Calcium Phosphate Apatite Formation on Biological Bone Surfaces via Low-Intensity Pulsed Ultrasound (LIPUS) for Osseointegration

Abstract

Background
Hydroxyapatite (HA) is widely utilized as a coating material for dental implants and artificial joints due to its excellent osteoconductivity. The author has previously demonstrated that low-intensity pulsed ultrasound (LIPUS) promotes osseointegration by facilitating the precipitation of bone-like calcium phosphate apatite on the surface of HA-coated materials through a physical crystallization mechanism. However, no previous studies have examined the effects of LIPUS irradiation on the biological bone that faced to the implant.

Purpose
This study aimed to investigate whether LIPUS stimulation could similarly promote the precipitation of bone-like calcium phosphate apatite on the surface of natural bone paired with an implant using this crystallographic mechanism.

Materials and Methods
Natural bone samples were immersed in simulated body fluid (SBF) and subjected to LIPUS irradiation. The resulting surface precipitates were analyzed using scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDS), and X-ray diffraction (XRD).

Results
The results indicated that hydroxyapatite precipitation on the natural bone surface was significantly greater than that observed on synthetic biomaterials.

Conclusion
These findings suggest that LIPUS irradiation promotes vigorous calcium phosphate precipitation on both implant and natural bone surfaces. This physical mechanism potentially enhances the process of osseointegration, providing a promising approach for clinical implant applications.

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Keywords

Hydroxyapatite, LIPUS (low-intensity pulsed ultrasound), Biological bone, Crystallization, Osseointegration.

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INTRODUCTION

Among bioactive materials, Hydroxyapatite (HA) exhibits excellent biocompatibility and is known to demonstrate Osteoconductivity, promoting the new formation and growth of surrounding bone within the body, and Osseointegration, direct bonding with bone. Currently, utilizing these properties, the development of medical implants such as artificial joints, dental implants, and artificial bones with hydroxyapatite coating on their surfaces is actively being pursued. The aim is to achieve early and strong bonding with natural living bon (1-3). However, in clinical practice, there is a demand for even faster integration between implants and living bone from the perspective of rehabilitation and postoperative recovery of Activities of Daily Living (ADL), and research is progressing to achieve earlier osseointegration.

Against these backgrounds, the author focused on Low-Intensity Pulsed Ultrasound (LIPUS), which is used in bone fracture treatment, and have reported that the ultrasound irradiation to bioactive material such as bioactive titanium and hydroxyapatite promotes the amount of bone-like hydroxyapatite crystal deposition on the material surface, leading to early bonding between the material and natural living bone via this HA layer in past in vitro and in vivo studies (4-7]. This ultrasound-induced enhancement of osseointegration suggests the involvement of a crystal-chemical crystallography mechanism regarding the precipitation and growth enhancement of bone-like calcium phosphate crystals, which is different from biological mechanisms such as osteogenesis. The possibility of a stable implantation and attachment between dental implants and living bone, as well as early achievement of osseointegration, is expected.

However, my previous studies have not examined the effects of LIPUS irradiation on the biological bone at the interface, rather than merely on the implant itself (7). Based on past clinical reports and basic research on fracture treatment using LIPUS (8–11), the mechanism of action underlying LIPUS's effect on bone union has primarily been discussed in terms of the biological response of osteocytes and fibroblasts to mechanical stimulation, While it is clear that this mechanism promotes osseointegration and bone healing in living bone, the effect of ultrasonic-induced biomineralization—specifically the promotion of calcium phosphate compound crystallization—as described in the author's previous research on artificial implants has not been confirmed.

Therefore, in this study, author conducted simulation experiments using simulated body fluids to investigate whether the irradiation of LIPUS onto the surface of biological bone specimens leads to the formation of calcium phosphate crystals that promote osseointegration.

