FULL PAPER *Anatomy*

Role of Collagen and Inorganic Components in Electrical Polarizability of Bone

Rumi HIRATAI[1,2], Miho NAKAMURA[1)]* and Kimihiro YAMASHITA[1)]

[1)]*Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, 2–3–10 Kanda-Surugadai, Chiyoda, Tokyo 101–0062, Japan*
[2)]*School of Veterinary Medicine, Rakuno Gakuen University, 582 Midorimachi, Bunkyoudai, Ebetsu, Hokkaido 069–8501, Japan*

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ABSTRACT. Hydroxyapatite (HA) has polarization capability and is able to accumulate electrical storage in bone. Experiments were conducted to measure the polarization capability of rabbit femurs. After preparing and polarizing bone samples using 2% KOH treatment (denoted 2% koh), 2% KOH and baking (2% koh+bake) and decalcification (decalcification) as well as untreated bone (untreated), stored charges were quantitatively determined using thermally stimulated depolarization current (TSDC) measurements. In TSDC spectra, untreated and 2% koh samples showed peaks at 100 and 500°C, while 2% koh+bake showed one peak at 580°C and decalcification one peak around 100°C. These evidences indicated that collagen and inorganic components play a major role in polarization of the bone at different temperature conditions.
KEY WORDS: bone, collagen, hydroxyapatite, polarizability.

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It was recognized by early studies that stimulation by mechanical loading, such as walking, has an influence on bone's remodeling [3]. According to Wolff's law proposed by Julius Wolff in 1892 [1], bone could functionally adapt to a new principal stress trajectories, in response to trauma or change of lifestyle, through reorienting its bony trabeculae. In the 1960s, it was conducted to apply mechanical force perpendicular to the long direction of a bone, which was fixed at one end [2, 3]. This experiment was based on the fact that the electrical potential could be measured by the addition of mechanical stress to the bone. It was found that a negative potential was generated on the concave side of the compressed bone [8–11, 14]. They termed this reaction a piezoelectric phenomenon.

Although it has been shown that mechanical stimulation of bones has an electrical influence, detailed mechanisms of how bones can sense mechanical stimulation, what types of electrical changes can happen and in what way bone remodeling is influenced are still unknown. The fact that electrical stimulation has an influence on bone formation is also known in the clinical field, and studies into various methods of exerting an electrical influence on bones, such as burying batteries in the body [19], electrically stimulating the body through the skin [5, 7] and applying electrical stimulation non-invasively to the body from outside [3], are progressing on a daily basis. However, most of the hypotheses regarding functional mechanisms are based on reactions with hormones, growth factors or cytokines [20, 21] rather than the direct function of electrical stimulation of bone cells or bone structures. Today, it is recognized that the electrical characteristics of dried bones can be mainly described by the piezoelectric phenomenon caused by mechanical stress [12] or the streaming potential generated by micro-flow of electrolytes containing fluid, such as blood serum, inside the bone [6]. The piezoelectric phenomenon is known to occur in dried bones, boiled bones and decalcified bones, and it is thought to be mainly caused by the structural distortion of collagen [13, 24]. It was classically believed that piezoelectric effects could not occur in hydroxyapatite (HA), as this material has a symmetrical hexagonal crystal structure [4]. Therefore, it was assumed that inorganic components similar to HA in bones did not exhibit piezoelectricity. However, it has been recently reported that piezoelectricity can be measured on the surface layer of HA [22]. In addition, we have reported that HA acquires polarization capability [25], which can semi-permanently accumulate electricity, when a high voltage is applied at elevated temperatures. We have also proved that polarized HA has a high bone affinity in living organisms [15, 16]. Charged conditions caused by polarization can be preserved for long periods and electricity is stored [18], while the electrical potential caused by piezoelectricity is reversible and the relatively high voltage is retained only for a short period of time on the outside surfaces of crystals.

We thought it should be possible to find conditions which cause polarization, enabling charge accumulation in bones. We also considered that inorganic components, which constitute 80% of bone material, played some role in this process. The purpose of this study is to investigate electrical properties and functions of collagen fibrils and mineral crystals of bone tissue using rabbit femurs.

