무균돼지뼈를 이용한 복합 골지지체의 제조와 생체적합성 평가

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Preparation and Biocompatibility of Composite Bone Scaffolds Using Gnotobiotic Pig Bones

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Abstract

Highly porous composite bioceramic bone scaffolds were developed using sintered gnotobiotic pig bones. These scaffolds consisted of poly-D,L-lactic acid (P(D,L)LA) and bioceramic materials of pig bone powder. The bone scaffolds were able to promote biocompatibility and possess interconnected pores that would support cell adhesion and proliferation adequately. The composite scaffolds were tested with dental pulp stem cells for cytotoxicity test. Cells seeded on the composite scaffolds were readily attached, well proliferated, as confirmed by cytotoxicity test, and cell adhesion assessment. The composite bone scaffold had no toxicity in cytotoxicity test on the extract of 0.013 g scaffold to 2 ml culture medium. The cells on the composite bone scaffold proliferated better than cells on the P(D,L)LA scaffolds.

Keywords : Pig bone powder, Composite bone scaffold, P(D,L)LA, Biocompatibility.

1. INTRODUCTION

Bone grafts have been used to fill bone defects caused by disease or trauma, such as bone fractures, infections, and tumors (Boer, 1988; Vacanti et al., 1993). Autografts have the distinct advantage of histocompatibility without the risks of disease transfer and are still the best material for bone repair. However, their limited availability necessitates the development of alternative bone substitutes. Although allogenic bone grafts have better availability than autografts and avoid the need for a second surgical procedure to obtain an autograft, the use of allogenic bone grafts may transmit diseases and cause immune responses, which can lead to the graft failure (Bonfiglio et al., 1972). The synthetic artificial biomaterials have high possibility of infection, and their stability of long term is not known yet (You et al., 1998).

Another possible alternative for treatment of bone defects is the use of xenogenous bone, which is morphologically and structurally similar to human bone. Xenogenic bone is usually of animal bone and is easy to obtain in lower cost in unlimited supply. At the material level, animal bone is composed of organic and inorganic components. The organic part contains mainly collagen and proteins, whereas the inorganic component is mainly hydroxyapatite (HA) with a small percentage of other elements being incorporated in the structure such as carbonate, magnesium and sodium

(Krishna et al., 2002). Heat treatment has been suggested as an alternative to obtain protein-free bovine bone (Lin et al., 1999). The crystalline phase composition of sintered bovine bone is similar to natural bone mineral which is composed of $Ca_{10}(PO_4)_6(OH)_2$ (HA). As with HA obtained from bovine bone, hydroxyapatite derived from powder processing route has great potential for bone substitute owing to its excellent biocompatible and osteoconductive properties (Tancred et al., 1998; Martin et al., 2000; Tadic et al., 2004). Pig bones are used for foods, natural organic fertilizers, and animal feeds as materials of low grade. The pig bones used for foods cause environmental pollutant problems. These wasted pig bones can be used as bio-materials for fabricating bone scaffolds. Pig bones are sintered and grinded into micro powders for ceramic biomaterials. A study on the use of bioceramic biomaterials using pig bone powder is required. Recently, studies on the development of bone scaffolds using animal and fish bones such as cow, tuna, and cod, etc were reported (Lee et al., 1997). A novel scaffold is required for cell growth and tissue regeneration. The structure and properties of three-dimensional bone composite scaffolds are of critical importance for their application in tissue engineering. It has been generally accepted, that the scaffolds need to be biocompatible, high porosity and high interconnectivity to increase the specific surface area for cell attachment, tissue ingrowth, facilitating a uniform distribution of cells and adequate transport of nutrients and possess good mechanical properties to match those of the tissues at the site of implantation (Freed et al., 1994; Hutmacher, 2000). The synthetic polymers well known are poly (glycolide acid) (PGA), poly (lactide acid) (PLA) and poly (D,L-lactic-co-glycolic acid) (PLGA). However, PGA and PLGA are apt to be biodegradable easily and may cause infection reaction. They may have bad effects in bone reconstruction due to their acidulation on tissue. They also have a difficulty in culturing cells due to their hydrophobic property. Hence, composite polymer-ceramic scaffolds are needed as bioceramics have advantages of hydrophilic and bio-inert properties. The researches on the development of composite bioceramic bone scaffolds are being studied. A study on the development of bioceramic bone scaffold using toothapatite was reported

(Chung et al., 2004; Kim et al., 2004).

