PREPARATION OF ACELLULARIZED BONE USING ULTRA HIGH PRESSURE TECHNOLOGY FOR TISSUE ENGINEERING

Yoshihide Hashimoto[1], Tsuyoshi Kimura[1], Kwangwoo Nam[1], Toshiya Fujisato[2], Akio Kishida [1]
[1]Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Tokyo, Japan
[2]Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, Osaka, Japan

Introduction

One of key factors affecting the effective application of tissue engineering is the development of scaffolds, which provide the physical support for adhesion and expansion of cells that can regenerate the lost of diseased tissue. Recently, several scaffolds consisting of synthetic materials or substance derived from natural tissue, such as poly(lactic acid) (PLA) (1), poly(glycolic acid) (PGA) (2), hyaluronic acid (3) and collagen (4), have been mainly studied. They have been able to form various structures, such as porous, fibrous and gel to be adapted to the objective tissue. It was reported that the shape and microscopic structure of them act an important role in tissue formation as well as the physical and physicochemical nature of scaffold (5). However, it is difficult to actualize the same shape and structure as the biological tissue.

As another approach for preparing resemble natural scaffold, there are decellularized tissues in which the cells and antigen molecules are removed to reduce the host immune response. The acellular tissue should be considered to have the same structure and composition as the natural tissue and the regeneration within the scaffold is expected to be modulated by newly cells. The ionic and non-ionic detergents, such as sodium dodecyl sulfate (6), sodium cholate (7) and Triton® X-100 (8), were utilized extensively to remove the donor cells and its components. Ionic detergents are able to solubilize effectively cellular membranes, but tend to denature proteins by protein-protein interactions, so the tissue structures are also disordered. On the other hand, non-ionic detergents disrupt lipid-lipid and lipid-protein interactions but not protein-protein interactions, so the structure in decellularized tissue by non-ionic detergents treatment was relatively maintained. It was also reported that the remaining of the detergents and the residual cellular components in the scaffold and the denaturing of tissue structure were the important problems.

We have also reported the novel methodology of tissue decellularization using ultra-high pressure (UHP) technology without detergent (9). This decellularization method involves two processes. As first step, cells, bacteria and viruses in tissue are physically disrupted by ultra high hydrostatical pressurization (10,000 atmospheres (atm) for 10 min). Subsequently, the residues of components in disrupted cells were removed by washing process (Figure 1). The decellularization of hear valve and vessel was completely achieved by UHP treatment.

In the present study, we have demonstrated the preparation of decellularized bone using UHP technology for tissue engineered bone. Porcine bones (femur and costa) containing bone marrow, which has complex micro-structure, were decellularized using UHP treatment. The decellularization and alternation of micro-structure in bone during UHP processing were investigated by histological study and SEM observation. The adhesion and expansion of cells reseeded on the acellular bone culture was examined in vitro. Also, the biocompatibility test of the acellular bone was carried out in vivo.

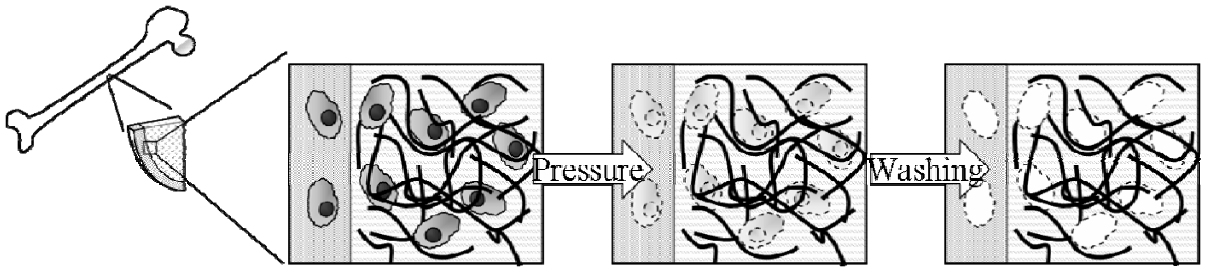

Figure 1. Illustration of the decellularization of bone by UHP treatment

Experimental Part

Materials

Porcine bones (femur and costa) were purchased from Tokyo Shibaura Zoki Co. Ltd. (Tokyo, Japan). Phosphate buffer saline (PBS) and medium were obtained from Invitrogen Co. (CA, USA). DNase I was purchased from Roche Co. (Germany). Other chemicals were purchased from Wako (Tokyo, Japan).

