Bone regeneration by freeze‐dried composite of octacalcium phosphate collagen and teriparatide

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Bone regeneration by freeze-dried composite of octacalcium phosphate collagen and teriparatide

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Abstract

Objective: Octacalcium phosphate (OCP) and collagen (col) composite (OCPcol) demonstrated superior bone regeneration properties, and its commercialization appears to be forthcoming. As a practical medical material for new combination products, we developed a freeze-dried composite with OCPcol and teriparatide (TPTD) (OCPcolTPTDf), and investigated its bone regenerative properties.

Materials and Methods: A disk of OCPcol was made by mixing OCP granules and atelocollagen for medical use. Then, OCPcolTPTDf was prepared by impregnation of the OCPcol disc with 1.0 μg or 0.1 μg of TPTD solution (OCPcolTPTDf 1.0 and OCPcolTPTDf 0.1, respectively) followed by lyophilization. In vitro release profiles of TPTD from OCPcolTPTDf were determined using an enzyme-linked immunosorbent assay. Implantation of OCPcolTPTDf or OCPcol was carried out for a rat critical-sized calvarial defect. And five defects in each group were collected after 12 weeks of implantation.

Results: The retention–release profiles of TPTD from OCPcolTPTDf supported a higher degree of retention of TPTD. Radiographic, histologic, and histomorphometric examinations indicated that regenerated bone was filled in most of the defects of the OCPcolTPTDf. Additionally, the OCPcolTPTDf groups showed significantly enhanced bone regeneration compared with the OCPcol group.

Conclusions: These results suggested that this newly developed bone regenerative composite could be a practical medical material.

Keywords: Bone tissue engineering, Calcium phosphate, Collagen, Parathyroid hormone

Running title: **Freeze-dried composite of OCPcol and teriparatide**

Introduction

Hydroxyapatite (HA: $Ca_{10}(PO_4)_6(OH)_2$) or β-tricalcium phosphate (β-TCP: $Ca_3(PO_4)_2$) is one of calcium phosphates (CaPs), and their synthetic type have already been developed and widely applied for bone substitutes of clinical cases (Habraken et al., 2016, Kokubo et al., 2003). Octacalcium phosphate (OCP: $Ca_8H_2(PO_4)_6 \bullet 5H_2O$) is one of CaPs and a synthetic bioresorbable material as well as β-TCP. OCP has been suggested to be a precursor of biological apatite in bones (Brown et al., 1962, Crane et al., 2006), and it has demonstrated significant bone formation abilities compared with other bone replacement materials (Kamakura et al., 2002). Although OCP has limitation of moldability and handling performance, development of OCP and collagen (col) composite (OCPcol) enabled to overcome its limitations (Kamakura et al., 2006). In preclinical studies, OCPcol demonstrated significant bone formation to OCP granules and other commercialized bone replacement materials in critical sized bone defects (Kamakura et al., 2007a, Kamakura et al., 2006, Tanuma et al., 2013). Additionally, OCPcol has achieved stable bone regeneration without cell transplantation, and the improvement of physiological bone remodeling is expected (Kamakura et al., 2006, Tanuma et al., 2013, Matsui et al., 2014). Moreover, OCPcol has good usability and cost performance (Iibuchi et al., 2010). After preclinical studies, a physician-initiated clinical study was carried out for extraction sockets of teeth and cyst holes, which demonstrated the safety and efficacy of OCPcol (Kawai et al., 2014, Kawai et al., 2017, Kawai et al., 2016). Recently, a sponsor-initiated clinical trial on OCPcol was completed, which was registered with the International Clinical Trials Registry in World Health Organization (JPRN-UMIN000018192), and commercialization on bone defects of oral region including sinus floor elevation and alveolar clefts is expected in 2018. However, independent use of OCPcol has limited bone regeneration, thus, the development of more trustworthy bone regenerative materials is awaited (Miura et al., 2012).

Metabolism and functions of calcium and phosphate is fundamentally regulated by parathyroid hormone (PTH) (Sibai et al., 2011). And teriparatide (TPTD) which is a recombinant form of PTH consisting of bioactive portion of N-terminal fragment comprising 34 amino acids (Niall et al., 1974). TPTD has a unique mechanism, and its continuous administration led to a decrease in bone volume, whereas its intermittent administration conducted to increase trabecular bone (Jilka et al., 1999, Tam et al., 1982). Furthermore, TPTD is the only authorized anabolic drug for the treatment of osteoporosis by the U.S. Food and Drug Administration (Morimoto et al., 2014).

Several preclinical studies were performed to repair the critical sized bone defects by applying scaffolds, such as an absorbable collagen sponge (Stancoven et al., 2013), demineralized bone matrix (Pensak et al., 2015, Stancoven et al., 2013), β-TCP (Yun et al., 2010), or poly-lactic acid (Jacobson et al., 2011) with intermittent subcutaneous administration of TPTD. Although high amounts of TPTD compared with that for the treatment for osteoporosis in clinical situations, were administrated in these studies, some researchers reported a significant effect (Jacobson et al., 2011, Yun et al., 2010), whereas others failed to find any effect (Pensak et al., 2015, Stancoven et al., 2013).

