Decellularization of Xenogenic Bone Grafts for Potential Use as Tissue Engineering Scaffolds

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***Abstract*-Xenogenic bone grafts have been studied as an alternative to autogenic and allogenic bone grafts in order to satisfy the high demand for orthopedic reconstruction. The aim of the present study was to develop an effective method for xenogenic bone decellularization and cells reseeding forwards bone regeneration applications. Decellularization of porcine cancellous bone blocks with either acetone or chloroform/methanol results in completely acellular bone scaffolds. Umbilical cord blood-derived cells were seeded on the acellular cancellous bone by centrifugal force 2000 rpm in 1 minute and repeated 4 times. After 7 days of in vitro culture, the cells adherence and proliferation within the scaffolds were confirmed by MTT and SEM. *In vivo* transplantation of acellular bone scaffolds also demonstrated that they did not cause immune response. The achieved results demonstrated that the acellular bone scaffolds are capable of supporting cell adherence and proliferation. Furthermore, *in vivo* study shows the potential use of acellular bone scaffolds as bone grafts in orthopedic surgery.**

Key words- Xenogenic Bone Graft; Decellularization; Scaffold; Bone Regeneration; Cancellous Bone; Umbilical Cord Blood-Derived Cells; Cell-Seeding

I. INTRODUCTION

Bone grafting is a replacement of bone loss or defect in order to repair the bone fractures or improve the bone function. In orthopedic surgery, bone grafting is a common technique. The bone graft substitute market is largely driven by the high demand for orthopedic reconstruction. According to the result of the Japanese Orthopedic Association Committee investigation on tissue grafting status from 1985 to 2004, there are a total of 163 564 tissue grafting, including 134 782 (82.4%) of bone grafting [1]. About 500 000 - 600 000 bone grafting were performed in United States, and ¾ of the bone grafts were allogenous [2, 3]. Lost bone can be replaced by autologous bone grafts, which is widely considered to be the golden standard for transplantation, however, obtainable quantities, additional surgery procedure and postsurgical pain at the donor site are still unresolved issues. Allograft bone shares the osteoconductive properties of the autograft, therefore, it is often used as a substitute for autograft. Allografts also carry some disadvantages such as expensive cost, immune response precipitation, disease transmission and limited availability [4]. Xenogenous bone grafts are alternative to autologous and allogenous bone grafts. The most remarkable advantage of xenograft is that there is a large quantity of bone grafts available which are usually produced from bovine or porcine origin. Xenogenous cancellous bone morphology and composition are similar to human cancellous bone, additionally, it has osteoconductivity ability [5]. However, the major disadvantage of xenogenous bone grafts is immune response which is also stronger than that to allografts. The cellular components from these xenografts are the main cause to human immune response and transplant rejection. Therefore, in order to use animal-derived tissues as substitute materials, decellularization is the first and most important issue that needs more attention. In the recent two decades, many methods have been studied for decellularization of tissues or organs in order to eliminate the antigenicity of the grafts, but still preserve the graft morphology and structure to support the society demand on orthopedic grafting. With these ideas, this study was conducted to develop a method for fabrication of ABSs, additionally, evaluate their biocompatibility including cytotoxicity, *in vitro* and *in vivo* compatibility.

II. MATERIALS AND METHODS

A. Decellularization of Porcine Bone

Fresh porcine spine bones were harvested from a local slaughter house and transported to the lab in Phosphate buffer saline (PBS) containing Penicillin (400 U/ml) and Streptomycin (400 µg/ml) at 4˚C. The decellularization process of the bones resulted in the acellular bone scaffolds (ABSs). Briefly, the samples were cleaned of adherent tissues and fat, trimmed creating 3mm x 4mm x 4mm bone blocks, and rinsed in Tris-NaCl solution for 6 hours. The demineralization process of the samples was performed using 0,6M HCl for 15 minutes. The demineralized bone blocks were decellularized in either acetone for 17 hours or chloroform/methanol solution for 6 hours, then rinsed in distilled water for 12 hours at room temperature. ABSs were chemically sterilized in absolute ethanol for 24 hours, then transferred into ethanol 80%, 70%, 20% solution within 24h for each step. The residual ethanol was eliminated by washing with sterile PBS for 24h.

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Decellularization efficiency was investigated by histology H&E (Hematoxyline and Eosin) staining and scanning electron microscopy (SEM). In addition, the porosity and pore size distribution of ABSs were determined by SEM analysis.