MATERIALS AND METHODS

Sample preparation: The bone samples were extracted from the 10-week-old, male, Japanese White rabbits. At least

*CORRESPONDENCE TO: NAKAMURA, M., Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, 2–3–10 Kanda-Surugadai, Chiyoda, Tokyo 101–0062, Japan.
e-mail: miho.bcr@tmd.ac.jp

one week prior to conducting the experiment, each rabbit was acclimatized to the environment by being housed separately in a temperature controlled facility having 12-hr light/dark cycle. The rabbits were allowed to food and water *ad libitum*. The animals utilized in this experiment all received human care. The experimental protocol was approved by the Animal Welfare Regulation Committee of the Tokyo Medical and Dental University. After being preserved at a temperature of −80°C, eight rabbit femurs were cut in half transversely. Some samples were immersed in 2% KOH solution for 4 days (2% koh), immersed in 2% KOH and then baked in electrical furnace at 800°C for 10 min (2% koh+bake). Other samples were decalcified by treatment with EDTA (decalcification). The epiphysis of these samples, as well as the bone with no treatment (untreated), was longitudinally cut and grinded into uniform shapes 4 × 7 × 0.45 mm for measurement with a diamond saw (Buehler, Lake Bluff, IL, U.S.A.) and diamond disk (5 μm). The bone samples were washed with deionized water and then dried at 40°C until use.

Dense ceramic samples of hydroxyapatite (HA) were prepared as a control. HA powder was synthesized from analytical-grade reagents of calcium hydroxide and phosphoric acid by the wet method [16], calcined at 850°C for 1 hr, pressed into a mold at 200 MPa and sintered in a saturated water vapor atmosphere at 1,250°C for 2 hr to suppress dehydration.

Characterization: Bone samples were characterized by X-ray diffraction (XRD), Fourier transform-infrared (FT-IR) and scanning electron microscopy (SEM).

XRD measurements for each powdered sample were performed for phase analysis at room temperature with CuKα radiation at 40 kV and 40 mA on a diffraction spectrometer (PW-1700, PHILIPS Co., Ltd., Amsterdam, Netherlands) equipped with a graphite monochromator.

FT-IR spectra of each sample were measured in the range of 400–4,000 cm^{-1} using an infrared spectrophotometer (I-2000, JEOL Co., Ltd., Tokyo, Japan). The samples were pulverized with a mortar and pestle for the KBr method of which KBr and the samples were mixed in a ratio of 20:1. Then, peak ratios of ν_3CO_3 and ν_2CO_3 to ν_3PO_4 were calculated from the measurements.

After the treatments, bone samples were dried and sputtered with platinum/palladium. Surface morphology of each sample was observed at higher and lower magnifications using a scanning electron microscope (SEM) (Hitachi Instruments Service Co., Ltd., Tokyo, Japan).

Polarization: For the polarization measurement, samples were pinched by platinum electrodes, and the outside of each electrode was covered by alumina and clamped. A direct current electric field was applied to the samples using stabilized DC power supply units (AE8800, ATTO Co., Ltd., Tokyo, Japan and E3260A, HEWLETT PACKARD Co., Ltd., Palo Alto, CA, U.S.A.) under the chosen polarization condition. Regards, the samples were treated at 400°C with an electric field of 5 kV/cm for 1 hr and at 37°C with an electric field of 5 kV/cm for 1 hr. To avoid polarization relaxation, the electric field was maintained, while the samples were cooled down to the room temperature. The control sample was also treated at 400°C for 1 hr, but without electric field.

TSDC measurement: Samples were once again pinched by platinum electrodes, and the outside of the electrodes was covered by alumina plates and clamped. TSDC measurement of the samples was accomplished using a min electric current measuring unit (4140B pA METER, HEWLETT PACKARD Co., Ltd., Palo Alto, CA, U.S.A.). The polarization relaxation current was measured, while the sample temperature was increased at a constant 5°C/min from room temperature to 650°C. The amount of stored charge in the samples was calculated by surface integration of the TSDC spectrum.

Change of mass: In order to obtain the remaining organic ratios, each prepared bone sample was baked at 1,000°C for 30 min with weight measured before and after baking.

Proportion measurement of collagen: The amount of hydroxyproline, a main component of collagen of each sample, was quantitatively determined by the following procedure. Steps for this measurement are described below. The sample was combined with 6 M HCl and heated at 110°C for 24 hr to enable amino acid analysis. The reaction mixture was then dried in a rotary evaporator and dissolved in 0.02 M HCl. Amino acid analysis was then performed on an L-8800 amino acid analyzer (Hitachi Instruments Service Co., Tokyo, Japan) using citrate buffers and a sodium chloride gradient. Amino acids in the eluate were monitored by post-column reaction with ninhydrin. After measurement, the weight percentage of collagen in each sample was calculated.

