INTRODUCTION

Implant treatment is widely used for oral rehabilitation and it has become more clinically predictable and reliable\(^1\). In recent years, numerous efforts have been exerted to make implant treatment more appealing for potential patients with compromised alveolar condition or severe systemic diseases\(^2\)\(^-\)\(^4\). One of these efforts has been to modify the titanium implant surface to shorten and improve the osseointegration process\(^5\)\(^,\)\(^6\).

Doxycycline is a broad-spectrum antibiotic of the tetracycline group, which is widely used for treating infectious disease including periodontitis\(^7\). Moreover, there are several non-antibiotic properties of doxycycline\(^8\). When doxycycline was first used in periodontal treatment, the efficacy was attributed to their antimicrobial properties. However, in recent years, several research revealed that the efficacy should be attributed to the anti-inflammatory properties. Thereupon, a new concept of sub-antimicrobial dose doxycycline (SDD) or low-dose doxycycline (LDD) as adjuvant therapy for periodontal disease has been approved by U.S. Food and Drug Administration and other national regulatory agencies in Europe and Canada\(^9\).

On the other hand, hydroxyapatite (HA) is widely used as bone substitute and surface coating of orthopedic and dental implants due to its high osseoconductivity\(^10\). A room temperature sputtering technology has been recently developed to deposit thin HA coating, which evoked much interest in the strong adhesion to Ti substrate, compact microstructure, well-controlled elemental composition, and preserved surface roughness of the underlying substrate. Due to its excellent characteristics, sputtered HA-coated surface would be a promising surface to improve the success rate in treating patients in the compromised alveolar bone condition\(^11\)\(^-\)\(^13\). Indeed, HA can be also used as a carrier of delivery system for therapeutic drugs such as antibiotics or anticancer drugs\(^14\)\(^-\)\(^16\).

The purpose of the present study was to examine the effect of doxycycline-treated HA surface on bone apposition. We compared bone appositions around doxycycline-treated HA coating implant (test group, DOX group) and HA-coated implant (control group, HA group) in the murine maxillae.

MATERIALS AND METHODS

Sample preparation

Twenty custom-made pure titanium self-tapping screw type implants (Matsumoto Industry, Chiba, Japan) with a length of 1.5 mm and diameter of 0.8 mm were used for this experiment (Fig. 1). After ultrasonic cleaning in ethanol for 5 min, HA coating was produced with magnetron frequency sputtering method on all the implants in an SPD-410H (ANELVA, Kawasaki, Japan) chamber\(^17\). The distance between the target and the substrate was about 60 mm, and the diameter of the target was 50 mm. Both the target and the substrate
were water cooled during the sputtering process. The sputtering chamber was evacuated to a pressure below $1 \times 10^{-5}$ Pa, using an oil diffusion pump and a liquid nitrogen trap. Argon gas (99.999%) was then introduced into the chamber. Before deposition, the target was pre-sputtered using Ar ions for 10 min, with the substrate covered with a target shield. The deposition was carried out using an Ar gas pressure of 0.5 Pa and a discharge power of 100 W. Then, the hydrothermal treatment was carried out at a temperature of 110°C and a pressure of 0.145 MPa in an electrolyte solution containing calcium and phosphate ions (Ca–P solution) in a stainless steel vessel for 24 h.

Subsequently, a hydrothermal treatment was performed at a temperature of 110°C in electrolyte solution containing calcium and phosphate ions for 20 h. Half of the implants were dipped into 5 mg/mL doxycycline aqua solution for 30 min, and washed by Dulbecco’s Phosphate-Buffered Saline (DPBS) for twice. All the procedures were operated in dark (test group, DOX group). The other implants were washed with PBS for twice (control group, HA group). Then, all the implants were dried at room temperature.