B. Cell Isolation and Culture

Mononuclear cells (MNCs) were obtained from human umbilical cord blood according to our protocol. Briefly, umbilical cord blood was collected from placentas of healthy full-term neonates. MNCs were separated from the whole cord blood by Ficoll–Hypaque (Sigma) density gradient centrifugation. The cells were then washed twice with PBS to remove the residual Ficoll. MNCs were cultured in DMEM/F12 medium supplemented with 10% FBS, Penicillin (100 U/ml) and Streptomycin (100 µg/ml) in 37^{o}C, 5% CO_2. Culture medium was changed every two days. After 4 passages, MNCs were harvested and seeded in the cancellous bone scaffolds.

C. Cell-Seeding in ABSs

Before cell seeding process, ABSs were then incubated in culture medium for 24 hours. Eppendorfs containing 1 ml of $2x10^5$ cells and one bone block were prepared. Cell seeding was performed by centrifugation method (2000 rpm, 4^{o}C for 1 minute). This process was carried out 0, 2, 4, 6, 8 or 10 times. The amount of cells successfully delivered into the porous scaffolds was calculated as the difference between the initial number of cells and the number of cells remaining in each eppendorf after centrifugation. Consequently, the essential centrifugation repeating was determined.

D. Cell Proliferation inside Seeded Scaffolds

Proliferation of MNCs inside ABSs was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tet-razolium bromide (MTT) assays at days 3, 6, 9 and 12. Additionally, MNC adhesion and its shape were observed by SEM and H&E staining.

E. In Vivo Biocompatibility Testing

18 five-week old mature white mice *Mus musculus* var. *Albino* with body weight of about 22 grams were used in this experiment. The mice were separated into 2 groups: the control group (8 mice) and experimental group (8 mice). In the control group, a skin incision was made and then sewn up in the mouse's back. In the experimental group, the mice were transplanted subcutaneously with ABSs. After 2, 5 and 8 days transplantation, the mice blood was collected for cytokine TNFα, IFNγ, IL-5, IL-4, IL-2 evaluation by BD CBA T_{h1}/T_{h2} Cytokine kit (BD Sciences). Mice in the experimental group underwent a surgery to take out the scaffolds for H&E staining.

III. RESULTS AND DISCUSSIONS

A. Decellularization of Porcine Bone

Before undergoing the treatment process, the bone blocks were red due to containing many kinds of cells including blood cells such as erythrocytes, leukocytes and bone cells such as osteoblasts, osteocytes, osteoclasts. After decelluarization process, cellular components were eliminated, the blocks became white which is supposed to be the natural color of bone extracellular matrix (ECM). Furthermore, H&E staining demonstrated that the bone blocks were successfully decellularized, there was the absence of red blood cell fragments and bone cells. H&E staining of bone specimen without being decellularized presented the key cellular components consisted of osteoblasts, osteocytes, the hematopoietic elements and adipocytes (Fig. 1). After being washed with Tris-NaCl solution for 6 hours, cancellous specimens are white, which proved that almost blood cells were cleaned. In comparation of decellularization efficiency between acetone and chloroform/methanol, acetone solution is more effective than chloroform/methanol solution. In some cases, chloroform/ methanol treated grafts have about 20-30 percent cells left, while acetone treated grafts achieved 100 percent cell elimination (data not shown).

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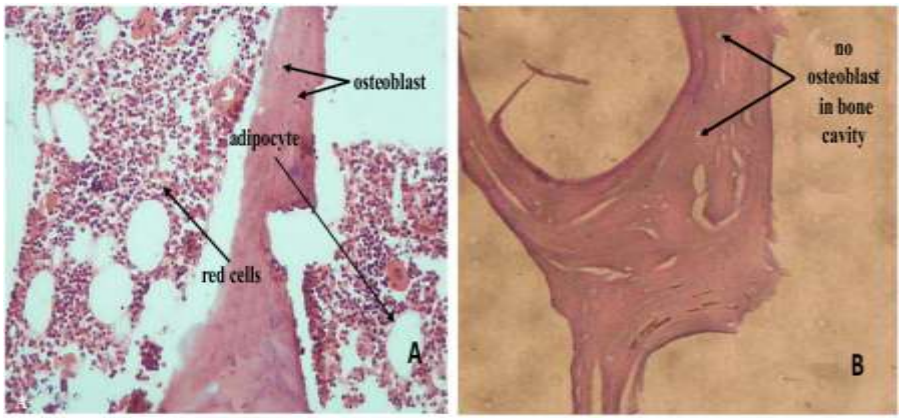