RESULTS

Characterization: Infrared absorption peaks corresponding to ν_2CO_3 and ν_3CO_3 modes were detected to confirm the existence of carbonic acid group in the untreated and 2% koh samples (Fig. 1). The strength of ν_2CO_3 and ν_3CO_3 in comparison with ν_3PO_4 signal was 13 and 58.8% for the untreated samples, 8.8 and 48.8% for the 2% koh samples and 0 and 13.3% for the 2% koh+bake sample, respectively (Table 1). Thus, the remaining organic substance was highest in the 2% koh+bake sample.

X-ray diffraction peaks detected for every sample were attributed to HA, as the patterns were able to be matched to publish data ICDD no.9-432, and demonstrated that the surfaces of the specimens consisted of a single phase of hexagonal HA (indicated by open circles) (Fig. 2). The 2% koh sample showed broad pattern indicating amorphous compositions which were similar to that of the untreated sample, while crystallization was observed in the 2% koh+bake sample. Figure 3 shows the resulting SEM images. Surface of the untreated sample was rough, and that of the 2% koh sample was almost uniform. Collagen fibers were exposed in the decalcification sample. Many small voids in the 2% koh+bake sample seemed to be the results of collagen removal.

TSDC measurement and the amount of stored charge: The amount of the stored charges of all samples (Fig. 5) was calculated from their TSDC spectra (Fig. 4). The stored

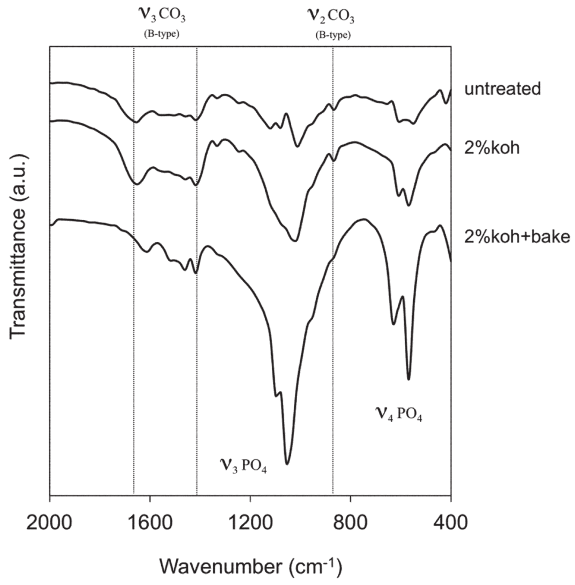

Fig. 1. FT-IR measurement results for the untreated, 2% koh and 2% koh+bake samples. Infrared absorption peaks in ν_2CO_3 and ν_3CO_3 modes confirmed the existence of carbonic acid group in the untreated and 2% koh samples.

Table 1. CO_3 ratio of each sample to ν_3PO_4 and amounts of the remaining organic substances in the untreated, 2% koh and 2% koh+bake samples

	Untreated (%)	2% koh (%)	2%koh+bake (%)
ν_2CO_3	13.0	8.8	0.0
ν_3CO_3	58.8	48.8	13.3
Remaining organic substance	62.0	65.0	97.0

charge of the unpolarized 2% koh sample was 0.04 μC/cm^2, but those of the untreated, 2% koh, 2% koh+bake and HA (or decalcified) samples treated at 400 vs. 37°C for 1 hr with an electric field of 5 kV/cm were 1.72 vs. 0.96, 138 vs. 1.63, 11.31 vs. 11.69 and 18 vs. 5.8 μC/cm^2, respectively. Besides, those of the 2% koh samples treated at 37°C for 1 and 10 min with an electric field of 5 kV/cm were 0.96 and 1.57 μC/cm^2. It was apparently that polarization in a harsher 400°C condition was not possible as the sample was burned and destroyed (Fig. 5). Peak of the TSDC spectrum of the untreated, 2% koh and 2% koh+bake samples was found in the vicinity of 100, 450–500 and near 580°C, respectively (Fig. 4). The second peak could not be observed, since the samples were destroyed at higher temperature.

