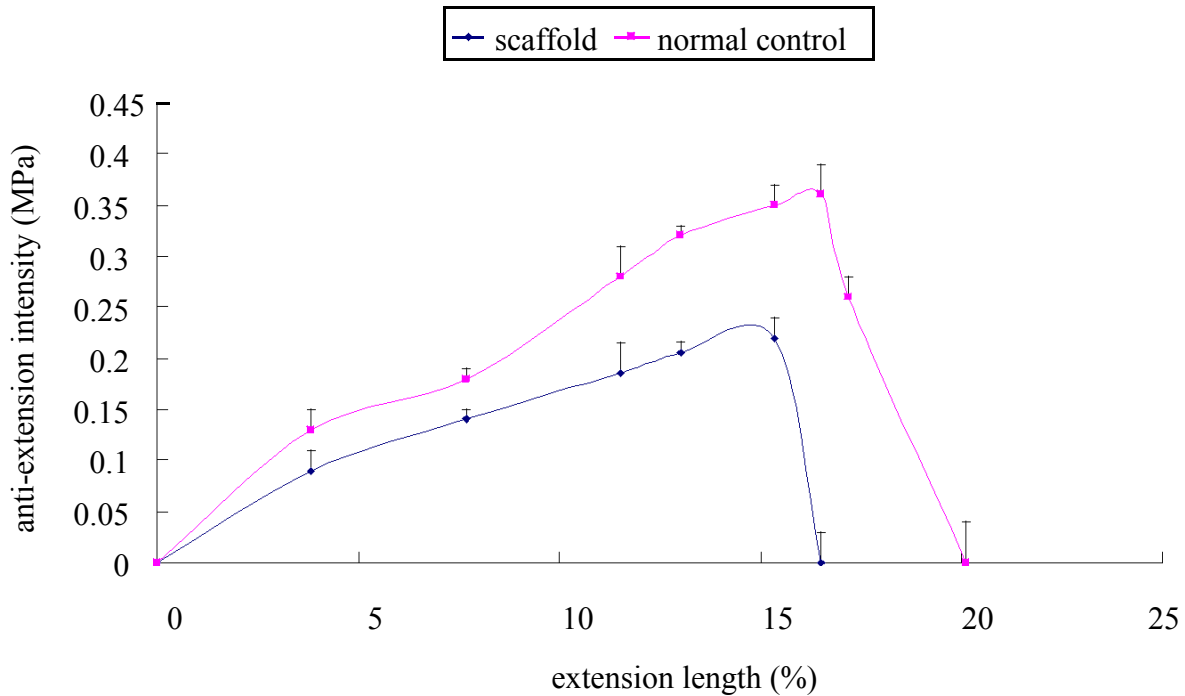


Figure 3. Anti-extension intensity of the scaffolds.

Table 1. Biomechanics test of the scaffolds ($\bar{x} \pm s$ n = 6).

	Area (mm ²)	Maximum load (N)	Anti-extension intensity (Mpa)	Elastic intensity (Mpa)	Extension length (%)
scaffolds	3.14±0.32	0.61±0.28*	0.24±0.09*	0.06±0.02*	17.2±2.3
Normal control	3.26±0.24	0.83±0.31	0.35±0.12	0.08±0.03	20.1±3.1

*0.01 < P < 0.05, compared with normal control.

integrity and production of compressive stress to surrounding tissue. Swelling behavior of a scaffold depends strongly on the pH value of the implantation site. The chitosan–alginate scaffold appeared to retain its overall size and cylindrical shape, while apparent swelling was noted on the chitosan scaffold. This flexibility makes the gelatin scaffold potentially applicable to a wider range of clinical situations. Swelling and degradation of pure chitosan involve the protonation of amino/imine groups and the mechanical relaxation of coiled chitosan chains (Bonar and Glimcher, 1970; Angermann and Jepsen, 1991; Begley et al., 1995; Sahithi et al., 2010).