Fig. 1 The H&E staining results of cancellous bone before (A) and after (B) decellularization by using aceton

The pictures taken by SEM showed that ABSs is a porous structure consisting of an interconnected network of the large pores. Pore size ranges from 250 μm to 1000 μm (1 mm) in diameter, which indicated that ABSs are high porosity (Fig. 2). Pore with large size as partially demineralized cancellous would support vascular ingrowth assisting a convenient condition for cell infiltration.

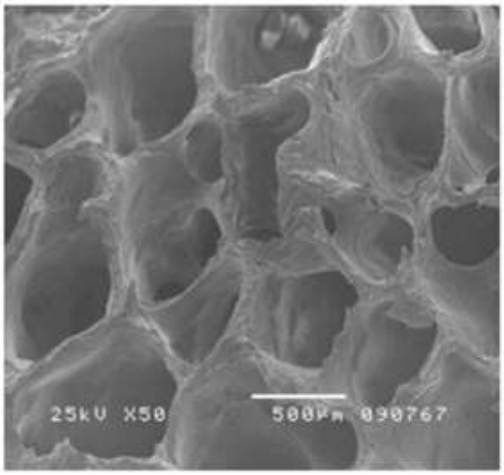

Fig. 2 The SEM result for identifying the spongy structure of the decellularized bone. The pore sizes were about 250-1000 μm. Distance between 2 pores is 50-100 μm

B. MNCs Culturing and Seeding on the Cancellous Bone by Centrifugation Force

MNCs derived from human umbilical cord blood were cultured in DMEM/F12 containing 10% FBS. After 2 hours, MNCs attached to substrate, and 7 days later, MNCs reached 80% flask surface (Fig. 3).

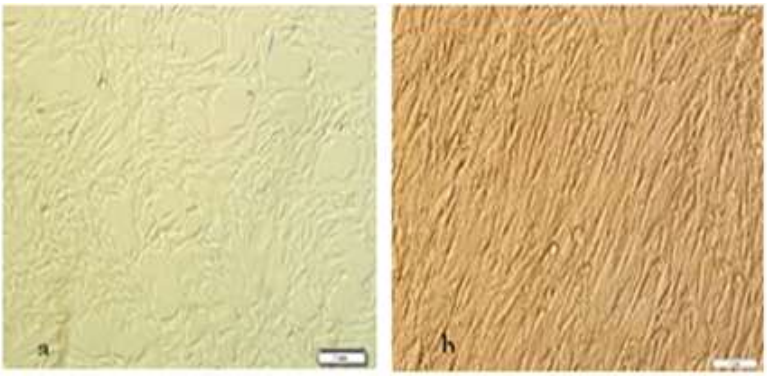

Fig. 3 MNCs after 7 days (a) and 10 days (b) culturing

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Cell penetration into the porous bone scaffold was assisted by centrifuge force. After centrifuging, the amount of the cells residing within the scaffold was determined by the difference of viable cells in control groups (without bone scaffold) and experimental groups (with bone scaffold) at one certain point time (0, 2, 4, 6, 8, 10 minutes) (Fig. 4). At 4 and 6 minutes, the difference is higher than other times. The number of cells seeded into scaffold at the times of 4, 6 minutes aren't statistically different. Thus, we compared the death rate of the cells between 4 and 6 minutes. It was found that, at 4 times, death rate is lower. After seeding into scaffold 7 days, MNCs attached and spread on scaffold surface.