Recently, it was reported that the local single administration of TPTD (20 μg) with a collagen sponge is effective for repair of a rat calvarial defect (Auersvald et al., 2017). It was revealed that OCPcol with a local single administration of TPTD (1 or 0.1 μg) which is a low dosage similar to that for the treatment for osteoporosis, enhanced bone repair of the critical-sized bone defect (Kajii et al., 2017), and its modality might be practical for improving effective bone regeneration safely. However, it would be more versatile as a medical material if a freeze-dried composite with OCPcol and TPTD (OCPcolTPTDf) was created. Also, β-TCP is a synthetic bioresorbable material which has been a commonly used in maxillofacial lesion (Trombelli et al., 2014). Although OCP is a synthetic bioresorbable material as well as β-TCP,

OCP has unique features unlike β-TCP. It stimulated osteogenesis by osteoblastic cells and/or committed osteoprogenitors (Anada et al., 2008). And the implanted OCP is more resorbable than the implanted β-TCP (Kamakura et al., 2002), and it can serve as a core for initiating bone regeneration if implanted in a bone defect (Kamakura et al., 2001). Therefore, β-TCP is applied as a control of OCP, and the difference of bone regeneration with freeze-dried composite TPTD with OCPcol or β-TCP was investigated if implanted in a rat calvarial critical-sized bone defect.

Materials and Methods

1. Preparation of OCPcol and β-TCPcol

The preparation of OCPcol and β-TCPcol was described previously (Kamakura et al., 2007a). Briefly, OCP was prepared by direct precipitation (Suzuki et al., 1991), and sieved granules (particle sizes of 300 - 500 μm) of OCP were produced. Commercially available sintered β-TCP (Osferion, Olympus Terumo Biomaterials Corp., Tokyo, Japan) was purchased and sieved granules (particle sizes of 300–500 μm) of β-TCP were prepared for the fabrication of β-TCPcol. And, it was purchased the powder of lyophilized atelocollagen (NMP collagen PS; Nippon Meat Packers, Tsukuba, Ibaraki, Japan) which was digested by pepsin from the porcine dermis. After OCP granules were mixed with concentrated collagen, and it was completed 77% of the weight percentage of OCP in OCPcol. Likewise, the same volume of β-TCP granules as well as OCP granules were added to concentrated collagen and mixed. The OCPcol or β-TCPcol mixture was then lyophilized, and a disk was molded (9 mm diameter, 1.5 mm thickness). The OCPcol or β-TCPcol disks were prepared by a dehydrothermal treatment (150°C, 24 h) in a vacuum drying oven.

2. Preparation of OCPcolTPTD composite and β-TCPcolTPTD composite

Firstly, TPTD solution was prepared. Chemically-synthesized lyophilized teriparatide acetate (TERIBONE^{TM} Inj. 56.5 μg; Asahi Kasei Pharma Corp., Tokyo, Japan) was reconstituted and made a solution of 56.5 μg/ml. The process for the preparation of OCPcol disks combined with 1.0 μg of TPTD (OCPcolTPTDf 1.0) was as follows: an OCPcol disk was placed in 48 well plate, and 17.7 μl of TPTD solution (56.5 μg/ml) was impregnated into the OCPcol disk. The OCPcolTPTDf 1.0 disks were then lyophilized, followed by sterilization using electron beam irradiation (22 kGy). Likewise, 17.7 μl of TPTD solution (5.65 μg/ml) was transferred onto an OCPcol disk to prepare OCPcol disks combined with 0.1 μg of TPTD (OCPcolTPTDf 0.1). OCPcol disks without TPTD (OCPcol) were also sterilized by electron beam irradiation (22 kGy). And the same procedures were performed for β-TCPcol disks, and β-TCPcol disks combined with 1.0 μg or 0.1 μg of TPTD (β-TCPcolTPTDf 1.0, β-TCPcolTPTDf 0.1, respectively) or β-TCPcol without TPTD (β-TCPcol) were prepared.

3. Retention/release of TPTD from OCPcolTPTDf or β-TCPcolTPTDf

To examine the pattern of retention/release from OCPcolTPTDf or β-TCPcolTPTDf, each disk of OCPcolTPTDf 1.0, OCPcolTPTDf 0.1, β-TCPcolTPTDf 1.0, or β-TCPcolTPTDf 0.1 was placed into a 12-well plate (SUMILON, SUMITOMO BAKELITE Co., LTD, Tokyo, Japan) and soaked with 2.0 ml of saline containing 0.05% benzalkonium chloride, which was used to suppress the adsorption of TPTD onto the plate surface. The plates were placed in an incubator at 37°C. The supernatants were collected, and the plates were replenished with fresh saline containing 0.05% benzalkonium chloride at 1, 3, 7, 14, and 28 days. The concentration of TPTD released into the saline at each time point was determined by analyzing the supernatant using an enzyme-linked immunosorbent assay (ELISA) with a TPTD Assay Kit

(PTH 1-34 Teriparatide PK ELISA, Somru Bioscience, Charlottetown, Canada) and TPTD standards. Two specimens were used per group.