The aim of this research is to prepare the composite ceramic bone scaffold using gnotobiotic pig bones and to assess the biocompatibility of the bone scaffold.

2. MATERIALS AND METHODS

A. Preparation of bone powder

Bone powder was obtained from gnotobiotic pig bones, which were raised in the Seoul National University Hospital. The pig bone soaked in oxygenated water for 24 h to eliminate the organic components and substances in the surface of pig bones. It was then cut into rectangular samples of approximate size 20 mm x 20 mm x 20 mm. Then, the bones were sintered in an electric furnace (ST-01045, Daihan scientific, Korea) at 1100°C for 2 h to eliminate the organic compounds of the bones. During sintering the bones, organic substances were completely eliminated and crystallization of bone minerals occurred. The sintered pig bones were pulverized by a miller (A10, IKA-WERKE, Japan). Particle size of sintered bone powder was classified using sieves of 20-500 μm (Sieve/Shaker, Daihan scientific, Korea). Especially, the particle size of bone powder used in this study was 150-200 μm. Then, the sintered pig bones were sterilized in an autoclave.

B. Preparation of composite bone scaffolds

The process for porous scaffolds prepared by using a solvent casting and particulate leaching method is shown in Fig. 1 (Ishaug et al., 1997). Composite polymer solution was made by mixing P(D,L)LA (Mw = 116,000, SIGMA Chemical, Germany) and dimethyl sulfoxide (DMSO, MERCK, Germany). The polymer P(D,L)LA was put into DMSO in 15 wt%, and it was sealed and stirred for 12 h. Bone powder was added into the solution, where the mixing weight ratio of P(D,L)LA and bone powder was 1 : 1. Salt particles (sodium chloride, Mw = 58.44, Amresco, USA) used as a porogen were in the range of 300 to 400 μm. It was put into a mold by 90 wt% of the solution of P(D,L)LA and bone powder.

Then, the solution was poured into the mold with the salt. A teflon mold (thickness : 4 mm) filled with the solution
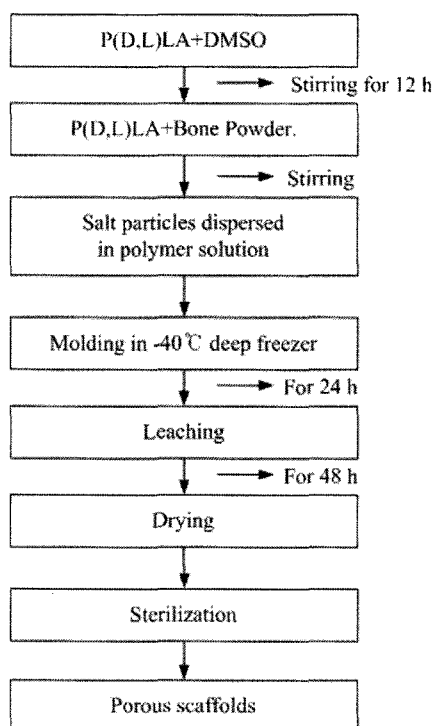
Fig. 1 Preparation of porous P(D,L)LA-bone powder scaffolds by using a solvent casting/particulate leaching method

was stored in a deep freezer of -40℃ to form a structure for 24 h. Refrigerated scaffolds were leached in distilled water bath at 37℃ for up to 48 h. The distilled water was replaced by approximately 6 h intervals. The leaching process is necessary to eliminate the foreign matters such as NaCl and DMSO which are harmful to cells. The NaCl and residual chemical such as DMSO were subsequently removed from the scaffolds by leaching the scaffolds in distilled water five times. The wetted scaffolds were stored in a desiccator (HSD-360, Hansung Industry Co., Korea) for 10 h under vacuum of 1 mmHg until use. It was sterilized by soaking into 70% ethyl alcohol, and washed by phosphate buffer saline (PBS) solution to eliminate the remaining ethyl alcohol. The bone scaffolds were exposed to ultraviolet for 24 h.