**Ultraviolet-visible spectroscopy**

The quantity of doxycycline attached on the HA surface was measured to assess the total amount of doxycycline on the HA coated titanium plate (grade 2 titanium plate, 20×20×1 mm³) with the same method mentioned above. The HA-coated titanium plates were dipped in 5 mg/mL doxycycline solution, washed with DPBS for twice, and then the plates were washed with 3 mL of 1 M hydrochloric acid. The solution was collected and then detected with an ultraviolet-visible spectrophotometer (Nano-Drop ND 2000, Nanodrop Technologies, Wilmington, DE, USA). The Nanodrop was calibrated using 5 standards of doxycycline solution at ambient temperature. The absorbance was measured at 238 nm, and a standard curve for calculation of doxycycline concentration was generated from the absorbance values. The quantity of doxycycline attached on the HA-coating surface was calculated according to the previously determined standard curve.

**Physical and chemical characterization of implant surface**

The implant surfaces were analyzed with SEM (JSM-5310 LV, JEOL, Tokyo, Japan) at 15 kV. The implants were osmium sputtered prior to analysis and fixed on the aluminum rack with conductive carbon tape. Acceleration voltage of 15 kV for 100 s with the working distance to 15 mm in the vacuum condition was applied to achieve magnifications of 1,500× and 5,000×. Energy dispersive X-ray spectroscopy (EDS) (EMAX-7000, HORIBA, Kyoto, Japan) was used for elemental analysis, Ca/P molar ratio and thickness of HA-coating surface. The data was calibrated by the PRZ Standard-less Quantitative Correction of the EMAX-7000 program (Version 1.32, HORIBA).

The surface roughness (Ra) of titanium and HA-coating was determined with a surface measurement tester (SURFCOM 130A) on commercial titanium plate (grade 2, 20×20×1 mm³) with and without the HA coating because the mini-implants were too small for the measurement.

**Animal care and surgical procedures**

The experimental protocol of the present study was approved by the Committee of Animal Experiments (Approval No. 0170383A) at Tokyo Medical and Dental University, Tokyo, Japan. The study was performed on twenty 4-week-old wild type male mice (C57BL/6NCrSlc). Animals were anesthetized preoperatively with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg). All the surgeries were performed under a stereomicroscope (olympusSZH10/ILLD, Olympus Optical, Tokyo, Japan).

The upper first right molar was extracted with a small needle (Terumo needle 27G×1, Terumo Medical, Tokyo, Japan) following the established protocol. Eight weeks after the tooth extraction, the soft tissue covering the extraction site was trephined off manually with a small needle (Terumo non-bevel needle 22 G×1 1/2”, Terumo Medical, Tokyo, Japan). In the light of the gingival tissue under the screw head might become compressive region which leads a serious problem such as implant head grows down into the gingiva, etc. To minimum the possibility mentioned above, approximately 1.2 mm deep implant sockets were prepared with endodontic K-files from K20 (diameter approximately 0.20 mm) to K80 (diameter approximately 0.8 mm) with gentle manual rotations in a step back manner to form a...
Fig. 2  Surgical procedure and healing of this study.
(a) Extracting the upper first molar with a 27 gauge needle as an elevator. (b) Loosening the teeth by lacerating the periodontium. (c) Extraction socket. (d) After 8-week healing period, the mucosa covering the extraction site was removed with a 22G non-bevel needle. (e) Preparing the implant socket using endo-files in a step-back manner. (f) The socket was prepared. (g) Placing the customized mini-implant into the socket. (h) The implant was completely placed. (i) The implant at 4 weeks after the placement.

apical end in alveolar bone, which furthermore, could also prevent the implants penetrating into the sinus leading to bleeding and inflammation. HA coated implant with or without doxycycline were placed into the preparation sites with the custom screw driver (Matsumoto Industry) until only the screw head was exposed in the oral cavity (Fig. 2). The mice were fed soft diet during the first week after implant placement. At 4 and 8 weeks the mice were euthanatized with excess dose of pentobarbital and the maxillae were collected.

Micro-computed tomography (micro-CT)
Micro-CT analysis (InsepXio, Shimadzu Science East, Tokyo, Japan) was done immediately after sacrificing the animals under the following conditions: Tube voltage 70 kv, tube current 30 μA, voxel size 0.03 mm/voxel. Volumetric data was reconstructed with Tri/3DBon software program (RATOC System Engineering, Tokyo, Japan).