4. Implantation procedure

Twelve-week-old of male Wistar strain rats (SLC Corp.; Hamamatsu, Shizuoka, Japan) were used in this study. All procedures (2013-Biomedical Engineering Animal -003) were permitted by the Animal Research Committee of Tohoku University, which followed the principles of laboratory animal care and national laws. Body weight of rats were measured and they were anesthetized by dexmedetomidine hydrochloride (0.15–0.4 mg/kg), butorphanol tartrate (2.5–5 mg/kg), and midazolam (2 mg/kg) through intraperitoneal injection. After a parietal skin incision and ablation of the periosteum of the calvarium, 9 mm in diameter of standardized critical-sized defect was trephined in the calvarium (Kajii et al., 2018).

Randomly selected thirty experimental rats were separated into six groups (1. OCPcolTPTDf 1.0, 2. OCPcolTPTDf 0.1, 3. OCPcol, 4. β-TCPcolTPTDf 1.0, 5. β-TCPcolTPTDf 0.1, and 6. β-TCPcol) and five defects were treated in each group. Each type of a disk was implanted into the trephine defect after saline irrigation of the trephine defect. Finally, the treated periosteum and skin were relocated and sutured, and subcutaneous injection of cephalexin (15 mg/kg) was made to prevent infection. These experimental animals were euthanized 12 weeks after implantation. The total amounts of TPTD in this study were 3.93 ± 0.08 μg/kg in the OCPcolTPTDf 1.0 group, 0.38 ± 0.01 μg/kg in the OCPcolTPTDf 0.1 group, 3.84 ± 0.14 μg/kg in the β-TCPcolTPTDf 1.0 group, and 0.38 ± 0.02 μg/kg in the β-TCPcolTPTDf 0.1 group, when adapted to the average weight of each group at implantation.

5. Micro-computed tomography (micro-CT) examination

At 4 and 12 weeks after implantation, examination of rat calvarium was made by using an *in vivo* micro-CT system (Latheta LCT-200; Hitachi Aloka Medical, Tokyo, Japan) during intraperitoneal injection of sodium pentobarbital (50 mg/kg) (Kajii et al., 2017b). After measuring body weight, the treated defects were scanned in 120 μm thickness for slices and 60 μm pixel size. The images of micro-CT were acquired in standardized conditions (50 kVp, 500 μA, 3.6 ms). After finishing the CT analysis of 12 weeks, the experimental animals were euthanized by intraperitoneal injection of overdosed sodium pentobarbital. Then, the samples were resected with the surrounding bone, and the tissue were immersed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4.

6. Radiographic analysis

Radiography of the skulls were taken by a microradiography unit (Softex M-60, Softex Co., Ltd., Ebina, Kanagawa, Japan) with X-ray film (FR; Fuji photo film, Tokyo, Japan) in standardized conditions (45 kV, 1.5 mA, 2 min.), in which the OCPcol disks before implantation showed no radiopacity

7. Tissue preparation and a quantitative micrograph analysis

After the radiographs had been taken, the samples were decalcified in 10% EDTA in 0.01 M phosphate buffer, pH 7.4 at 4°C for 2–4 weeks. After embedding in paraffin, coronal section of the center of the defect was prepared. Hematoxylin and eosin (HE) staining was made and taken photographs by a photomicroscope (Leica DM2500; Leica Microsystems Japan, Tokyo, Japan). The histomorphometric analysis used in this study have been previously described (Kamakura et al., 2007a, Kamakura et al., 2002). The percentage of newly formed bone in the

defect (n-Bone%) was calculated as the area of newly formed bone/area of the original defect × 100. n-Bone% was computed by public-domain software (ImageJ 1.43).

8. Statistical analysis

Statistical analysis of n-Bone% was performed by using software (Excel v. X.; Microsoft Co., Redmond, WA), and the values of means ± standard deviation (SD) are reported. The chi-squared test and Bartlett's test were used to examine whether each group had a normal distribution and the homogeneity of variance across samples. To compare means among groups, one-way analysis of variance (ANOVA) was used and $p < 0.05$ was accepted significance. If the mean values were significantly different, a post-hoc test was performed by Tukey–Kramer multiple comparison analysis.

Results

1. Retention/release of TPTD from OCPcolTPTDf or β-TCPcolTPTDf

The release profiles of TPTD from OCPcolTPTDf or β-TCPcolTPTDf are shown in Figure 1. The release pattern of TPTD from OCPcolTPTDf 1.0 or β-TCPcolTPTDf 1.0 resulted in approximately 40% initial release within 1 day, followed by the release of smaller amounts over 7 days. Then, the release of TPTD then plateaued until 28 days, with a total of 46–49% of TPTD released. In the cases of OCPcolTPTDf 0.1 or β-TCPcolTPTDf 0.1, about 20% initial release was observed within 1 day, followed by smaller amounts over 7 days. The release of TPTD then plateaued until 28 days, with a total 24–25% of TPTD released.