C. Property measurement of scaffolds

The microstructure of the scaffolds fabricated in this study was observed by the scanning electron microscope (JSM-5410LV, JEOL, Japan). All scaffolds were coated with gold using a sputter-coater (JFC-1100E, JEOL, Japan). The porosity of scaffolds prepared was examined by a

Mercury porosimetry (Poremaster 60, Quantachrome, USA). Compressive properties of the scaffolds were separately assessed on the cylinder-shaped P(D,L)LA scaffolds and P(D,L)LA-bone powder composite scaffolds (thickness of 4 mm and diameter of 10 mm) using a mechanical tester with a compression interface diameter of 5 mm (TA-XT2i, Stable Micro System Ltd. London, England). Compression tests were conducted at a crosshead speed of 2 mm/min using a 20 kg load cell. Young's modulus was calculated from the linear elastic portion of the stress-strain curve. At least four specimens were tested for each scaffold, and the average and the standard deviation were calculated. Additionally, water contact angle of fabricated scaffolds was tested using a contact angle analyzer (Phoenix 600, SEO, Korea) for the P(D,L)LA and P(D,L)LA-bone powder, respectively. A contact angle analyzer linked to a computer was used to measure contact angles in a wet. Distilled water was used as a wetting medium.

D. Cytotoxicity assessment of composite bone scaffolds

Bone scaffolds prepared in this study may cause a bad effect to human body due to the toxic matter extracted from the scaffold to culture mediums and extracellular matrix. Therefore, as a former study of in-vivo test, biocompatibility of the scaffold should be investigated through the cytotoxicity assessment. First, it was assumed that when the amount of 0.013 g scaffold was inserted to a body, the amount of toxic matter extracted from the scaffold was a toxic quantity of 100%. A control group was set to be a scaffold made by only P(D,L)LA, and treatment groups were set to be composite scaffolds of P(D,L)LA and bone powder. Extract concentrations in each group were 0, 20, 40, 60, 80, and 100%. Each treatment was repeated 10 times. The extraction was performed in the culture condition of 37℃, 5% CO_2 concentration and 95% humidity. A cytotoxicity test was performed by a MTT test. Cells used in experiment were dental pulp stem cells, and seeding cell number was 15,000 unit per 1 well. The optical density (OD) was measured at 540 nm wave length using a ELISA reader (VERSAmax reader, Molecular device Co., USA). The optical density value was used in terms of IC_{50} in this study. IC_{50} was

defined as 'optical density for 50% concentration inhibition' in this study. The IC_{50} value was as same as the optical density when the total cell number was reduced to 50%. This definition is expressed as followings:

$$OD \text{ for } IC_{50} = \frac{OD \text{ for } 0\% \text{ Extract}}{2} \tag{1}$$

Where, IC_{50} : optical density (OD) for 50% concentration inhibition

E. Cell adhesion assessment of composite bone scaffolds

Most of animal cells are adhesion type. Scaffolds which cells would be cultivated must have a hydrophilic property. Cell adhesion assessment was performed in two ways. First, morphology of scaffolds and cell adhesion images were observed by a scanning electron microscope. Cells which proliferated and adhered to scaffolds were cultivated for two weeks before the observation. Secondly, adhesion ratio of cells was measured to investigate how many cells were adhered to a scaffold. The adhesion ratio was measured by a XTT test, and test results were obtained from the optical density measurement.

F. Statistical analysis

The statistical analysis (SAS User's Guide, 1990) was carried out using the SAS Statistical Analysis System for Windows v8.2 (SAS Institute, Inc., Cary, NC, USA). Statistical significance between control and treatment groups was analyzed using t-test.