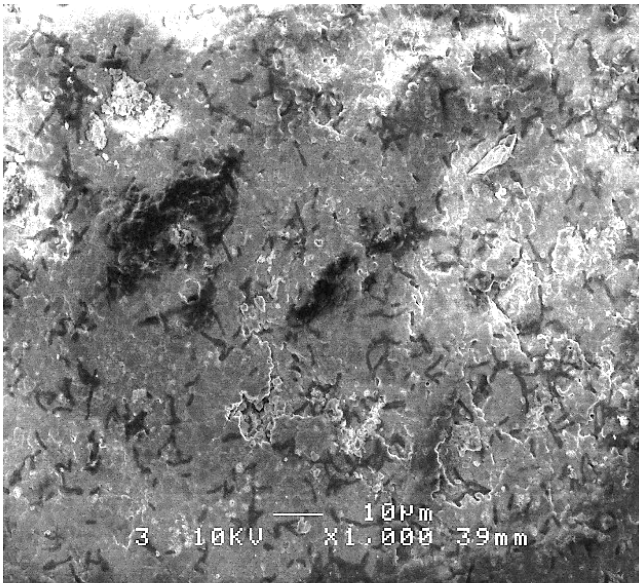

Fig. 1 The SEM image of natural bone specimen surface in this study (×1000).

MATERIALS AND METHODS

In this study, as a method to evaluate the apatite precipitation on natural bone tissue under LIPUS irradiation, natural bone specimens immersed in a simulated body fluid (SBF) were subjected to ultrasound irradiation as in the previously reported simulation experimental method (7), the changes in calcium phosphate apatite deposition on the specimen surface were observed and compared.

Experimental Materials

In this experiment, living bone from meat pigs was used as a biological specimen. The bone was harvested immediately after slaughter and frozen to preserve its condition. This biological specimen needs to be a type of hydroxyapatite material, allowing for comparison with previous data obtained using synthetic hydroxyapatite.

Cortical bone was excised from the femur, and after thawing, soft tissues such as the periosteum was removed.

After that, specimen surfaces were sequentially polished with emery paper #300 to #1500 to adjust the surface roughness. From the processed bone specimens described above, samples with dimensions of 30 mm × 20 mm × 5 mm were sectioned. Figure 1 shows SEM images of the surface of the biological bone specimen used.

Simulated Body Fluid (SBF) Soaking Test

As a basic osseointegration test, the simulated body fluid (SBF) soaking method was performed according to Kokubo's study (12) in order to evaluate the apatite-forming ability of bioactive materials, in the same manner as the previous study.