2. Micro-CT analysis and radiographic examinations

Figure 2 indicates the central part of the defect of coronal sections. Although OCPcol disks had negligible radiopacity (Kamakura et al., 2007b), implanted OCPcol was radiopaque

because of conversion of the apatitic phase or regenerating bone (Iibuchi et al., 2010). In the OCPcolTPTDf 1.0 and OCPcolTPTDf 0.1 groups, radiopaque figures were observed in most of the defect including the central part at 4 weeks (Fig. 2). The uniform plate-like radiopacity was continuous with the original bone. The level of radiopacity increased with time, indicating repair of the defects. The boundary between the margin of the defect and the pre-existing bone became indistinguishable, and the radiopacity was comparable to that of the pre-existing bone (Fig. 2 and 3).

In the OCPcol group, most of the defect was occupied by scattered small radiopaque masses at 4 weeks after implantation (Fig. 2). These became uniform, small scattered radiopaque masses, which were intermingled in the defect at 12 weeks after implantation. The radiopacity was comparable to that of the pre-existing bone (Fig. 2 and 3). In the β-TCPcolTPTDf 1.0 group, granulous radiopacity was predominant in the defect at 4 weeks. Although this pattern increased with plate-like radiopacity, which extended from the defect margin at 12 weeks, the central part of the defect seemed to remain radiolucent with granules of β-TCP (Fig. 2 and 3). In the β-TCPcolTPTDf 0.1 group, granulous radiopacity was predominant in the defects at 4 and 12 weeks (Fig. 2 and 3). In the β-TCPcol group, granulous and cluster-like radiopacity were intermingled at 4 weeks, and the radiopacity increased at 12 weeks (Fig. 2 and 3).

3. Histological results of implants

Figures 4 and 5 indicates the side of the skin is upper and side of the dura mater is lower. In the OCPcolTPTDf 1.0 and OCPcolTPTDf 0.1 groups, newly formed bone was occupied in most of the defect, and the thickness of the new bone was similar to that of the pre-existing bone (Fig. 4). In addition, the boundary between the margin of the defect and the pre-existing bone was indistinguishable, and the implanted OCPcol was almost resorbed (Fig. 4 and 5). A

cortical bone-like structure was associate with some part of regenerated bone, and invasion of blood vessels and the formation of bone marrow was observed. In the OCPcol group, newly formed bone was filled in a large part of the defect, and most of the implanted OCPcol was resorbed. Some of the new bone which had united with implanted OCPcol, indicated a mosaic pattern (Fig. 4 and 5). In the β-TCPcolTPTDf 1.0, β-TCPcolTPTDf 0.1, and β-TCPcol groups, newly formed bone was filled in a large part of the defect, although most of the regenerated bone originated from the margin of the defect. A part of the implanted β-TCP granules was surrounded and replaced by newly formed bone. In addition, the implanted β-TCP granules, which were surrounded by fibrous connective tissue, were abundant in the bone defect (Fig. 4 and 5).

4. Histomorphometric examination

The n-Bone% in the OCPcolTPTDf 1.0, OCPcolTPTDf 0.1, and OCPcol groups were 58.6 ± 2.7%, 56.1 ± 3.0%, and 42.3 ± 4.3%, respectively. Furthermore, those of the β-TCPcolTPTDf 1.0, β-TCPcolTPTDf 0.1, and β-TCPcol groups were 36.8 ± 4.3%, 27.5 ± 10.0%, and 32.4 ± 9.1%, respectively. The mean values of n-Bone% indicated significant differences (ANOVA, $P = 6.3 \times 10^{-8}$), and Tukey–Kramer multiple comparison analysis showed significant differences between the OCPcolTPTDf 1.0 group and the other four groups (OCPcol, β-TCPcolTPTDf 1.0, β-TCPcolTPTDf 0.1, and β-TCPcol), between the OCPcolTPTDf 0.1 group and the other four groups (OCPcol, β-TCPcolTPTDf 1.0, β-TCPcolTPTDf 0.1, and β-TCPcol), and between the OCPcol and β-TCPcolTPTDf 0.1 groups (Fig. 6).

Discussion

Radiographic analyses including micro-CT, histologic, and histomorphometric analysis indicated that OCPcolTPTDf significantly enhanced bone regeneration more than OCPcol alone. In the OCPcolTPTDf groups, radiopaque figures were observed in most of the defect at 4 weeks, which increased and extended throughout the whole defect with time. New bone had the same thickness as the original bone, and it extended over the central part of the defect. In addition, some of the bone showed a cortical bone-like structure after maturation. In the OCPcol group, the newly formed bone was smaller than in the OCPcolTPTD groups, although it had integrated with implanted OCPcol. OCP stimulated bone regeneration by osteoblastic cells and/or committed osteoprogenitors (Anada et al., 2008) and TPTD enhanced osteogenic and osteoclastic activities (Morimoto et al., 2014). Therefore, OCPcol combined with TPTD through lyophilization could exhibit a synergistic effect with OCPcol and TPTD by increasing bone regeneration.