Change of mass: The untreated, 2% koh and 2% koh+bake samples were burned at 1,000°C for 30 min, and the bone mass ratio between before and after burning was calculated for each sample. Those ratios were 62% for untreated, 65% for 2% koh and 97% for 2% koh+bake samples (Table 1).

Proportion measurement of collagen: The amount of hydroxyproline, a main component of collagen, was quantitatively determined for each sample, and the weight proportion of collagen was calculated. These results were 13.84% for untreated, 13.86% for 2% koh, 0% for 2% koh+bake and 74.2% for decalcification samples (Fig. 6).

DISCUSSION

In this experiment, untreated, 2% koh and 2% koh+bake bone samples were prepared from rabbit femur, and all could be polarized after treatment at 400°C with an applied electric field of 5 kV/cm for 1 hr. These are conditions under which HA can be polarized. However, in addition, all the samples could be polarized at a lower temperature of 37°C, at which HA cannot be polarized [23].

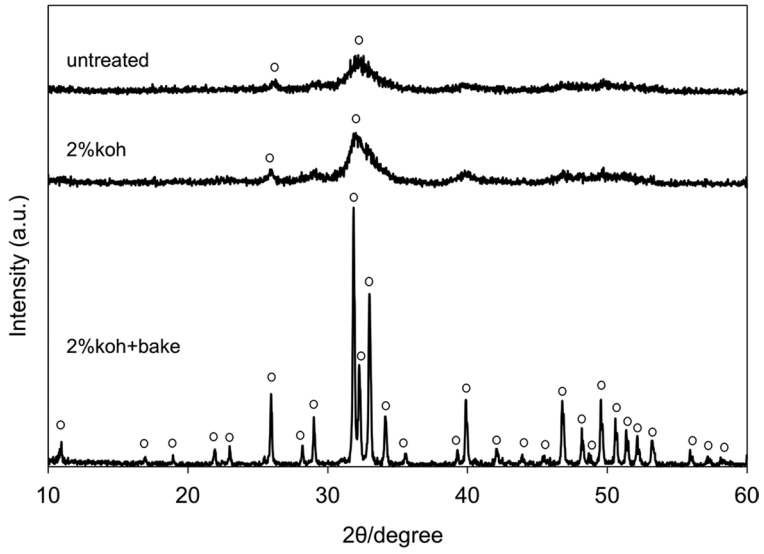

Fig. 2. XRD patterns of untreated, 2% koh and 2% koh+bake samples. The bone samples consisted of a single phase of hexagonal HA (indicated by open circles).

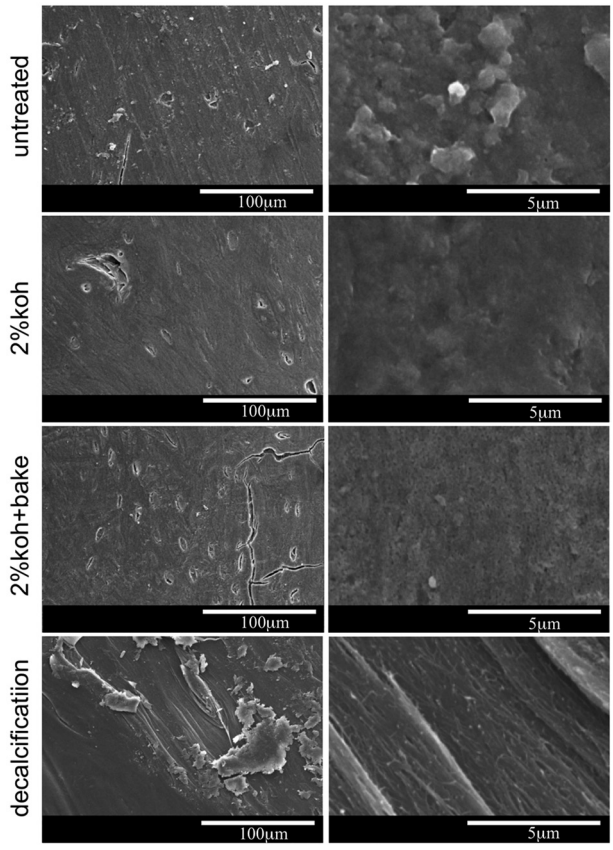

Fig. 3. SEM observation of the untreated, 2% koh, 2% koh+bake samples and decalcification samples. Surface of the untreated sample was rough, and that of the 2% koh sample was almost uniform. Collagen fibers were exposed in the decalcification sample. Many small voids in the 2% koh+bake sample seemed to be the results of collagen removal.