Histological preparation and histomorphometric analysis
The specimens were fixed in 10% neutral formalin solution (Wako Chemical, Tokyo, Japan) for 48 h and then, dehydrated with ascending grades of alcohol and methyl methacrylate (MMA Technovit 7200, Heraeus Kulzer, Hanau, Wehrheim, Germany). The specimens were embedded in methyl methacrylate (Technovit 7200) and polymerized. Then, the ground section of mesiodistal direction including the implant was prepared with a cutting-grinding unit (Exakt, Apparatebau, Norderstedt, Germany) and with a micro grinding polishing unit (Le Cube, PRESI, Brié-et-Angonnes,
Fig. 3  Schematic image of NBA around the implant.

France). The ground sections, from 70 to 80 μm thickness, were stained with 0.1% toluidine blue (TB).

Histological images were taken with an optical microscope (Biozero-8000, Keyence, Osaka, Japan) and the data were processed using BZ-Analyzer software (BZ-Analyzer, Keyence). The bone-implant contact (BIC) was analyzed extending from the implant shoulder to the apical end. For the analysis of the bone area around each implant, the sum bone areas between all the threads in both mesio and distal side of the implants were defined as new bone areas (NBA) (Fig. 3).

BIC was defined as the length of bone surface in direct contact with the implant surface and calculated as percentage of bone surface in direct contact to all the threads part of implant surface. The mean value of BIC and NBA in HA and DOX groups was compared.

**SEM and EDS line analysis of undecalcified sections**

The undecalcified sections of HA and DOX group at 4 and 8 week time point were examined by SEM at 15 kV and The implants were carbon sputtered prior to analysis and fixed on the aluminum rack with conductive carbon tape. Elemental analysis of each implant was carried out by EDS under an accelerating voltage of 15 kV for 100 s using line analysis with the working distance of 15 mm in the vacuum condition without conductive coating.

**Statistical analysis**

Statistical analyses were performed using a commercial available software (SPSS 18, IBM, Armonk, NY, USA). The mean and standard deviation of BIC and NBA among all groups were analyzed via the paired t-test and one-way analysis of variance (ANOVA). The level of significance was set at \( p < 0.05 \).

**RESULTS**

**Physical and chemical characterization of HA coating**

SEM images revealed a smooth morphology of the titanium surface with machined texture as compared to HA-coating surface. The HA-coated surface showed even-sized apatite-like particles covering the titanium substrate surface (Fig. 4).

The physical and chemical parameters were listed in Table 1. Thickness of HA coating was thin and the morphology of the substrate was preserved after the coating, which was revealed by SEM. The roughness of HA-coated surface was slightly higher comparing to the one of machined titanium surface. Ca/P ratio of the coating was similar to HA.

**Loading efficiency of doxycycline on the HA coated surface**

The amount of doxycycline loaded on HA-coated surface was measured at 263 nm with uv-vis spectrophotometer after dissolving the HA with hydrochloric acid. The loading efficacy of doxycycline on the HA coated surface was 225±4.62 ng/mm² (n=6). The surface area of the custom-made mini-implant was 0.612 mm². Thus, the amount of the loaded doxycycline per the implant was 138±2.83 ng.

**Visual observation after the surgery**

Post-operatively, all animals tolerated the surgical procedures without any obvious complication. After 4 weeks, the overall wound after tooth extraction and implant placement properly healed (Fig. 2). No tissue dehiscence or visible infections were observed during the study period, and all samples were subjected to radiological and histological analyses.

**Radiographic observation (micro-CT)**

The new bone formation around the implant was obvious whereas implant penetration to the sinus was not found. The contact of the bone to the implant and the bone between the threads of the implant were observed in both groups (Fig. 5).

**Histological observation**

At 4-week time point in HA-coating group, the new formed trabecular woven bone was evident around the implant. Direct contact of the bone to the implant surface was obtained (Fig. 6a). The newly formed bone which was clearly distinguishable from the native bone with relatively light stains, gained direct contact with the implant surface.