The β-TCPcolTPTDf groups (β-TCPcolTPTDf 1.0, β-TCPcolTPTDf 0.1) showed insufficient bone regeneration compared with the OCPcolTPTDf groups, suggesting that OCPcolTPTDf could enhance bone regeneration more than β-TCPcolTPTDf. Bone regeneration with OCPcolTPTDf originated from the OCPcolTPTDf itself and the margin of the defect, whereas that with β-TCPcolTPTDf was predominantly from the margin of the defect. This could be related to OCPcol nucleated bone regeneration if implanted into rodent critical sized calvarial defects (Kamakura et al., 2007a, Kamakura et al., 2006). Moreover, no significant differences in the newly formed bone were observed between the β-TCPcolTPTDf groups and β-TCPcol group. In these groups, the implanted β-TCP regenerated bone was intermingled with regenerated bone and fibrous connective tissue in the created defect, although granulous radiopacity changed to plate-like radiopacity with time. In addition, newly formed bone predominantly developed from the margin of the defect. It was considered that

β-TCP have not actively involved in the differentiation of osteoblasts, and the degradation of β-TCPcol which might be released TPTD was slower than OCPcol. Hence, it might be difficult to regenerate bone by β-TCPcol if TPTD was released around β-TCPcol, because there were few osteoprogenitor cells around β-TCPcol. It was correlated the histological finding that most of the regenerated bone originated from the margin of the defect in three β-TCPcol groups. This indicated that the synergistic effect could be restricted by the combination with β-TCPcol and TPTD. Consequently, a combination with OCPcol and TPTD could be specific for the synergistic effect of bone regeneration.

The release profiles of TPTD from OCPcolTPTDf 1.0 or β-TCPcolTPTDf 1.0 indicated that half of the TPTD was released within 7 days, and the other half remained at 28 days. In addition, that of OCPcolTPTDf 0.1 or β-TCPcolTPTDf 0.1 demonstrated about one-quarter of the TPTD was released within 7 days, and three-quarters remained at 28 days. These results resembled the release profile of bone morphogenic protein-2 (BMP-2) from Zein polydopamine TiO2 BMP-2, which could have triggered early differentiation markers and activated the expression of late osteogenic markers (Babitha et al., 2017). The release pattern of OCPcolTPTDf or β-TCPcolTPTDf suggested that TPTD was retained in OCPcol or β-TCPcol after initial release. It indicated that secondary release of TPTD from implant would be depended on the degradation of OCPcol or β-TCPcol. Because OCPcol was more resorbable than β-TCPcol (Kamakura et al., 2007a), the retained TPTD in OCPcol might be released more than that in β-TCPcol. Consequently, these procedures might enhance bone regeneration of OCPcolTPTDf more than β-TCPcolTPTD. In addition, this study demonstrated that bone regeneration was enhanced more with OCPcolTPTDf than with OCPcol. There could be a similar synergistic effect of OCPcol impregnated with TPTD, as reported recently (Kajii et al., 2017). However, bone regeneration with β-TCPcolTPTDf showed no significant differences compared with β-TCPcol, suggesting that there may be no synergistic effect of β-TCPcol and

TPTD. It was reported that OCP is capable of inducing the differentiation of osteoblastic cell lineages (Anada et al., 2008), and OCP incubated in rat serum proteins adsorbed bone formation-related proteins (Kaneko et al., 2011). In addition, it was observed a decrease in the concentration of Ca^{2+} and increase in inorganic phosphate during apatitic conversion from OCP (Anada et al., 2008). Therefore, it might be related to the difference in bone regenerative properties between OCPcol and β-TCPcol, and the behavior of osteogenic markers with OCPcolTPTDf should be investigated further.

In this study, no significant difference was observed between bone regeneration with OCPcolTPTDf 1.0 and that with OCPcolTPTDf 0.1 as well as bone regeneration with OCPcol with the local single administration of TPTD solution (1.0 μg or 0.1 μg) (Kajii et al., 2017). It could be considered that OCPcol combined with TPTD could not enhance bone regeneration in a dose-dependent manner. It suggested that the dose of 0.1 μg of TPTD in this defect might be sufficient to regenerate bone by OCPcolTPTDf. It is well known that usually administered doses of TPTD to patients with osteoporosis is about 1 μg/kg/week (Teribone™) or 0.2–0.5 μg/kg/day (Forteo®). And osteosarcoma might be occurred, if TPTD was subcutaneously given at 13.6 μg/kg/day for 2 years (total amount of about 10,000 μg/kg) (Watanabe et al., 2012). Because TPTD administered in this study (total amount of approximately 0.38–3.93 μg/kg) was distinctly lower dose than other previous studies as a single or total amount, OCPcolTPTDf should be a safe material for practical use with excellent bone regeneration. In order to be acceptable for clinical applications as OCPcolTPTDf, the influence of the dose of TPTD should be further investigated in larger animal studies.