Hank's balanced solution (Lonza®; USA) was used as an

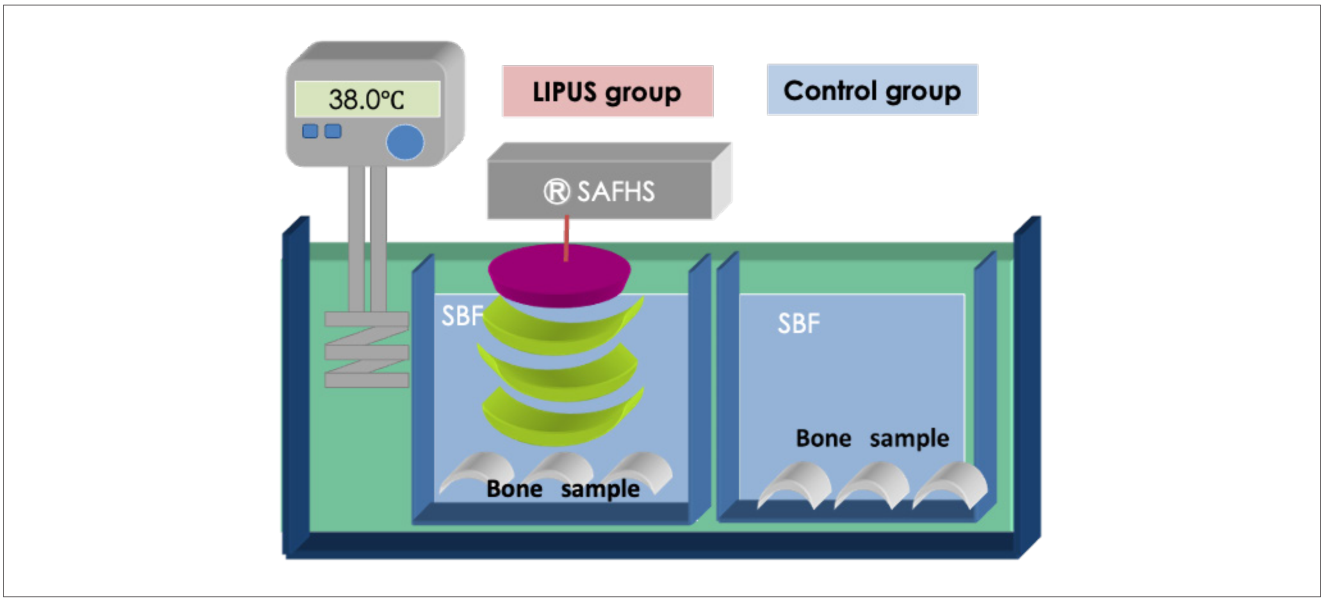

Fig. 2 Diagram of the pulsed ultrasound waving radiation on natural bone specimens in simulated body fluid (SBF).

SBF solution, and maintained at pH over 7.0 and 37˚C and replaced every two days. Ultrasound radiation was applied by using Sonic Accelerated Fracture Healing System (SAFHS; Smith & Nephew, Memphis, TN, USA; Teijin Pharma, Tokyo, Japan). The treatment head module delivered ultrasound waves with 1.5 MHz, 200 µsec signal term, and spatial average intensity of 30 mW/cm2. A total of 20 natural bone samples were soaked in SBF and subjected to ultrasound stimulation for 20 min daily during the operation term for three days, one week, and two weeks, respectively. As a control, the same specimens were left in SBF without ultrasound radiation under the same experimental conditions (Figure 2).

Five or seven specimens were removed from the SBF under the above conditions after a fixed immersion period (3 days, 1 week, 2 weeks), and the state of bone-like precipitates on the surface of the biological bone specimens was observed by Scanning Electron Microscopy (SEM) and X-Ray Diffraction (XRD).

RESULTS

SEM Images of the Sample Surfaces after Up to 2 Weeks of Immersion in SBF

Figure 3 shows a comparison of the results of SEM observations of the surfaces of specimens in the ultrasonic-irradiated group and the non-irradiated group (control group) after up to 2 weeks of immersion in SBF. In both groups, an increase in precipitates was observed with longer immersion time in SBF; however, crystal precipitation was clearly more pronounced in the LIPUS-irradiated group. The morphology of the precipitates in both groups appeared as irregular, stacked polygonal crystals, which differed significantly from the precipitate morphology previously reported on artificial hydroxyapatite implant surfaces.