Decellularization of bone

The femur was cut cylindrically (10 x 3 mm). The costa was shaped cubically (4 x 4 x 4 mm). They were stored in phosphate buffer saline (PBS) containing penicillin (100 U/ml) and streptomycin (100 μg/ml) at 4 ˚C for 24 hours. They were transferred to sterile plastic package filled with PBS and then pressed hydrostatically at 10,000 atm, 10 or 25 ˚C for 10 min using high pressure machine (Kobe steel Co. Ltd., Kobe, Japan) to disrupt the cells in them. The pressuring rates were 2000 and 666 atm/min at 10 and 25 ˚C, respectively for 10 min. The pressurized bones were immersed in medium containing DNase I (0.25 mg/ml) and then were shaken at 37 ˚C for 2 weeks to remove the residues of the disrupted cells in bones.

Histological evaluation

The acellular bones were fixed in 10 % natural buffered formalin at room temperature for overnight. They were demineralized using EDTA and then dehydrated stepwisely using 50, 60, 70, 80, 90, 99, 100 % ethanol. They were immersed in xylene and then embedded in paraffin. Paraffin sections were cut at 4μm and deparaffinized using xylene. The tissue sections were stained with Hematoxylin and Eosin (H-E) and

Adhesion and proliferation of cells reseeded on acellular bone

Rat bone marrow (rBM) cells were collected from the femur of rats and then cultured in α-MEM (20 % FBS). Acellular bones were placed in 24-well plates. The rBM cells (1×105) were seeded statically on the acellular bone and incubated at 37 ˚C in humidified 5% CO2 in air atmosphere for 1, 3, 5 days. They were fixed in 2.5% glutaraldehyde at room temperature for overnight. They were dried as described above and coated with gold for scanning electron microscopy (SEM) observation.

Biocompatibility of acellular bone

Wister rat (male, 250g body weight, 7-week-old) was used. Native and decellularized bones including bone marrow were implanted subcutaneously in rat. After 2 and 4 weeks implantation, they were explanted and subjected to histological staining (H-E staining).

Results

Figure 2 shows the relationship between pressure and temperature during pressurization. The optimal pressurization condition, in which a tissue was not boiled and frozen, was found in the case of pressurization at the pressurizing rate of 666 atm/min. Figure 3 shows the H-E staining of the decellularized bone and bone marrow of femur by UHP treatment. The removal of cells in both of them was completely achieved by UHP treatment. The porous structure of bone and the fibrous collagen structure along with lipid droplets in bone marrow were well maintained. Also, the decellularized costa was prepared by UHP treatment. The spread cells adhered on the bone were observed by SEM observation for the non-treated costa, whereas they were not observed by SEM observation for the decellularized costa by UHP treatment as well as femur. These results indicate that the UHP treatment is effective method for the decellularization of bone.

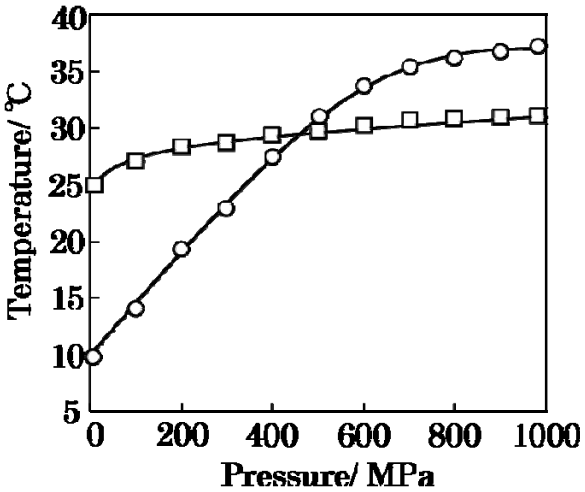

Figure 2. Pressure-temperature profile during UHP treatment of 10,000 atm at the pressurizing rate of (○) 2,000 and (□) 666 atm/min.