In this study, it was confirmed that an OCPcol and TPTD composite (OCPcolTPTDf) could enhance bone regeneration as well as OCPcol with the single local administration of TPTD (OCPcolTPTDd) (Kajii et al., 2017), and these two materials significantly enhanced bone regeneration more than OCPcol alone. Compared with OCPcolTPTDd, OCPcolTPTDf

has several advantages. Because there is no need to prepare a solution of TPTD during the implantation procedure, the operation will be simpler and more reliable. In addition, OCPcolTPTDf has a good storage stability and high versatility. This newly developed OCPcol and TPTD composite (OCPcolTPTDf) could be a readily usable medical material and a therapeutic alternative for difficult bone defects or urgent surgery.

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Conflict of Interest

Some of the authors (S.K. and K. S.) have obtained a patent on OCPcol in Japan (#5046511). And some of the authors (A. I., F. K., H. T., K. S., and S. K.) have obtained a patent on OCPcolTPTDf.

References

Anada, T, Kumagai, T, Honda, Y, Masuda, T, Kamijo, R, Kamakura, S, Yoshihara, N, Kuriyagawa, T, Shimauchi, H. Suzuki, O (2008). Dose-dependent osteogenic effect of octacalcium phosphate on mouse bone marrow stromal cells. *Tissue Eng Part A 14*: 965-78. doi:10.1089/tea.2007.0339

Auersvald, CM, Santos, FR, Nakano, MM, Leoni, GB, de Sousa Neto, MD, Scariot, R, Giovanini, AF. Deliberador, TM (2017). The local administration of parathyroid hormone encourages the healing of bone defects in the rat calvaria: Micro-computed tomography, histological and histomorphometric evaluation. *Archives of oral biology 79*: 14-19. doi:10.1016/j.archoralbio.2017.02.016

Babitha, S, Annamalai, M, Dykas, MM, Saha, S, Poddar, K, Venugopal, JR, Ramakrishna, S, Venkatesan, T. Korrapati, PS (2017). Fabrication of a biomimetic ZeinPDA nanofibrous scaffold impregnated with BMP-2 peptide conjugated TiO2 nanoparticle for bone tissue engineering. *Journal of tissue engineering and regenerative medicine*. doi:10.1002/term.2563

Brown, WE, Smith, JP, Frazier, AW & Lehr, JR (1962). Crystallographic and Chemical Relations between Octacalcium Phosphate and Hydroxyapatite. *Nature 196*: 1050-+. doi:DOI 10.1038/1961050a0

Crane, NJ, Popescu, V, Morris, MD, Steenhuis, P & Ignelzi, MA, Jr. (2006). Raman spectroscopic evidence for octacalcium phosphate and other transient mineral species deposited during intramembranous mineralization. *Bone 39*: 434-42. doi:10.1016/j.bone.2006.02.059

Habraken, W, Habibovic, P, Epple, M & Bohner, M (2016). Calcium phosphates in biomedical applications: materials for the future? *Materials Today 19*: 69-87. doi:10.1016/j.mattod.2015.10.008

Iibuchi, S, Matsui, K, Kawai, T, Sasaki, K, Suzuki, O, Kamakura, S & Echigo, S (2010). Octacalcium phosphate (OCP) collagen composites enhance bone healing in a dog tooth extraction socket model. *International journal of oral and maxillofacial surgery 39*: 161-8. doi:10.1016/j.ijom.2009.12.006

Jacobson, JA, Yanoso-Scholl, L, Reynolds, DG, Dadali, T, Bradica, G, Bukata, S, Puzas, EJ, Zuscik, MJ, Rosier, R, O'Keefe, RJ, Schwarz, EM. Awad, HA (2011). Teriparatide therapy and beta-tricalcium phosphate enhance scaffold reconstruction of mouse femoral defects. *Tissue Eng Part A 17*: 389-98. doi:10.1089/ten.TEA.2010.0115

Jilka, RL, Weinstein, RS, Bellido, T, Roberson, P, Parfitt, AM & Manolagas, SC (1999). Increased bone formation by prevention of osteoblast apoptosis with parathyroid hormone. *J Clin Invest 104*: 439-46. doi:10.1172/JCI6610

Kajii, F, Iwai, A, Tanaka, H, Matsui, K, Kawai, T & Kamakura, S (2018). Single-dose local administration of teriparatide with a octacalcium phosphate collagen composite enhances bone regeneration in a rodent critical-sized calvarial defect. *J Biomed Mater Res B Appl Biomater* 106: 1851-7. doi:10.1002/jbm.b.33993

Kajii, F, Iwai, A, Tanaka, H, Matsui, K, Kawai, T & Kamakura, S (2018). Influence of electron beam irradiation doses on bone regeneration by octacalcium phosphate collagen composites. *Journal of tissue engineering and regenerative medicine 12*: e1186-e1194. doi:10.1002/term.2505

Kamakura, S, Sasaki, K, Homma, T, Honda, Y, Anada, T, Echigo, S & Suzuki, O (2007a). The primacy of octacalcium phosphate collagen composites in bone regeneration. *J Biomed Mater Res A 83*: 725-33. doi:10.1002/jbm.a.31332

Kamakura, S, Sasaki, K, Honda, Y, Anada, T, Matsui, K, Echigo, S & Suzuki, O (2007b). Dehydrothermal treatment of collagen influences on bone regeneration by octacalcium

phosphate (OCP) collagen composites. *Journal of tissue engineering and regenerative medicine 1*: 450-6. doi:10.1002/term.58

Kamakura, S, Sasaki, K, Honda, Y, Anada, T & Suzuki, O (2006). Octacalcium phosphate combined with collagen orthotopically enhances bone regeneration. *J Biomed Mater Res B Appl Biomater 79*: 210-7. doi:10.1002/jbm.b.30531

Kamakura, S, Sasano, Y, Homma, H, Suzuki, O, Kagayama, M & Motegi, K (2001). Implantation of octacalcium phosphate nucleates isolated bone formation in rat skull defects. *Oral diseases 7*: 259-65.