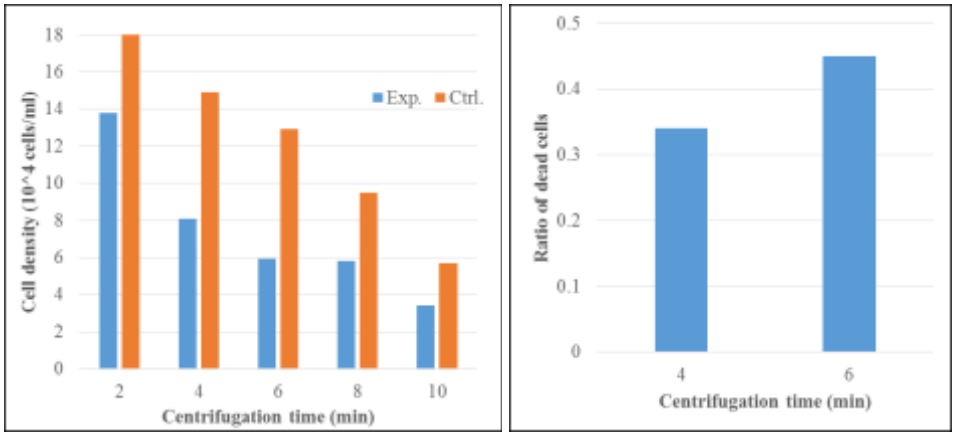

Fig. 4 The quantity of viable cells after centrifugation (A) and the ratio of dead cell after centrifuging for 4 and 6 minutes (B)

C. In Vitro Biocompatibility

Although centrifuge force was applied to the porous scaffold to assist the penetration of cells, there were still a small amount of cells leaking out of scaffold and proliferating on the culture surface. It is found that an increase in cell number occurs on the seventh day, which proved that the cancellous bone scaffold has no toxic effect on cell culture. Besides, cell distribution through the cancellous bone scaffold was detected by H&E staining and SEM at day 7 and 14 days after cell seeding (Figs. 5 and 6). Therefore, it is demonstrated that the bone blocks after being treated with chemicals for demineralization and decellularization still have ability to support cell attachment and proliferation.

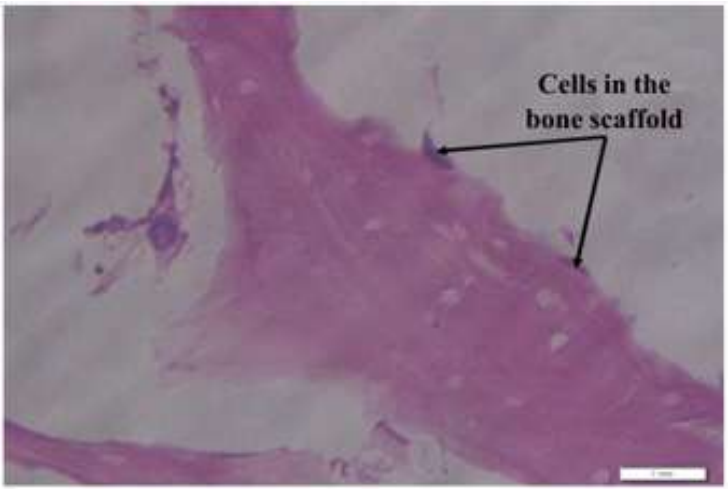

Fig. 5 The H&E staining result of cell-seeded scaffold (400X)

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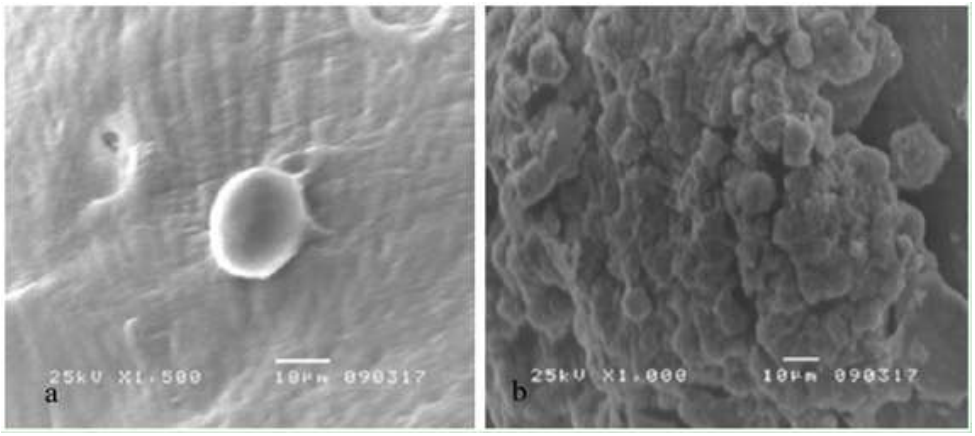

Fig. 6 MNCs attachment on the scaffold surface were imaged by SEM after 2 week culturing ((a): one cell, (b): cell cluster)

MTT assay reflected the activity of a mitochondrial dehydrogenase which is a component of living cell, this enzyme transforms yellow MTT into dark blue formazan that is determined photometrically. Therefore, this assay is able to assess cell proliferation in the scaffolds. In day 0, the cells were seeded on the scaffold at a density of 20,000 cells/ml by centrifuging at 2000 rpm for 4 minutes.