At 4-week time point in Dox group, new bone had fulfilled the space between the threads and the contact of implant to the bone was close and there were less light stained areas of the newly formed bone in the threads than that in 4-week HA group (Fig. 6b). Well-established contact to the implant surface and less light stain indicated a more stable and mature condition of the newly formed bone comparing to HA group of same time point.

At 8-week time point in HA group, the woven bones
Fig. 4  SEM images of uncoated titanium implant surface (a, b) and HA-coated surface (c, d). A dense layer of HA was deposited on the surface of titanium textured surface (c) and in the high magnification image, apatite-like crystal particles are clearly evident (d).

Table 1  Physical and chemical parameters of the implant surface (n=8)

| Sample    | $R_a$ (μm) | Ca/P   | Thickness (μm) |
|-----------|------------|--------|----------------|
| HA-coated | 1.25±0.26  | 1.72±0.21 | 1.12±0.177     |
| Ti        | 1.13±0.39  | Null   | Null           |

Fig. 5  Micro-CT images of HA and Dox groups at 4 and 8 weeks.
Fig. 6  Histological images of HA and Dox groups at 4 and 8 weeks. TB staining. The magnification of the images in the red boxes was showed in the right side of each Figure.

Fig. 7  SEM images and EDS line scanning cross the implant interface. HA group (a, e) and Dox group (b, f) at 4 weeks. HA group (c, g) and Dox group (d, h) at 8 weeks.

became more mature (Fig. 6c). Newly formed bone areas were also able to be observed; however, less than at 4 weeks of same group.

At 8-week time point in Dox group, the woven bone was well located between the threads, and its contact to the implant was still firm (Fig. 6d). Bone status was more mature and stable comparing to the 4-week Dox group.

SEM images and element analysis of bone-implant interface
At 4 weeks, in HA group the bone contacted to the
implant surface; however, some areas abutted to the surface were not well mineralized (Figs. 7a, e).

At 4 weeks, in DOX group the bone firmly attached to the implant surface and lacunae in the new bone indicated active bone remodeling (Figs. 7b, f).

In 8-week HA-coating group, there was less contact between the new bone and implant surface comparing to the 4-week time point. The lacunae vary in size were visual in the NBA (Figs. 7c, g).

At 8 weeks in DOX group, bone tissue around the implant was quite dense and mature with organized osteocytes obtaining distinct layers along the surface, Lacunae were fewer contrast to that in 8-week HA-coating group; in some areas, the contact to implant was absent (Figs. 7d, h).

**BIC**

BIC (%) basing on the histological sections was presented in Fig. 8. The mean values (+standard deviation) for BIC were: HA-4w 66.06% (+4.21%), DOX-4w 74.68% (+2.25%), HA-8w 55.27% (+2.52%) and DOX-8w 78.76% (+3.32%) (Fig. 8). At 4 and 8 weeks BIC of Dox group was significantly higher than the one of HA group. In HA group BIC at 8-week was lower than the one at 4 weeks whereas in Dox group, the significantly difference between 4 and 8 weeks was not detected.

**NBA**

NBA (μm²) of the both groups at 4 and 8-week time point was presented in Fig. 9. Mean NBA measurements (+standard deviation) were: HA-4w 6,435.65 μm² (+378.7), DOX-4w 7,166.63 μm² (+847.65), HA-8w 4,900.35 μm² (+73.52) and DOX-8w 7,966.38 μm² (+397.1). At 8 weeks NBA of Dox group was significantly higher than the one of HA group.

**DISCUSSION**

In the present study histological observation clearly demonstrated new bone formation around the HA-coated surface, reconfirming high osseoconductivity of HA-coated surface. Notably, decrease of BIC in HA group from 4 weeks to 8 weeks (Fig. 8) indicates bone remodeling around the implant.

The biocompatibility of HA has been well documented both in vitro and in vivo studies. In different experimental systems, it has been shown that a mineralized bone matrix can be newly formed in close contact with the HA coating; however, the proof of osseoinductivity of HA has still been controversial. It has been demonstrated that HA might act as a solid-state matrix for adsorption, storage and controlled release of bone morphogenic proteins (BMPs) which locally initiate bone formation19-21).