Kamakura, S, Sasano, Y, Shimizu, T, Hatori, K, Suzuki, O, Kagayama, M & Motegi, K (2002). Implanted octacalcium phosphate is more resorbable than beta-tricalcium phosphate and hydroxyapatite. *J Biomed Mater Res 59*: 29-34. doi:DOI 10.1002/jbm.1213

Kaneko, H, Kamiie, J, Kawakami, H, Anada, T, Honda, Y, Shiraishi, N, Kamakura, S, Terasaki, T, Shimauchi, H. Suzuki, O (2011). Proteome analysis of rat serum proteins adsorbed onto synthetic octacalcium phosphate crystals. *Analytical biochemistry 418*: 276-85. doi:10.1016/j.ab.2011.07.022

Kawai, T, Echigo, S, Matsui, K, Tanuma, Y, Takahashi, T, Suzuki, O & Kamakura, S (2014). First clinical application of octacalcium phosphate collagen composite in human bone defect. *Tissue Eng Part A 20*: 1336-41. doi:10.1089/ten.TEA.2013.0508

Kawai, T, Suzuki, O, Matsui, K, Tanuma, Y, Takahashi, T & Kamakura, S (2017). Octacalcium phosphate collagen composite facilitates bone regeneration of large mandibular bone defect in humans. *Journal of tissue engineering and regenerative medicine 11*: 1641-1647. doi:10.1002/term.2110

Kawai, T, Tanuma, Y, Matsui, K, Suzuki, O, Takahashi, T & Kamakura, S (2016). Clinical safety and efficacy of implantation of octacalcium phosphate collagen composites in

tooth extraction sockets and cyst holes. *Journal of tissue engineering 7*: 2041731416670770. doi:10.1177/2041731416670770

Kokubo, T, Kim, HM & Kawashita, M (2003). Novel bioactive materials with different mechanical properties. *Biomaterials 24*: 2161-75.

Matsui, A, Matsui, K, Handa, T, Tanuma, Y, Miura, K, Kato, Y, Kawai, T, Suzuki, O, Kamakura, S. Echigo, S (2014). The regenerated bone quality by implantation of octacalcium phosphate collagen composites in a canine alveolar cleft model. *The Cleft palate-craniofacial journal : official publication of the American Cleft Palate-Craniofacial Association 51*: 420-30. doi:10.1597/12-096

Miura, K, Matsui, K, Kawai, T, Kato, Y, Matsui, A, Suzuki, O, Kamakura, S. Echigo, S (2012). Octacalcium phosphate collagen composites with titanium mesh facilitate alveolar augmentation in canine mandibular bone defects. *International journal of oral and maxillofacial surgery 41*: 1161-9. doi:10.1016/j.ijom.2012.05.020

Morimoto, T, Kaito, T, Kashii, M, Matsuo, Y, Sugiura, T, Iwasaki, M & Yoshikawa, H (2014). Effect of Intermittent Administration of Teriparatide (Parathyroid Hormone 1-34) on Bone Morphogenetic Protein-Induced Bone Formation in a Rat Model of Spinal Fusion. *The Journal of bone and joint surgery. American volume 96*: e107. doi:10.2106/JBJS.M.01097

Niall, HD, Sauer, RT, Jacobs, JW, Keutmann, HT, Segre, GV, O'Riordan, JL, Aurbach, GD. Potts, JT, Jr. (1974). The amino-acid sequence of the amino-terminal 37 residues of human parathyroid hormone. *Proceedings of the National Academy of Sciences of the United States of America 71*: 384-8.

Pensak, M, Hong, SH, Dukas, A, Bayron, J, Tinsley, B, Jain, A, Tang, A, Rowe, D. Lieberman, JR (2015). Combination therapy with PTH and DBM cannot heal a critical sized murine

femoral defect. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society 33*: 1242-9. doi:10.1002/jor.22896

Sibai, T, Morgan, EF & Einhorn, TA (2011). Anabolic agents and bone quality. *Clin Orthop Relat Res 469*: 2215-24. doi:10.1007/s11999-010-1722-9

Stancoven, BW, Lee, J, Dixon, DR, McPherson, JC, 3rd, Bisch, FC, Wikesjo, UM & Susin, C (2013). Effect of bone morphogenetic protein-2, demineralized bone matrix and systemic parathyroid hormone (1-34) on local bone formation in a rat calvaria critical-size defect model. *J Periodontal Res 48*: 243-51. doi:10.1111/jre.12001

Suzuki, O, Nakamura, M, Miyasaka, Y, Kagayama, M & Sakurai, M (1991). Bone formation on synthetic precursors of hydroxyapatite. *Tohoku J Exp Med 164*: 37-50.