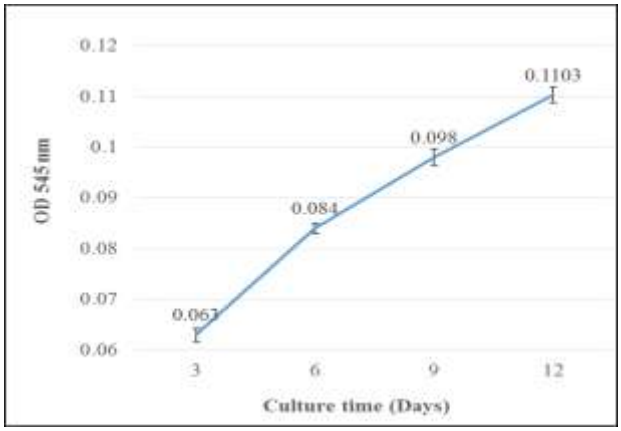

Fig. 7 Representative data indicating the proliferation rate performed by MTT assay. The Y axis showed Optical density (OD) of the formazan product which reflects increasing cell numbers

The graph in Fig. 7 presented the result of an MTT assay for cell proliferation within the cancellous bone scaffold after periods of 3, 6, 9, and 12 days. The cell number increased sharply 1.31-fold from day 3 to day 6, then, the increasing speed slowed down 1.19-fold on day 9 and 1.12-fold on day 12 (the radio increasing was achieved by compared the observed day with the day before it). There was a significantly higher OD on day 12 compared with day 3, which was 1.72-fold (statistical significance). This result showed that after 12 days seeding on the bone scaffold, the cells attached and proliferated on the scaffold. Additionally, in a study of Guangpeng L. on human bone marrow stromal cells proliferation on allogenous cancellous bone [5], cell numbers increased 1.9-fold after 14 day culturing, which was similar with the experimental result of this study.

D. In Vivo Biocompatibility

The cancellous bone scaffolds were transplanted subcutaneously as an in vivo biocompatibility study model. After day 2, all the mice in both control and experimental groups were still alive and began healing at the surgeon' side. Especially for the experimental group, the mice lived well and had no over inflammation symptom due to immune intolerance or graft rejection. As a result, it was demonstrated that the bone scaffolds were completely eliminated cellular components and antigenicity. On day 2, 5 and 8, the mice in experimental groups underwent a surgery to take out the bone scaffolds. On day 2, the scaffolds were normal and undamaged, fibroblast attachment around the scaffolds was observed, however, there was no migration of fibroblasts or lymphocytes into the scaffold. Calcification, a small number of fibroblasts and lymphocytes were detected in the matrix on day 5, evidence of blood vessels was also found. On day 8, formation of vascular network within the porous scaffolds was clearly detected, calcification was increased as much as on day 5.

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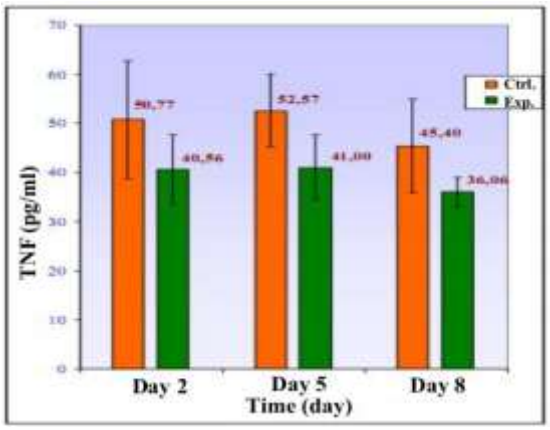

Fig. 8. TNFα level in control and experimental group after cancellous bone transplantation

Furthermore, five types of cytokine including TNFα, IFNγ, IL-5, IL-4, IL-2 were investigated in this experiment by isolating serum of mice in the experimental group (Exp.) and control group (Ctrl.) with BD CBA Mouse T_{h1}/T_{h2} Cytokine kit (BD Sciences) (Table 1). If the inflammation occurs, the cytokine concentration will increase. IL-5 and IL-4 are produced by T_{h2} cells (T helper-2 cells) due to chronic inflammation response. In the early days of transplantation, the level of these two cytokine is not high, which totally matched the experimental result. In contrast, TNFα, IFNγ and IL-2 are secreted by T_{h1} cells (T helper-1 cells) and responsible for pernicious inflammation.

