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Analysis of heat-treated bovine cortical bone by thermal gravimetric and nanoindentation

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ABSTRACT

Xenograft bone has been widely used as a bone grafting material because it gains advantages in biological and mechanical properties as compare with the use of an allograft bone. Heat-treatment of bone is recognized as one of the simple and practical methods to lower the human immunodeficiency virus (HIV) infection and overcome the risks of rejection and disease transfer during the bone transplantation. Therefore, understanding the change of bone's organic matrix after heat treatment has become a significant topic. In this study, thermal gravimetric analysis (TGA) was used to investigate the condition of organic constituents of a bovine cortical bone. In order to well characterize the microstructural and mechanical property of the bone after heat treatment, nanoindention technique was also employed to measure the localized elastic modulus (E) and hardness (H) of its interstitial lamellae and osteons lamellae at the temperatures of 23 °C (RT), 37 °C, 90 °C, 120 °C and 160 °C, respectively.

The TGA results demonstrated that heat-treated bones had three stages of weight loss. The first stage was the loss of water, which started from RT to 160 °C. Follow by a weight loss of organic constituents starting from 200 °C to 600 °C. Upon reaching 600 °C, the organic constituents were decomposed and mineral phase loss started taking place until 850 °C. From the nanoindentation results, it showed the values of E and H measured for the interstitial lamellae were higher than that of the osteons lamellae. This phenomenon indicates that the interstitial lamellae are stiffer and easy to be mineralized than osteons lamellae. For a specimen heat-treated at 90 °C, the values of E and H of interstitial lamellae and osteons lamellae were similar to a non-heat-treated specimen. For a specimen heat-treated at 120 °C, its interstitial lamellae had higher E and H values than osteons lamellae. When a specimen was heat-treated at 160 °C, both interstitial lamellae and osteons lamellae demonstrated a slight decrease of their E and H values. An ANOVA statistical analysis was used to analyze the difference in elastic properties and hardness in various temperature ranges.

1. Introduction

Bone is defined as a natural composite material which is primarily responsible for mechanical, biological, chemical, and protective functions for human body [1–4]. As a composite material, the major bone tissue is composed of 20–25 wt% of organic constituents, 70 wt% of inorganic constituents, and 5 wt% of water constituents. About 98% portion of type 1 collagen and noncollagenous protein form the organic constituent (osteoid) [5,6]. The organic constituent of bone provides tensile properties and flexibility. The inorganic constituent consists of mineral-crystalline calcium hydroxyapatite which mainly responses for compression and stiffness properties [5,7–9]. Type 1 collagen comprises of two α1 chains and one α2 chain intertwined into a triple helix [4,10]. Some studies of type 1 collagen have been characterized on the protein structure while some other studies were focused on the molecules thermal stability [10,11] such as collagen responses under temperature change in hydrated and dehydrated state and the denaturation of collagen under oxidative damage [4]. In addition, some studies have shown that the biomechanical properties of bone are associated greatly to the composition of organic constituent and the spatial arrangement [1–2,6,12–17].

Recently, heat-treated bovine bone has been proposed as a substitute for bone transplant material because it mainly composes of organic and inorganic constituents such as collagen, protein and hydroxyapatite (HA), hence it has great potential to enhance bone growth [18]. Although the autograft is the most preferred bone for

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grafting due to the great amount of bone cells and proteins to simulate the bone growth [19], however, autograft bone is limited in volume and additional surgery is needed which adds discomfort and pain to the recipient [20]. Todoh et al. [21] have reported that, Young's modulus of cortical bone along the bone axis, after degeneration at 200 °C, to be about 12 GPa (using the four-point bending test), whereas that of an intact specimen was 20 GPa. Catanese III et al. [20] also reported that the elastic modulus (mean ± SD) remained similar to that of an intact cortical bone along the bone axis, being 16.3 GPa for compression and 16.3 GPa for tension when the cortical bone was heated up to 350 °C. Furthermore, the cortical bone was found to have maintained 63% of its intact strength in compression after being heated up to 350 °C, this makes it well suited for compressive load-bearing applications as mentioned by Catanese III et al. [20]. Shin et al. [22] mentioned that heat-treated bone at 60 °C showed a decreased in strength ratio at 18 weeks after transplantation, yet, after transplantation for 48 weeks, the strength ratio has increased to 94.5%.