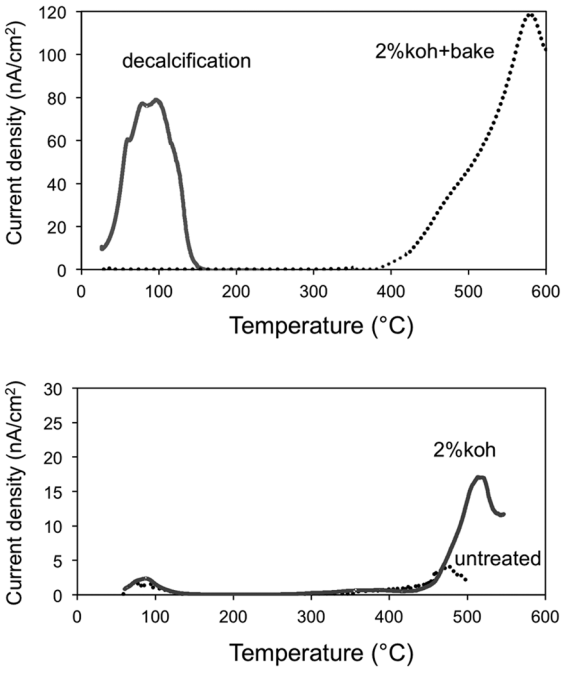

Fig. 4. TSDC spectra of the untreated, 2% koh, 2% koh+bake samples and decalcification samples. The untreated and 2% koh samples exhibited two peaks in the vicinity of 100 and 500°C. The single peak of the 2% koh+bake sample was near 580°C, and that of the decalcified samples was in the vicinity of 100°C.

The 2% koh sample exhibited stored charge of 138 μC/cm^2 after being subjected to the strongest treatment conditions, a very high degree of charge storage compared to the other samples. This sample could also be polarized at lower

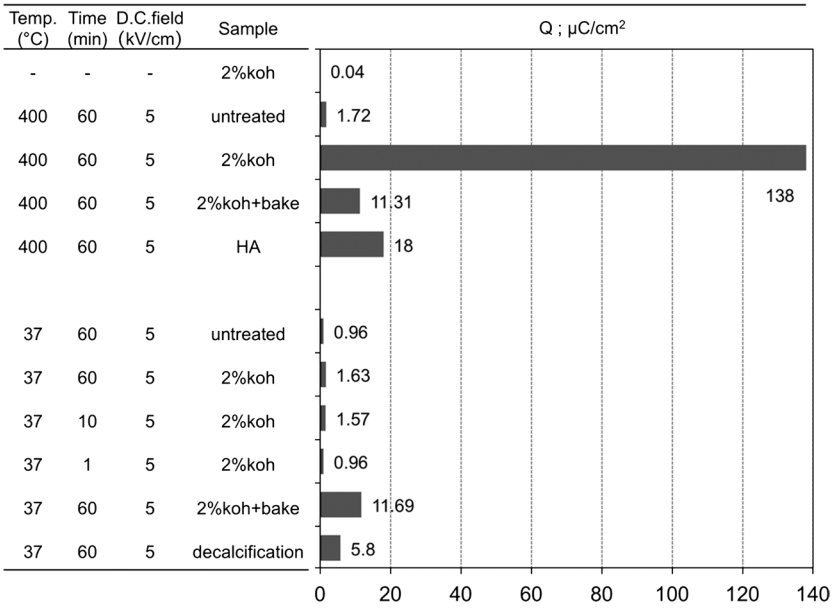

Fig. 5. The amount of stored charges calculated from TSDC spectra of each sample.