Overall, there was no significant difference of cytokine level between the control group and experimental group. Cytokine level was about 10-55 pg/ml in the control group and 15-50 pg/ml in the experimental group. This result showed that the cancellous bone has no negative effect to the secretion of cytokine in mice immune system.

TABLE 1 CYTOKINE CONCENTRATION (PG/ML) IN SERUM OF MICE AFTER TRANSPLANTATION (EXP.) IN COMPARISON WITH THE CONTROL GROUP (CTRL.). LIMIT OF DETECTION IS 10 PG/ML. ND-NOT DETECTED

Cytokine concentration (ρg/ml)	Day 2		Day 5		Day 8	
	Ctrl.	Exp.	Ctrl.	Exp.	Ctrl.	Exp.
TNFα	50.8	0.56	52.6	41	146.1	36.1
IFNϒ	46.7	51.3	55.8	252	15.07	15
IL5	ND	25.2	1.11	46.2	16.69	24
IL4	ND	ND	ND	ND	ND	ND
IL2	13.4	ND	11.8	16.1	ND	ND

In order to determine inflammation caused by the bone scaffolds, TNFα level in the control group and experimental group was compared. If the TNFα level in experimental group was higher (statistical significance), inflammation caused by the bone scaffolds was determined.

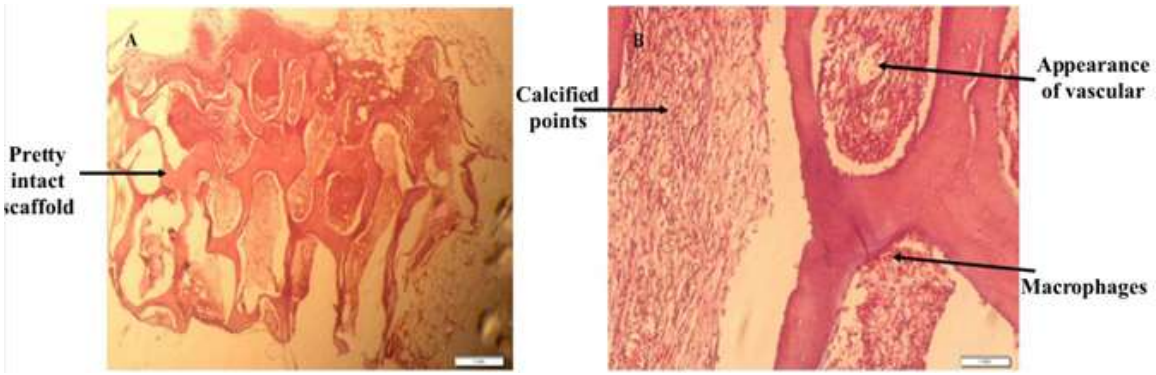

Fig. 9 The HE staining results of scaffolds after 8 days grafting, A (40X), B (100X). Macrophages and lymphocytes continued to migrate into the scaffolds and new vascular network were found

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There was no difference of TNFα level between control and experimental group (no statistical significance) after 2 days of cancellous bone transplantation, which proved that the scaffolds did not caused immune response, then the TNFα level was equivalent to TNFα level in the control group. On day 5, cytokine concentration in the experimental group slightly increased, but still did not make any difference compared with the control group. In both control and experimental groups, TNFα level on day 5 was higher than TNFα level on day 2, which was the evidence of inflammatory immune. When comparing with H&E staining result on day 5, TNFα result was acceptable due to the appearance of blood vessels and macrophages and lymphocyte migration within the scaffolds. Consequently, the inflammation response was caused by the mice body, not by the cancellous bone. On day 8, there was a decreasing inflammation response reflected by the decline of TNFα level.

