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Quality assessment for processed and sterilized bone using Raman spectroscopy

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Abstract To eliminate the potential for infection, many tissue banks routinely process and terminally sterilize allografts prior to transplantation. A number of techniques, including the use of scanning electron microscopy, bone graft models, and mechanical property tests, are used to evaluate the properties of allograft bone. However, as these methods are time consuming and often destroy the bone sample, the quality assessment of allograft bones are not routinely performed after processing and sterilization procedures. Raman spectroscopy is a non-destructive, rapid analysis technique that requires only small sample volumes and has recently been used to evaluate the mineral content, mineral crystallinity, acid phosphate and carbonate contents, and collagen maturity in human and animal bones. Here, to establish a quality assessment method of allograft bones using Raman spectroscopy, the effect of several common sterilization and preservation procedures on rat femoral bones were investigated. We found that freeze–thawing had no detectable effects on the composition of bone minerals or matrix, although heat treatment and gamma irradiation resulted in altered Raman spectra. Our findings suggest Raman spectroscopy may facilitate the quality control of allograft bone after processing and sterilization procedures.

Keywords Bone allograft · Raman spectroscopy · Sterilization · Quality assessment

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Introduction

Bone grafts represent one of the earliest devised reconstructive approaches for the musculoskeletal system and remain among the most common orthopaedic procedures, being used for the repair of fractures, arthrodeses, and cystic defects, and skeletal deficits after traumatic loss or tumor resection.

To eliminate potential infection during allografting, many tissue banks routinely process and terminally sterilize allografts prior to transplantation. A number of techniques have evaluated the various

properties of preserved and sterilized allograft bone, such as the use of scanning electron microscopy (SEM) for collagen structure (Voggenreiter et al. 1994), bone graft models for osteoinduction and bone absorption (Kühne et al. 1992), and compression or bending tests for mechanical properties (Anderson et al. 1992; Currey et al. 1997; Hamer et al. 1996; Nguyen et al. 2007). Although, these methods provide important information about the condition of allograft bone, they require lengthy assay periods or destruction of the bone; thus, bone tissues after processing and sterilization procedures are not evaluated prior to grafting. To permit the assessment of bone graft tissue, the development of more rapid and non-destructive evaluation methods has been needed.

Raman spectroscopy is a non-destructive, rapid analysis technique that requires only small sample volumes and is nearly resistant to interference from water. The spectroscopy technique has found wide application in biomedical fields and has recently been used to evaluate the mineral content, mineral crystallinity, acid phosphate and carbonate contents, and collagen maturity in human and animal bones (Akkus et al. 2004; Carden and Morris 2000; Freeman et al. 2001). As the frequency assignments for all major Raman spectral peaks of bone minera have been established, we speculated that Raman spectroscopy would be useful for the quality control of allograft bones after processing and sterilization procedures.

Here, to establish a quality assessment method of allograft bones using Raman spectroscopy, the effects of several common processing and sterilization procedures on rat femoral bones were investigated.

Materials and methods

Bone samples

The experimental protocol was approved by the animal care committee of Kitasato University School of Medicine. Femora were harvested from 8-week-old male Wistar rats (Charles River Japan, Inc., Yokohama, Japan), which were maintained at the animal facility of Kitasato University and fed rodent Diet CE-2 (CLEA Japan, Inc.), containing 1.18 g calcium and 1.03 g phosphorus/100 g of feed. Bones collected from 36 rats were divided equally into the following 6 treatment groups: (1) Fresh, in which bones were untreated; (2) FT1, in which bones were frozen using an ultra-low temperature freezer at −80°C (MDFU581; Sanyo Electric Co., Ltd., Osaka, Japan) for 6 weeks and then thawed at 37°C for 1 h; (3) FT3, in which bones were subjected to three cycles of freezing for 2 weeks at −80°C and then thawing at 37°C for 1 h; (4) 60°C, in which bones were heated at 60°C for 10 h; (5) 80°C, in which bones were heated at 80°C for 10 min; and (6) Radi, in which bones were subjected to 25 kGy cobalt-60 gamma-irradiation. The excised and treated femoral bones were fixed for 48 h in 4% paraformaldehyde, which was then replaced with phosphate buffered saline (PBS), and the bones were then refrigerated at 4°C until Raman spectroscopy analyses.