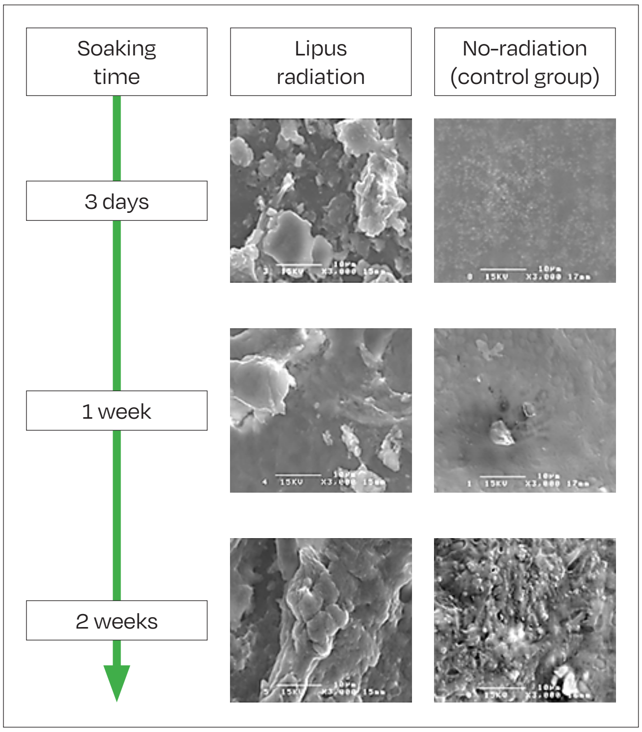

Fig. 3 SEM micrographs of the A and B specimen surface radiated by LIPUS (3000 ×).

X-Ray Diffraction (XRD)

The deposits on the specimen surface in LIPUS group after 3 days of immersion in SBF were identified by X-Ray Diffraction (XRD). The XRD peak patterns are shown in Figure 4. The XRD of the deposits showed a broad diffraction pattern, and no clear diffraction peaks were

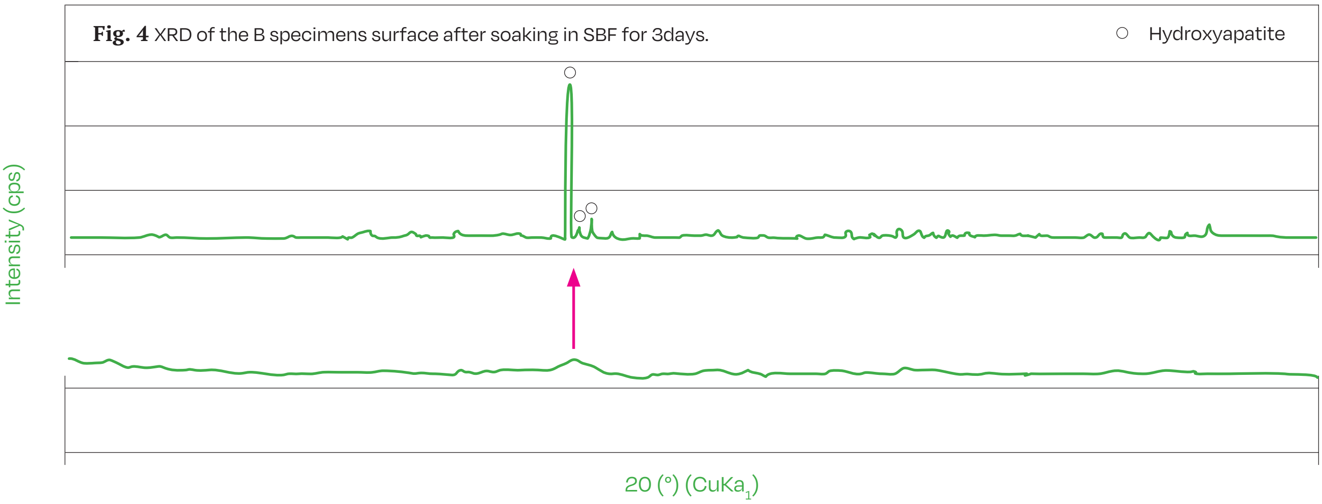

Fig. 4 XRD of the B specimens surface after soaking in SBF for 3days.