IV. DISCUSSIONS

A method for preparing decellularized scaffolds is supposed to be successful when it meets these following standards: completely eliminating cellular components, highly preserving the extracellular matrix and the natural tissue ultrastructure (e.g. pore size distributions), supporting cell attachment, proliferation and migration into the scaffold [6]. In this study, porcine bone was physically cut into small blocks, then decellularized by sequentially mechanical shaking with use of acetone solution, which achieved both a complete decellularization and preservation of the mechanical function and tissue structure of the porcine bone blocks. Besides the cellular components, there is a high level of adipocytes in cancellous bone tissue, which can prevent the chemical sterilization into the scaffold due to protective barrier formation. Therefore, for the consideration of sterilization, and storage for transplantation, adipocytes elimination is extremely necessary. Lipid or other component of cell originated form lipid can be dissolved in non-polar solvents such as acetone, chloroform, etc. Chloroform in combination with methanol has been used during delipidation procedure [6], however, the residual chloroform remained in the scaffolds after treatment causes toxicity, which should be concerned when in use. Sharing the same effect with chloroform, acetone also gives a good result in delipidation, in addition, acetone evaporates naturally at normal condition, hence, it does not leave any redundant in the scaffolds. HE staining result of treating by chloroform/methanol and acetone confirmed that acetone showed more effectiveness in decellularization and delipidation. In the cooperation with other decellularization methods including HHP 980 Mpa, 30^{o}C in 10 minutes [7] and lyophilization [8-10], acetone treating in combination with mechanical shaking shows the advantage of simple manipulation, in addition, the success in complete decellularization and preservation of ECM structure of the bone scaffolds.

The spongy bone scaffolds still remain the ability of promoting cell attachment and proliferation, which is an important property of the scaffolds for clinical application. In fact, the property of cell tolerance depends on the existence of the special proteins on the surface of the scaffolds after treated. The partial mineralization process results in the exposure of proteins, particularly the bone morphogenetic proteins (BMPs) and other bioactive molecules [11-13], because these kinds of protein are required for cell attachment and bone healing procedure. When transplanted into a defected area, BMPs are secreted from the bone scaffold matrix, which leads to the induction of mesenchymal derived cell (such as monocytes and fibroblasts) migration, proliferation and differentiation. These cells will secrete collagen matrix which is then mineralized into bone component and replaced the scaffolds [14]. The partial mineralization was carried out before the decellularization, which can badly affect the exposed proteins. However, the result of cell attachment and proliferation in vitro as well as in vivo proved that the bioactive proteins of the bone scaffold are preserved during the treatment. In vitro condition, MSCs were able to grow and develop within the acellular bone scaffolds, which was shown in HE staining and SEM (Figs. 5 and 6). The potential of using xenogenic acellular bone scaffolds in clinical application is provided by the evidence of immune system acceptance when being in vivo transplanted on mice. In the comparison with the control group, when being transplanted with the acellular bone scaffolds, mice in the experiment group had the same was inflammation – this is a protective attempt by the body to remove the injurious stimuli and to initiate the healing process. The inflammation response did not last long which indicated that the transplanted acellular bone scaffolds were not rejected by the recipient's immune system. From that perspective, the acellular bone scaffolds were completely eliminated all the cellular antigens by effective procedure of decellularization. Moreover, HE staining of the scaffolds after 5-day transplantation in vivo showed the migration of lymphocytes, macrophage migration inside the scaffolds. Especially, the presence of vascular structure in the scaffolds (Fig. 9) conformed the regeneration capacity of the bone scaffolds, because vascular formation means that there is certainly oxygen and nutrients distribution deep inside the scaffolds, therefore, the bone healing and reconstruction will be accelerated.

V. CONCLUSIONS

Xenogenous bone from various sources and large quantity can be used as biomaterials in the orthopedic reconstruction field after decellularization process. The concept of decellularization is referred as the extraction of cellular components from the tissue. This study described the successful decellularization of bone by chloroform/methanol followed by acetone treatment in combination with mechanical shaking. HE staining and SEM observation confirmed the complete removal of cells in bone while the ECM integrity is preserved. The acellular bone scaffolds after process do not present cytotoxicity, which is confirmed by the

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significant adhesion and expansion of cells reseeded on the scaffolds. *In vivo* transplantation did not show severe inflammation or graft rejection, thus, the acellular bone scaffolds obtained from the described method would potentially used as an alternative in bone grafting.

CONFLICT OF INTEREST

The authors declare that there is no personal or financial conflict of interests in the current research.

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