Confocal laser Raman spectroscopic measurements

Confocal laser Raman microspectroscopy was used to determine the composition and relative intensities of bone minerals and matrix in the mid-shaft anterior cortex of the left femora 15 mm from the proximal end. Using a custom-made fixture, the proximal half of femora was placed on the microscope stage such that the transverse cross section at mid-shaft was oriented perpendicularly to the laser beam incident from a 80× microscope objective. Locations of interest were positioned with an accuracy of 0.1 μm using a motorized XY stage and an optical camera. The intracortical compartment was subjected to measurements using a Nicolet Almega XR Dispersive Raman microscope system equipped with the OMNIC Atlus imaging software program (ThermoFisher Scientific, Inc., MA, USA). An area of less than 1 μm^2 can be mapped using Atlus software for samples visualized on the confocal video microscope. A high-brightness, low-intensity laser operating at 780 nm was used as the excitation source with a laser power of 35 mW. Each spectrum shown is the sum of 10 s measurements. The peak areas or heights were calculated with the following Raman shift wavenumbers (Table 1): PO_4^{3-} v_1, 981.9–925.7 cm^{-1}; CH_2 wag, 1,446–1,455 cm^{-1}; PO_4^{3-} v_4, 631.0–545.8 cm^{-1}; CO_3^{2-} v_1, 1,087.9–1,052.1 cm^{-1}; amide I, 1,716.3–1,541.2 cm^{-1}; amide III, 1,298.1–1,214.4 cm^{-1}; hydroxyproline, 855 and 878 cm^{-1}; and proline, 919 cm^{-1}; as described in Akkus et al. (2004). Crystallinity, a parameter of mineral maturation, was determined as the inverse of

the width of the phosphate symmetric-stretch band (PO_4^{3-} v_1 at 959 cm^{-1}) at half the maximum intensity value, as previously described (Yerramshetty and Akkus 2008).

Statistical analysis

Data were summarized to allow comparisons between the Fresh and other five treatment groups. All data values are expressed as the mean ± standard deviation (SD). The group means were compared by one-way analysis of variance (ANOVA) followed by Tukey's HSD post-hoc multiple comparison tests using a commercially available statistical package (SPSS 11.0 J for Windows; SPSS, Inc., Tokyo, Japan).

Results

Raman spectral analysis of the anterior cortex of rat femora in the treatment group revealed the resolvable mineral factor to be carbonated apatite. The peak wave numbers of the main mineral and matrix components were nearly identical to those originally reported by Tarnowski et al. (2002), as shown in Fig. 1 and Table 1.

We next compared the mineral-to-matrix ratios in the bone samples for all six experimental groups based on the Raman spectral analysis results. All three mineral–matrix ratios, $v1$/amide I, $v1$/amide III, and $v1/CH_2$, were significantly decreased in the 60 and 80°C treatment groups ($P < 0.05$) (Fig. 2a–c, respectively). The $v1/CO_3$ ratio also tended to be lower in the 60 and 80°C treatment groups (Fig. 2e). No differences in the hydroxyproline/proline ratio were observed between the Fresh and other five treatment groups (Fig. 2d). The crystallinity of bones in the Radi group was significantly higher than that of bones in the Fresh group (Fig. 2f).

Table 1 Raman spectroscopy results for rat cortical bone between 350 and 1,750 cm^{-1}

Raman shift (cm^{-1})	Assignment
1,716–1,541	Amide I
1,446–1,455	CH_2 wag
1,298.1–1,214.4	Amide III
1,087.9–1,052.1	CO_3^{2-} v_1, type B carbonate substitution
981.9–925.7	PO_4^{3-} v_1
631.0–545.8	PO_4^{3-} v_4

Discussion

Using confocal laser Raman spectroscopy, we evaluated rat femoral bones after being subjected to procedures routinely used for the preservation and sterilization of allografts. We found that although heat treatment and irradiation resulted in altered Raman spectra, freeze–thawing had no detectable effects on the composition of bone minerals or matrix. This finding suggests that Raman spectroscopy would be useful for quality assessment of allograft bones after processing and sterilization procedures.

The freezing of tissues at −80°C is generally used for long-term preservation in tissue banks and is also considered to reduce the immunogenicity of allografts (Gitelis and Cole 2002; Heyligers and Klein-Nulend 2005; Weyts et al. 2003). However, repeated freeze–thawing may affect the properties of tissue; compared to a single freeze–thaw treatment, the repeated freeze–thawing of tissues, including the palate, liver, and skin, produces more extensive and thorough tissue destruction (Gage and Baust 1998). In present study, no notable changes in any Raman spectra were observed in the FT1 and FT3 treatment groups, and even repeated freeze–thawing did not affect the Raman spectra of femur samples. This finding suggests that the freeze–thaw procedure for bone preservation may not affect collagen structure. Our results are consistent with those of Voggenreiter et al. (1994), who examined the effects of preservation and sterilization on cortical bone grafts using SEM and found that compared to fresh bone, cryopreservation and irradiation had no deleterious effects on the bone surface structure compared to fresh, untreated bone. In addition, in a previous study, we did not observe significant differences in the fibril occupation ratio or cycle length of intr aperiodic bands of collagen fibrils between the control and freeze–thawed tendons (Park et al. 2009). Taken together with the results of our present study, these findings suggest that the freeze-thaw cycle does not affect collagen structure.