3. RESULTS AND DISCUSSION

A. Preparation and characterization of composite bone scaffolds

Composite bone scaffolds were prepared using pig bone powder and P(D,L)LA as shown in Fig. 2. The composite bone scaffolds exhibited highly porous and interconnected structures. The exterior surface and cross-section morpholo-gies of the scaffolds exhibited a highly porous structure with interconnection that would support cell adhesion and
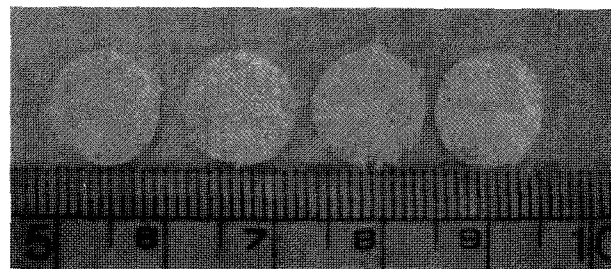

Fig. 2   A macroscopic image of composite bioceramic scaffolds prepared with pig bone powder and P(D,L)LA using a solvent casting and NaCl leaching method.

proliferation, adequately.

Fig. 3 shows the SEM micrographs of the pores in the cross-section surface morphology of a P(D,L)LA and bone powder composite scaffold, while Fig. 4 shows the SEM micrographs of the pores of a P(D,L)LA scaffold. It was found that the pore size range of scaffolds was 200-400 μm, and the interconnection of the scaffold pores was highly
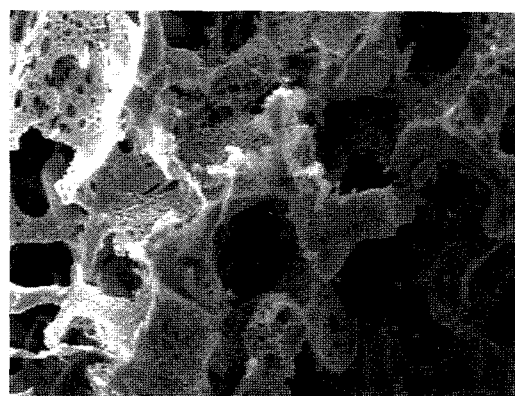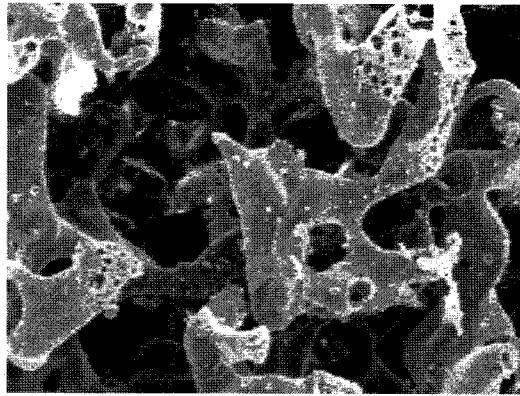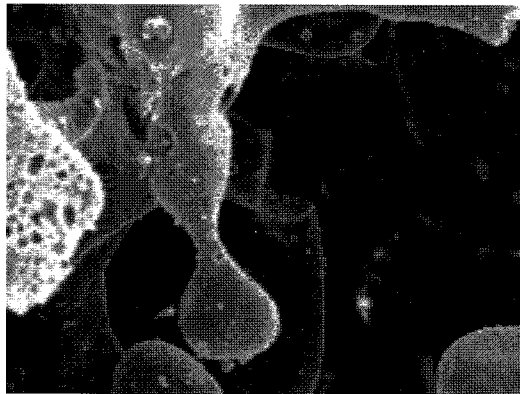

(a)
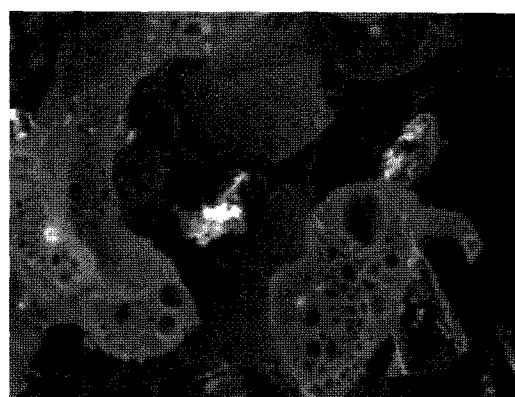
(b)

Fig. 3   Scanning electron micrographs (SEM) of the pores in the cross-section surface morphology of the P(D,L)LA and bone powder composite scaffolds. (a) SEM x 50 image and (b) SEM x 100 image.