The results of the biomechanics test (Figures 2 and 3; Table 1) showed a gradual increase in mechanical strength with increasing implantation periods. The elastic intensity value of the gelatin composites including new bone formation was 0.06 ± 0.02 MPa after 60 days of implantation. All animals survived for the entire duration of the experiment without any local or general compli-

cations. No wound infection, scalp effusion, distributed wound healing, hematoma or festers occurred at the surgical site of the bone. The surgical incisions that had been made on the rabbit rapidly healed. Gross observation of the whole vertebra bone at 60 days post-surgery revealed that the gelatin scaffold was intimately incorporated with the surrounding host bone (Figure 4A - K). No gelatin scaffold was extruded, indicating that neither body fluid nor blood swept the gelatin scaffold from the implantation site, nor was the gelatin scaffold biodegraded. This result showed that the gelatin scaffold not only was easily molded onto the vertebra bone defect without any fixation, but also adhered strongly to the peripheral osseous tissues after it had been implanted. Additionally, no abscess or inflammation of the peripheral osseous tissues at the implantation site was observed, suggesting that the implantation of the gelatin scaffold in the vertebra bone defect did not cause histopathology or exhibit malbiocompatibility with the peripheral osseous

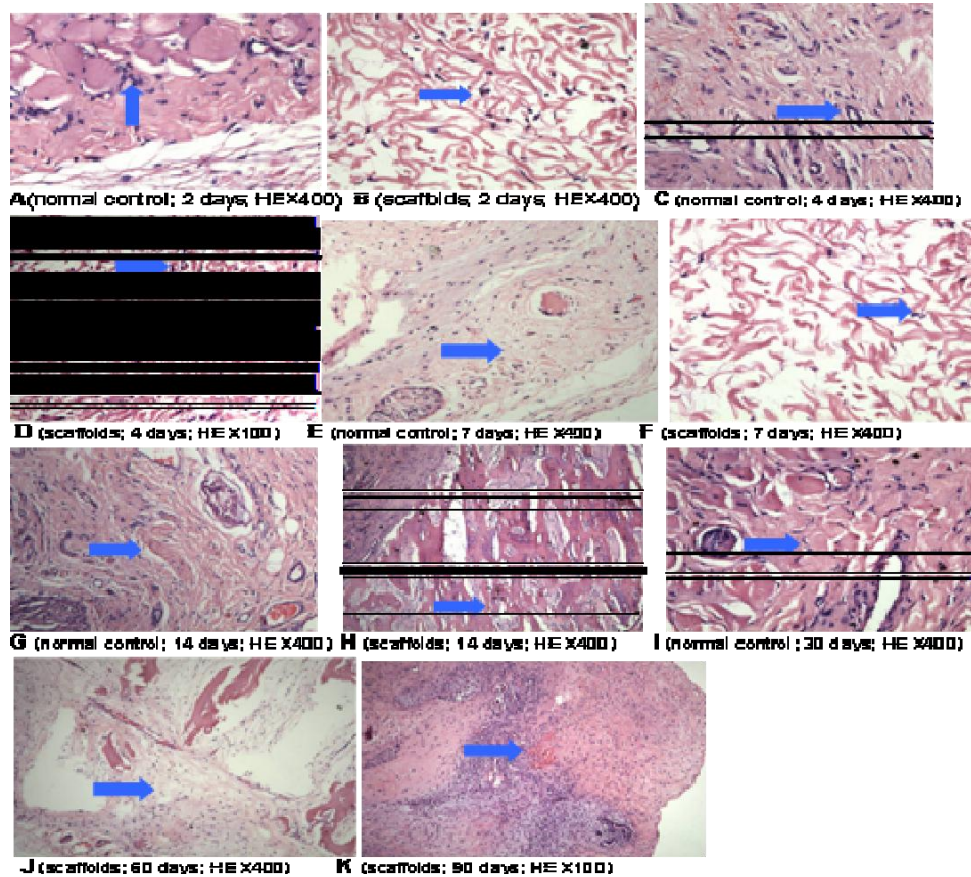


Figure 4. H&E stained section at varying time after implantation.

tissues.

Conclusion

For this study, microscopical and histochemical analysis of bone regeneration processes in rabbits was performed. Similar results concerning surface morphology, swelling ratio and degradation rate of demineralized bone matrix gelatin scaffolds were obtained. In conclusion, our data support the idea that no gelatin scaffold was extruded, indicating that neither body fluid nor blood swept the gelatin scaffold from the implantation site, nor was the gelatin scaffold biodegraded. Additionally, no abscess or inflammation of the peripheral osseous tissues at the implantation site was observed. Due to their more rapid growing rate, demineralized bone matrix gelatin scaffolds can be used to engineer tissue that meets individual needs for bone reconstruction.

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