To avoid the transmission of bacteria, human immunodeficiency virus, and hepatitis C virus during

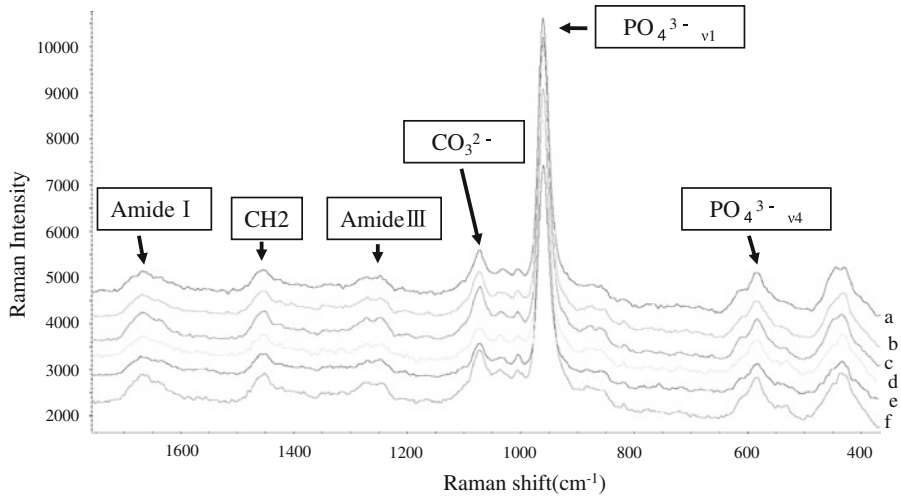

Fig. 1 Typical Raman spectra of cortical bone for each treatment group. *a* Fresh group, *b* FT1 group, *c* FT3 group, *d* 60°C group, *e* 80°C group, and *f* radi group. Peaks corresponding to the assignments listed in Table 1 are indicated. Raman intensity is shown in arbitrary units (a.u.)