In recent years, studies concerning the effect of HA on bone formation are progressively focused on molecular signal pathway level. Thorfve reported that HA coating is a strong activator for the Wnt signaling pathway, and that, to some degree, may explain its high bone induction capacity22). HA with micro-/nano-topography surface can also activate extracellular signal-related kinases (ERK), and p38 mitogen-activated protein kinase (MAPK) signaling pathways in bone marrow stromal cells (bMSCs)23). Furthermore, biomaterials composites with HA can promote bone marrow mesenchymal stem cells to induce osteogenic differentiation both in vitro and in vivo by activating integrin and BMP/Smad signaling pathway24). Therefore, it is reasonable that HA promotes bone regeneration in rat critical-sized calvariae defect models.

In this study, a simple protocol to use HA film as carrier of doxycycline was established.

Tetracyclines have been applied to HA for prolonged release of antibiotics. Tetracyclines were at first found to cause permanent discoloration of the teeth due to its high affinity for divalent metallic cations in teeth. The discoloration is influenced by the dosage used, length of treatment or expose, stage of teeth mineralization and activity of the mineralization process.

The adsorption and orientation of tetracyclines on HA was mainly due to Van Der Waals forces when tetracyclines were resolved in ethanol, p-dioxane and chloroform. The adsorption isotherms of tetracycline are reversal and ethanol and p-dioxane. The process of resorption does not affect the chemical integrity of tetracycline.
The releasing mechanism seems to be diffusion even though the formation of a chelate may also be involved\(^{20}\). Interestingly, it has been reported that combination of doxycycline and HA promotes adhesion, proliferation, and differentiation of bMSCs in vitro\(^{14}\).

Matrix metalloproteinases (MMP) are considered as key initiators of collagen degradation, thus contributing to bone resorption in bone remodeling\(^{20}\). During the process of bone remodeling, osteoblasts can initiate bone resorption by synthesizing the neutral proteinases including MMPs which can degrade osteoid. MMPs such as MMP-1, MMP-13 and MMP-14, which can degrade Type I collagen of demineralized bone, are also produced by osteoclasts. The inhibition of the MMP is the most widely documented and well characterized non-antibiotic property of doxycycline\(^{27-29}\).

It was shown that doxycycline also brought about the beneficial effect by inhibiting local and systemic oxidative stress\(^{30,31}\). In addition, recent studies have shown that doxycycline inhibits osteostageogenesis\(^{32}\), which makes it favorable to bone regeneration. Chang and Yamada’s study showed that collagen membrane combined with doxycycline utilized for guided tissue regeneration and local delivery system could enhance the new bone formation intensively\(^{33}\). It was also reported that doxycycline treated TiZr implant surface increased bone formation markers in vitro and in vivo\(^{40}\).

The morphometric data clearly showed that the doxy-treated implant surface was surrounded by more new bone within the threads than untreated group and had a higher BIC in both 4 and 8 weeks. Furthermore, the SEM and EDS analyses revealed that, although osseointegration of the implant was successfully achieved in the both groups, new bone formation of DOX group was superior to the one of HA group.

The different bone responses at 4 weeks were at least due to the difference of bone formation and mineralization on the HA-coated surfaces treated with or without doxycycline.

It has been reported that doxycycline prevents periodontal tissue breakdown by inhibiting local and systemic oxidative stress\(^{30}\), which in turn helps promote bone regeneration by activating osteoblastogenesis\(^{36}\).

Overall, it is very likely that the stimulation of bone apposition in Dox group in the present study was due to the various effects of doxycycline, including inhibitions of MMP, osteoclastic differentiation and survival, and stimulations of proliferation, differentiation and function of osteoblasts. Furthermore, the positive effect of doxycycline on bone apposition might be partially due to its anti-oxidative effect.

**CONCLUSION**

The results of the present study indicate that HA-coating implant surface treated with doxycycline promotes bone apposition around the implant.

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