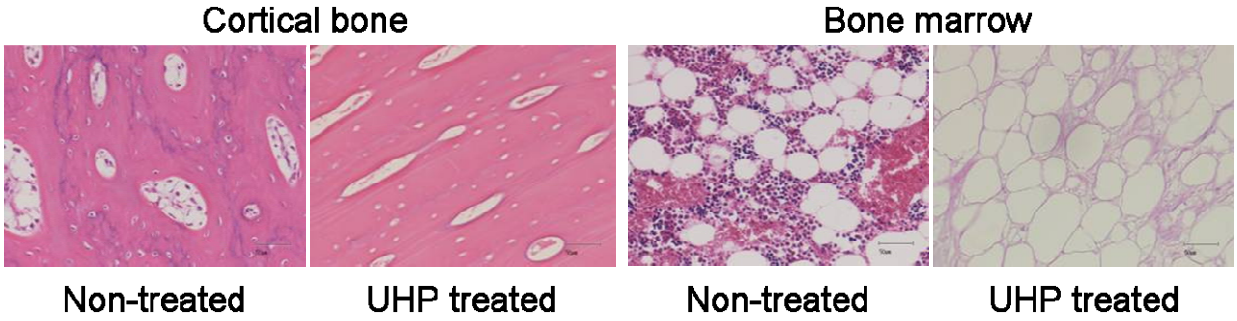

Figure 3. H-E stainings of cortical bone and bone marrow decellularized by UHP treatment

The MC3T3 cells (1 x 10^5 cells) were reseeded on the decellularized bone in vitro. After cultivation for 3 days, SEM observation revealed that the reseeded cells were adhered on the surface of the decellularized bone in the outside and the inside of bone (Figure 4). The amount of DNA extracted from the cultured cells on the acellular bone increased with prolongation of cultivation time (Table 1). These results suggest the effective cellular adhesion and expansion on the decellularized bone by pressurization.

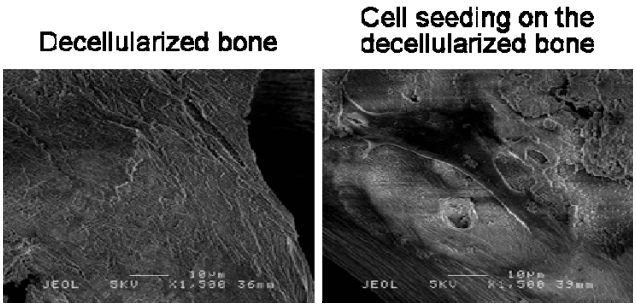

Figure 4. SEM photographs of the cell adhesion on the decellularized bone.

Table 1. Amount of DNA extracted from the cells reseeded on the acellular bone.

Culture time (day)	Amount of DNA (μg)
1	0.62
3	2.13
5	3.09

The decellularized femur was implanted subcutaneously in rats to examine the immune reactivity of them. After 2 and 4 weeks implantation, they were explanted and subjected to histological study (H-E staining). In the case of native bone after 2 weeks, the strong inflammatory response was observed by light microscopic observation. After 4 weeks implantation, the gradual collapse and the fibrous encapsulation of bone marrow was observed. On the other hand, the decrease of inflammatory cells and very thin fibrous encapsulation were observed around the decellularized femur. Also, the re-construction of tissue by infiltration of cells to decellularized bone marrow was slightly observed after 4 weeks, suggesting the capability of the decellularized bone as bio-scaffold.

Discussion

This report described the successful decellularization of bone using ultra high pressure technology. H-E staining and SEM observation confirmed the complete removal of cells in bone treated by UHP processing. The significant adhesion and expansion of cells reseeded on the acellular bone was also confirmed without the cytotoxicity. It is expected to utilize the decellularized bone as culture matrices because the detergents, which are generally cytotoxic remaining in tissue scaffold, were not used at all in this procedure. The inhibition of immune reaction was exhibited for the decellularized bone by UHP treatment, so the decellularized tissue would be useful as bone regeneration.

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