(a)

(b)

Fig. 4 Scanning electron micrographs (SEM) of the pores in the cross-section surface morphology of the P(D,L)LA scaffolds. (a) SEM x 50 image and (b) SEM x 100 image.

good. The porosity of the scaffolds was in the range of about 80-85%. Also, it showed the polymer frame of the scaffold contained the bioceramics of pig bone powders.
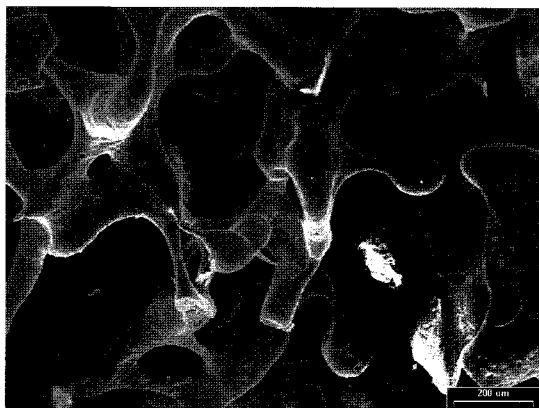
Fig. 5 shows the surface morphology of a P(D,L)LA-

Fig. 5 Scanning electron micrograph of the pores on the surface morphology of the P(D,L)LA and bone powder composite scaffolds, SEM x 100 image.
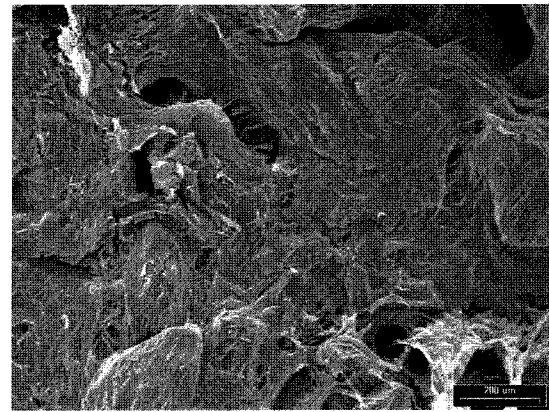

Fig. 6 Scanning electron micrograph of dental pulp stem cells after culturing on the composite bone scaffold for 2 weeks, SEM x 100 image.

bone powder scaffolds, while Fig. 6 shows a SEM micrograph of dental pulp stem cell after culturing of two weeks on the P(D,L)LA-bone powder scaffolds. When cells were seeded on the scaffolds for two weeks, cells were well cultivated on the scaffolds. It was performed to see how many cells were adhered to the scaffolds.

The P(D,L)LA-bone powder composite scaffolds exhibited enhanced mechanical properties as compared to the P(D,L)LA scaffolds. The compressive Young's moduli of the specimens were 0.12 ± 0.03 MPa and 0.23 ± 0.02 MPa for the P(D,L)LA-bone powder and P(D,L)LA scaffolds, respectively. This difference was found to be statistically significant with a p-value of 0.0028. These meant about 100% increase in the compressive strengths, demonstrating the positive effects of the P(D,L)LA-bone powder fabrication process in enhancing the mechanical properties of the scaffolds. Similar observations have been made for other scaffold materials reported by Hou et al. (2003).

The wetness behaviors of the P(D,L)LA-bone powder scaffolds and the P(D,L)LA scaffolds were assessed by a contact angle analyzer. The water contact angles were $71 \pm 3°C$ and $103 \pm 4°C$ for the P(D,L)LA-bone powder and P(D,L)LA scaffolds, respectively. The water contact angles were significantly different (p = 0.0034). The P(D,L)LA-bone powder scaffolds were found to be significantly more hydrophilic than the P(D,L)LA scaffolds.