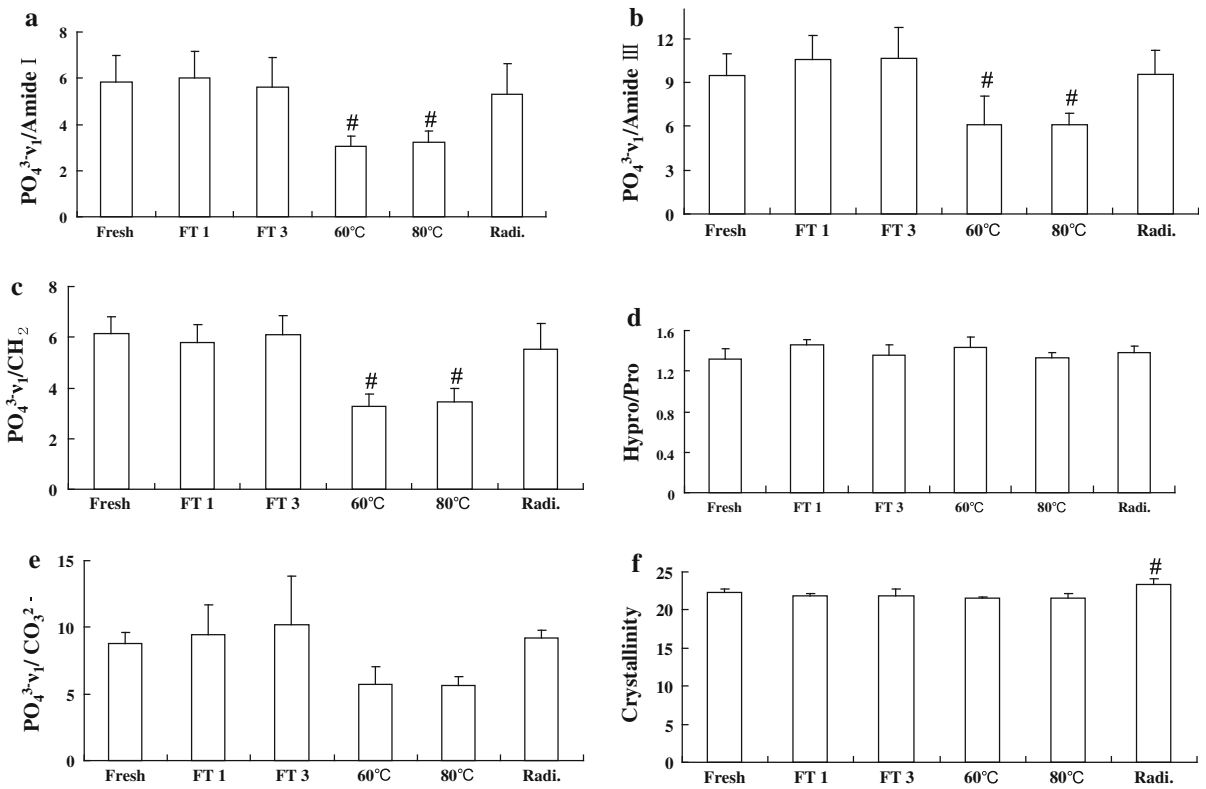

Fig. 2 Comparison of the mineral–matrix ratios and crystallinity for femur bone samples of the different treatment groups. **a** *v*1/Amide I, **b** *v*1/Amide III, **c** *v*1/CH_2, **d** hydroxyproline (Hypro)/proline (Pro), **e** *v*1/CO3, and **f** crystallinity. All data are presented as the mean ± SD. One-way analysis of variance (ANOVA) was followed by Tukey's HSD post-hoc multiple comparison test. $^{\#}P < 0.05$ versus the fresh group

grafting, two protocols involving heating at 80°C for 10 min and 60°C for 10 h are regulated as standard treatments for allograft bone by the Japanese Orthopaedics Association (1999). Knaepler et al. (1991) reported that osteoinduction properties and mechanical strength of bone were preserved at

temperatures lower than 80°C. Similarly, Izawa et al. (2001) demonstrated that bone morphogenetic proteins and osteoinduction potential are preserved after heat treatment at 60°C for 10 h, indicating that the quality of heat-treated bone is adequately retained. However, Kühne et al. (1992) reported that deep-frozen allografts more rapidly integrated than allografts subjected to moderate heat-treated treatment. In our present study, Raman spectroscopic analysis revealed that the mineral-to-matrix ratios, namely $\nu1$/Amide I, $\nu1$/Amide III, and $\nu1/CH_2$, were significantly decreased in bone of the 60 and 80°C treatment groups. Otomo et al. (2004) also reported a decrease of mineral–matrix ratios in the bone of ovariectomized rats (Otomo et al. 2004). Our finding of lowered mineral-to-matrix ratios following heat treatment indicates that the decreased quality of heated bones may also explain the slower integration period often observed for heat-treated allografts (Kühne et al. 1992).

Gamma irradiation from cobalt-60 sources, which has been widely used to terminally sterilize bone allografts, is well known to adversely affect the mechanical and biological properties of bone allografts in a dose-dependent manner (Nguyen et al. 2007). The mechanical properties of allograft bone markedly decrease when the gamma dose is increased above 25 or 60 kGy for cortical and cancellous bone, respectively (Nguyen et al. 2007). Tissue banks operating in countries that were using the ionizing radiation technique for the sterilization of tissues, particularly in Asia and the Pacific and in the Latin American regions, used a target dose of 25 kGy (Pedraza et al. 2011). They tried to follow ISO 11137 (IAEA 2007) and ISO/TR 13409 ISO/TR 13409(1996) to validate the process (Pedraza 2006). Therefore, this dose is most frequently used for sterilization treatment by tissue banks, although opinions are divided as to the optimal dose. In our present study, the use of 25 kGy gamma irradiation did not change the resulting Raman spectrum of treated femurs, but did increase the crystallinity. A previous study reported that the crystallinity of human bone increases with aging (Hanschin and Stern 1995). Yerramshetty and Akkus (2008) also demonstrated that tissue strength and stiffness increased with increasing crystallinity, whereas the ductility reduced. Thus, increased crystallinity in irradiated allograft bone may represent an adverse effect of irradiation sterilization methods. However, Jinno et al. (2000) reported that the irradiation process did not consistently or significantly affect the incorporation of syngeneic or allogeneic grafts. Therefore, changes in the crystallinity of bone may have little effect on the incorporation of syngeneic or allogeneic grafts.

Several studies have reported that Raman spectroscopy is useful for the evaluation of bone quality, which is the combined function of various patients characteristics, including age, and disease status, such as osteoporosis or osteoarthritis (Ager et al. 2005; Akkus et al. 2004; Krafft et al. 2009; Otomo et al. 2004). However, the bone quality of allograft bones after processing and sterilization procedures are not routinely evaluated because standard techniques, including mechanical testing and bone graft models, are time consuming and often lead to the destruction of the sample. We found that the freeze-thaw process did not affect the resulting Raman spectrum. As Raman spectroscopy allows the rapid evaluation of allograft bone quality without complicated tissue preparation, this approach may allow the quality control of allograft bones after sterilization procedures. Notably, we found that sterilization processes, particularly heating and gamma irradiation altered the Raman spectrum of femoral bones, suggesting that these treatment processes change the quality of bone allografts. Our analyses also suggest that Raman spectroscopy may be useful for the evaluation of bone sterilization techniques.

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