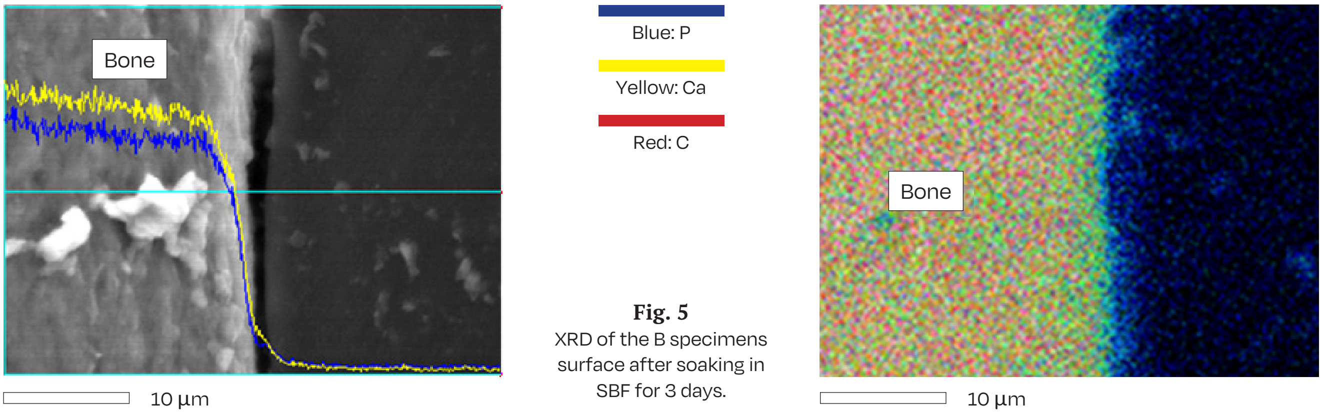

Fig. 5
XRD of the B specimens surface after soaking in SBF for 3 days.

observed. However, some diffraction peaks attributable to Hydroxyapatite (HA) were partially observed. Although there are concerns about overlapping with diffraction lines from the living bone specimen surface, which is the base matrix, because of the small amount of deposits, the presence of diffraction peaks characteristic of HA suggests that the deposits are calcium phosphate with an imperfect HA crystal structure. These results were consistent with previous reports on X-ray diffraction (XRD) studies of biological bone (13).

Energy dispersive X-ray spectrometer (EDS)

The cross section observation results of specimen (cross section) after 3 days of SBF immersion under LIPUS irradiation, analyzed by energy dispersive X-ray spectrometer (EDS), are shown in Fig. 5.

On the surface of biological bone specimens, the distribution of Ca-P atoms (green; A mixture of blue (P) and yellow (Ca) color) was observed, which may be due to calcium phosphate, a bone composition, but at the topmost layer, Ca (yellow color) was slightly less concentrated and P (blue color) was strongly identified, suggesting the crystal growth of PO4- derived hydroxyapatite. This phenomenon is considered to occur when the environmental phosphate ion concentration is high in the surface layer of the bone sample, which suggests the possibility of high phosphate ion concentration in SBF. The red color (C) originates from the collagen tissue within the bone sample.

Measurement of the Mass of Ca-P Crystallization

The amount of calcium phosphate crystal deposition on the surface was quantified by measuring the weight difference of the specimens before and after immersion in SBF. Figure 6 shows the measured mass change as hydroxyapatite-like precipitation (crystalline calcium phosphate) on the surfaces of the LIPUS group and control group after SBF soaking.

In both groups, the amount of Ca-P precipitation increased with longer SBF soaking times. However, the specimens in the LIPUS group exhibited a markedly greater amount of precipitation compared to those in the control group.

DISCUSSION

The author has previously reported on the effects of LIPUS irradiation in promoting the formation of an initial apatite layer as a method to enhance osseointegration on the surface of the biomaterial hydroxyapatite. Figure 7 shows SEM images of the surface of an apatite sample in which apatite was implanted into actual rabbit bone