B. Cytotoxicity assessment

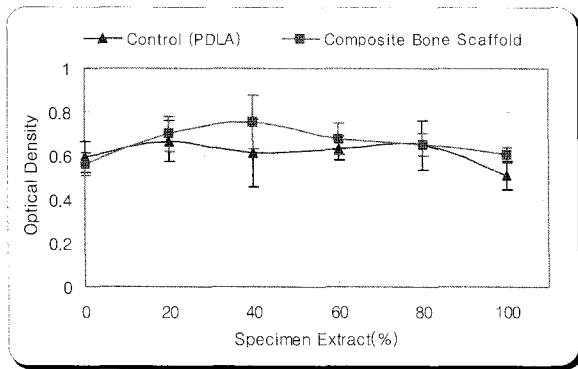Fig. 7 shows a result of MTT cytotoxicity tests for the

Fig. 7 Result of MTT cytotoxicity test for the extracts of a P(D,L)LA scaffold as a control group and a composite scaffold.

extracts of a P(D,L)LA scaffold as a control group and a composite scaffold as a treatment group. From this result the toxicity of samples in 2 ml of culture medium could be estimated in case that 0.013 g of a scaffold was inserted in human body. The optical densities at the 0% extract concentration of the control group and the treatment groups were 0.56 ± 0.11 and 0.59 ± 0.07, respectively. So, the average value of two results, 0.58, was an optical density at the 0% extract concentration. And IC_{50} was calculated with this value. Any values in the graph did not reach to this value, 0.29, as IC_{50}. Also, the optical densities at the 100% extract concentration were similar to those at the 0% extract concentration in the two scaffolds. Therefore, it was concluded that the bone scaffold had no toxicity relatively. It was observed that the optical densities of the composite ceramic bone scaffold as the a treatment group were more higher than those of the scaffold made by only P(D,L)LA as the control group even if the difference was a little. It meant that cell number on the composite bone scaffold was higher than that on the P(D,L)LA scaffolds.

C. Cell adhesion assessment

Fig. 8 shows the results of a XTT test. A cell adhesion test was conducted on a culture dish as a control group. The composite bone scaffold had high adhesion ratio of 77-90%, compared with the control. From the cytotoxicity test and cell adhesion test results, it was concluded that the P(D,L)LA-bone powder scaffold had very good biocompatibility. The optical densities on the culture dish and on
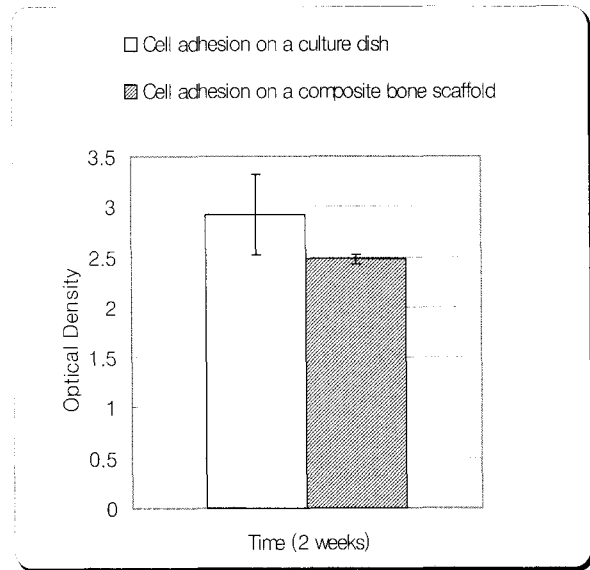

Fig. 8 Result of cell adhesion measured by XTT test for 14 days.

the composite bone scaffolds were 2.92 ± 0.39 and 2.47 ± 0.05, respectively. This difference was found to be statistically significant with a p-value equal to 0.001 (Fig. 8).

4. CONCLUSIONS

Composite bioceramic bone scaffolds were prepared using the gnotobiotic pig bone powder and the P(D,L)LA. The composite bone scaffolds were hydrophilic and had porous structures with interconnection throughout the entire scaffold, which resulted in good cell adhesion and proliferation. The P(D,L)LA-bone powder scaffolds had higher compressive strength than the P(D,L)LA scaffolds. The composite ceramic bone scaffolds had no toxicity in a cytotoxicity test on the extract of 0.013 g scaffold to 2 ml culture medium. The cells on the composite bone scaffolds proliferated better than those on the P(D,L)LA scaffold. The P(D,L)LA-bone powder scaffolds showed up very high adhesive property to cells in a cell adhesion test. It was found out that the composite P(D,L)LA-bone powder scaffold prepared in this study had very good biocompatibility from the cytotoxicity and cell adhesion tests.

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