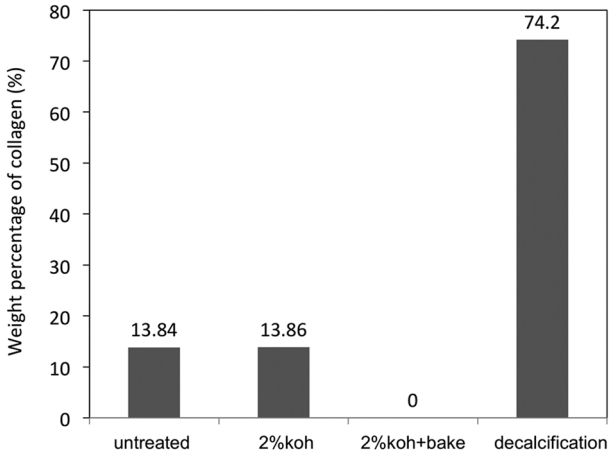

Fig. 6. The proportion of collagen weight calculated from the measurement of hydroxyproline.

temperature conditions with shorter treatment time. At 37°C, this sample was treated for 1 hr with the electric fields of 5 kV/cm for 10 min with 5 kV/cm and for 1 min with 1 kV/cm (Fig. 5). These results mean that the polarization of bones is possible even at body temperature. It was also found that the 2% koh sample had almost the same quantity of CO_3 as the untreated sample (Fig. 1, Table 1) and structurally was in a broad amorphous state with no crystallization observed by XRD. Those findings indicated that the 2% koh sample was not crystallized and could retain similar characteristics to that in the untreated sample (Fig. 2). In addition, the 3% higher in weight ratio of the 2% koh sample either before or after burning than the untreated sample, and a similar weight proportion of collagen in both samples (Fig. 6) would indicate a similar condition of collagen components in bone structure of both samples. However, this experiment still cannot explain clearly why the amount of the stored charge of the untreated sample was much lower than that of the 2% koh sample.

The 2% koh+bake sample showed similar results in IR and XRD measurements to those of HA. This sample could be polarized at the usual high temperature conditions, giving stored charge of 11.31 μC/cm^2. This value was similar to that of HA, which gave 18 μC/cm^2. On the other hand, the 2% koh+bake sample could be polarized under the lower temperature conditions, giving stored charge of 11.96 μC/cm^2, similar to the high temperature treatment, while HA could not be polarized at temperatures below 200°C. The 2% koh+bake sample had a mass ratio of 97%. This sample also showed 0% collagen content. These findings suggest that this sample was rendered a completely inorganic material by the sample treatment. Thus, the polarization capability of this sample is due to inorganic components of the bone. However, XRD measurement showed that the sample was crystallized in the same way as HA, while IR measurement revealed an extremely low CO_3 content. This meant that the sample had not retained the properties which were similar to those of bones.

The high weight proportion of collagen in the decalcification sample (74.2%) would indicate a successful removal of inorganic components from the bone; thus, leaving mainly organic components *in situ*. It is a matter of fact that such decalcified bone containing almost purely collagen could not tolerate a high temperature treatment. Thus, induction of polarization could merely perform only in a lower temperature. This statement indeed supports the concept that polarization of the bone relies largely on its organic components [17].

Double peaks of the untreated and 2% koh samples were found in the vicinity of 100 and 500°C. In contrast, the 2% koh+bake sample had only single peak in the region of 580°C (Fig. 4). At high temperature, it is certainly that collagen in the rabbit's bone could not be preserved; therefore, there was only one TSDC peak at the vicinity of 500°C. For the decalcified bone, a TSDC peak was found near the 100°C region. Similar polarization pattern of collagen portion of decalcified bone of various animal species had also been shown by Mascarenhas *et al.* [17]. The study in the decalcified bone also confirmed the polarizability of its collagen content. From these findings, it is conceivable that collagen is responsible for the polarization at around 100°C while the inorganic components are responsible at around 500°C.

Along with the already described phenomena regarding piezoelectric activity and streaming potential, this experiment could found evidences of polarization which enable electrical storage of the bone. Polarization of the bone could be induced even at 37°C, but not that of the HA. The study in decalcified bone could confirm that not only inorganic components but also collagen played a major role in this particular. However, the explanation for the polarization of bone at low temperature, but not of HA is very limited. The presence of residual CO_3 or the structural orientation of the bone would be a fundamental of this induction. Electrical storages by polarization from the electrophysiological standpoint would be a result of mechanical stimulation important to remodeling of the bones which lead to the known phenomena. In terms of clinical applications, polarization induction of autografting bone would possibly enhance new bone formation around the implanted area of bone defects upon the effects of the osteogenic cells in bone remodeling [16].

In conclusion, our experiment could exhibit the phenomenon of polarization with certain capability of electrical storages in rabbit femurs. The bones could be polarized even under low temperature conditions, but this phenomenon did not occur with HA. In addition, both collagen and inorganic components could play significant roles in polarization in different temperature conditions.

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