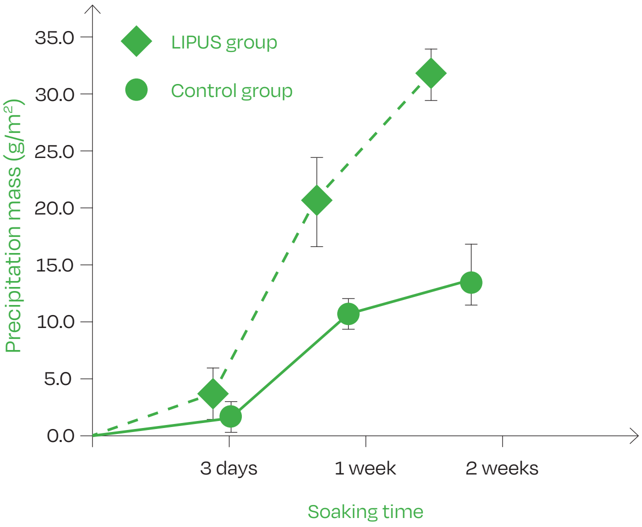

Fig. 6 The soaking time dependence of mass of P/Ca precipitation formation on the specimen's surfaces in the LIPUS and control groups.

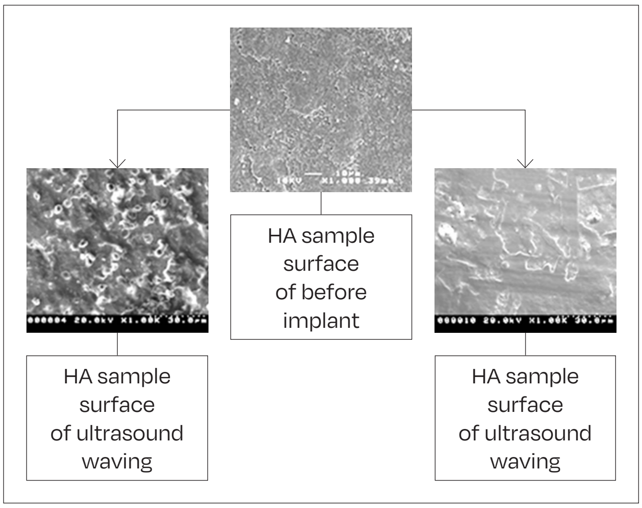

Fig. 7 SEM image of the hydroxyapatite implant surface of control group and LIPUS group in previous study. (×1000) (M.Kobayashi, J Osseointegration 2020;12(2):1.)

to evaluate the effects of LIPUS irradiation. Even in an in vivo environment, the enhancement of bone matrix formation on the apatite surface is evident.

The mechanism by which LIPUS induces apatite precipitation on the surface of bioactive materials in vivo is thought to be as follows: Moderate ultrasonic micro-vibrations amplify fluctuations in the concentrations of PO42- and Ca2+ ions and thermal fluctuations in the body fluids surrounding the implant, thereby promoting the nucleation of calcium phosphate crystals on the surface of the bioactive material. Furthermore, it is presumed that the circulation and agitation induced by ultrasound also effectively accelerated crystal growth.

Although this process of apatite precipitation is a purely physical crystallization mechanism not influenced by biological factors such as cells, the focus of this study was to determine whether LIPUS's ability to promote the formation of bone-like hydroxyapatite through this mechanism also occurs in living bone tissue that comes into contact with dental implants. The results of this experiment confirmed that even in SBF, which does not contain sufficient cellular nutrients, the crystal growth of calcium phosphate in the bone matrix apatite of the sample — as a physical phenomenon — increases over time, consistent with previous reports on biomaterials.

Clinically, this suggests that LIPUS irradiation immediately after dental implant placement promotes apatite crystal growth in biological bone, leading to the formation of strong osseointegration at an earlier stage.

Although clinical reports on the application of LIPUS for dental implant placement have already been published (14–16), the mechanisms described in these reports are theorized to involve the promotion of biological reactions for bone regeneration by LIPUS, such as those involving osteoblasts and fibroblasts. Of course, it is necessary to wait for LIPUS to promote these biological reactions for bone regeneration; however, based on the results of this study, early postoperative LIPUS irradiation of HA-coated implants to fill the gap between the implant and the bone and enhance bonding strength at an earlier stage is considered a highly beneficial treatment method, particularly in terms of the overall treatment duration, including rehabilitation.

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