Nanoindentation, also known as depth-sensing indentation, is one of the promising novel techniques recently used to quantify the nano- and microscale mechanical properties in tissues and other biomaterials [23]. This technique evolves from conventional Vickers micro-hardness testing, with additional capability of making small indentations at precise positions on microstructural features, while concurrently monitoring the loads and displacements of the indenter on the specimen surface. The nanoindentation is well suited to examine the microstructual features of material surface to provide a spatial resolution which is less than 1 μm [21,24–28]. Since the bone tissue varies at different structural levels (from microsturcture of 10 to 500 μm to sub-nanostructure of 1 nm) [21], the mechanical properties of microstructural units of bone tissue down to the osteon level can be explored by using nanoindentation technique. Rho et al. [26] employed the nanoindentation technique to indirectly measure Young's modulus of bone through the observation of the return path of load–displacement curve. It has been reported that Young's modulus, measured in the longitudinal direction are 22.5 GPa for the osteons and 25.8 GPa for the interstitial lamellae. Higher elastic moduli of 24.7 GPa and 30.1 GPa, for the osteons and the interstitial lamellae of cortical bone in the longitudinal direction, respectively, have also been reported by Wang et al. [27]. In addition, the hardness values of the cortical bone in the longitudinal direction ranging from 0.81 to 0.89 GPa for the osteons and the interstitial lamellae had also been reported by Wang et al. [27].

In order to understand the influence of heat degradation on bovine cortical bone, thermal gravimetric analysis (TGA) is conducted to examine the weight loss percentage of organic matrix. Nanoindentation technique was further used to determine the mechanical properties of pristine and pre-heat treated single osteons and interstitial lamellae at various temperature ranges of femur bovine cortical bone.

2. Materials and methods

Two types of frozen bovine cortical bone, femur and rib (~3–4 years old) were procured from local slaughterhouse and were prepared promptly after sacrifice within 24 h for thermal gravimetric measurements. Bone pieces were then machined into around 2 mm × 3 mm × 2 mm dimension by a low-speed diamond saw (Metkon, resin bonded diamond cut-off wheels) with continuous deionized water irrigation to prevent the thermal damage when machining the specimens. All the specimens were then place into an ultrasonic bath to remove surface debris for 5 min. Thermal gravimetric analysis (TGA) was performed using TA instruments Q500 thermal analyzer. Specimens were heated from room temperature to 850 °C at a heating rate of 10 °C/min in a stream of nitrogen (50 cm^3/min).

Bone specimens were divided into four groups, they were:

Group 1 and 2: Untreated with PBS (phosphate-buffered saline) femur bone (UFB) and untreated with PBS (phosphate-buffered saline) rib bone (URB), respectively. The cortical bone from the bovine femur was extracted and prepared as described above. All specimens were frozen at −10 °C before the experiment.

Group 3 and 4: Treated with PBS (phosphate-buffered saline) femur bone (TFB) and treated with PBS (phosphate-buffered saline) rib bone (TRB), respectively. The bone specimens were extracted and stored in PBS during preparation time and frozen at −10 °C before the experiment. From each set, at least five TGA runs were performed to avoid the variability of weight loss from various specimens. The data curves showed the average of weight loss for each set.

For the nanoindentation tests, 15 specimens were heat-treated at four different temperatures in an oven for one hour and compare with the pristine specimen, which was only dried at room temperature. The pre-set temperatures were 37 °C, 90 °C, 120 °C, and 160 °C. The bone specimens were mounted in the resin block without being vacuumed into epoxy resin to provide support and allow them to cure for 24 h at room temperature (23 °C). Araldite GY251 epoxy resin (with hardener HY956 mixture in the ratio of 5:1) was used to provide support for the bone specimens. All indentations in this study were conducted away from the bone edge and resin boundary to enhance the accuracy of data. Fan et al. [29] and Hoffler et al. [30] have reported that the use of epoxy resin, as a support of bone specimen only penetrate into trabecular pores but not the tissue, consequently, its influence to nanoindentation results were kept to minimum.

The surfaces of all mounted specimens were finely polished before the nanoindentation test in order to avoid the confounding effect of surface roughness that may affect the nanoindentation results. The specimens were grounded by different grades of silicon carbide papers (60, 320, 800, 1200 and 2000 grits) under soft water jet and then further polished manually by soft synthetic flock polishing cloths with different grades of the diamond powder (15 μm, 6 μm and 1 μm). After grinding and polishing, the specimens were placed into an ultrasonic bath to remove the surface debris for 10 min. For the sake of a clear lamellae microstructure, the preparation of the entire specimen was examined under an optical microscope to obtain a smooth surface for indentation.

2.1. Nanoindentation test

TriboScratch (Hysitron, Inc., Minneapolis, USA) was used to carry out the experiments at room temperature (~23 °C) throughout the study. A sharp Berkovich (three-sided pyramid) diamond indenter tip was embedded in the transducer to measure the nanoindentation modulus and hardness. The experimental specimens were glued on a stainless steel stage held on an x–y–z table and located under the indenter tip, the Berkovich indenter tip was slowly driven towards the specimen surface as the test started in a constant displacement rate and a permanent hardness impression was made after the surface contact. Each nanoindentation test was conducted to a maximum load of 30 mN at a loading and unloading rate of 0.6 mN/s to produce a surface contact depth and the hardness impression was held for a period of 5 s at the maximum load to eliminate any creep behavior. The data obtained from indentation load–displacement test was analyzed to calculate the elastic modulus, E, and the hardness, H, using the method of Oliver and Pharr, where the indenter area function have been well documented [31]. This method is based on the measurement of the contact stiffness S, from the upper portion of the unloading data to