Tam, CS, Heersche, JN, Murray, TM & Parsons, JA (1982). Parathyroid hormone stimulates the bone apposition rate independently of its resorptive action: differential effects of intermittent and continuous administration. *Endocrinology 110*: 506-12. doi:10.1210/endo-110-2-506

Tanuma, Y, Matsui, K, Kawai, T, Matsui, A, Suzuki, O, Kamakura, S & Echigo, S (2013). Comparison of bone regeneration between octacalcium phosphate/collagen composite and beta-tricalcium phosphate in canine calvarial defect. *Oral surgery, oral medicine, oral pathology and oral radiology 115*: 9-17. doi:10.1016/j.oooo.2011.12.029

Trombelli, L, Franceschetti, G, Stacchi, C, Minenna, L, Riccardi, O, Di Raimondo, R, Rizzi, A. Farina, R (2014). Minimally invasive transcrestal sinus floor elevation with deproteinized bovine bone or beta-tricalcium phosphate: a multicenter, double-blind, randomized, controlled clinical trial. *J Clin Periodontol 41*: 311-9. doi:10.1111/jcpe.12210

Watanabe, A, Yoneyama, S, Nakajima, M, Sato, N, Takao-Kawabata, R, Isogai, Y, Sakurai-Tanikawa, A, Higuchi, K, Shimoi, A, Yamatoya, H, Yoshida, K. Kohira, T

(2012). Osteosarcoma in Sprague-Dawley rats after long-term treatment with teriparatide (human parathyroid hormone (1-34)). *J Toxicol Sci 37*: 617-29.

Yun, JI, Wikesjo, UM, Borke, JL, Bisch, FC, Lewis, JE, Herold, RW, Swiec, GD, Wood, JC. McPherson, JC, 3rd (2010). Effect of systemic parathyroid hormone (1-34) and a beta-tricalcium phosphate biomaterial on local bone formation in a critical-size rat calvarial defect model. *J Clin Periodontol 37*: 419-26. doi:10.1111/j.1600-051X.2010.01547.x

Figure legends

Figure 1: Release profile of TPTD from OCPcol or β -TCPcol:

The release of TPTD from OCPcolTPTDf 1.0 or β-TCPcolTPTDf 1.0 resulted in an initial release of approximately 40% within 1 day, and a total 46–49% of TPTD was released within 28 days. In the case of OCPcolTPTDf 0.1 or β-TCPcolTPTDf 0.1, the initial release was approximately 20% within 1 day, and a total 24–25% of TPTD was released within 28 days.

Figure 2: Coronal sections examined using micro-computed tomography at the center of the created defects:

In the OCPcolTPTDf 1.0 and OCPcolTPTDf 0.1 groups, most of the defect was occupied by radiopaque figures with a thickness similar to that of the pre-existing bone. This radiopacity increased with time and repaired the defects. In the OCPcol group, most of the defect was occupied by scattered small radiopaque masses at 4 weeks after implantation. These masses enlarged and fused with each other over time. In the β-TCPcolTPTDf 1.0, β-TCPcolTPTDf 0.1, and β-TCPcol groups, granulous radiopacity was predominantly detected in the defect at 4 weeks. This pattern expanded to form a plate-like radiopacity, which extended from the margin of the defect at 12 weeks. Arrow heads: Defect margin, Bars: 3 mm

Figure 3: Radiographic examination:

In the OCPcolTPTDf 1.0 and OCPcolTPTDf 0.1 groups, most of the defect was occupied by uniform radiopacity. The boundary between the margin of the defect and the pre-existing bone became indistinguishable. In the OCPcol group, uniform and small scattered radiopaque masses intermingled in the created defect. In the β-TCPcolTPTDf 1.0, β-TCPcolTPTDf 0.1, and β-TCPcol groups, plate-like and granulous radiopacity intermingled in the created defect. Bars: 4 mm

Figure 4: Histological overview of implants:

In the OCPcolTPTDf 1.0 and OCPcolTPTDf 0.1 groups, newly formed bone was occupied in most of the defect, and the thickness of the new bone was similar to that of the pre-existing bone.

In addition, the boundary between the margin of the defect and the pre-existing bone was indistinguishable. In the OCPcol group, newly formed bone was filled in a large part of the defect. In the β-TCPcolTPTDf 1.0, β-TCPcolTPTDf 0.1, and β-TCPcol groups, most of the defect was filled with regenerated bone, whereas the most of the regenerated bone originated from the margin of the defect. Hematoxylin eosin stain, Arrow heads: Defect margin, Bars: 3 mm

Figure 5: Histological findings of implants:

In the OCPcolTPTDf 1.0 and OCPcolTPTDf 0.1 groups, most of the defect was filled with newly formed bone, and most of the implanted OCPcol was resorbed. In the OCPcol group, most of the implanted OCPcol was resorbed, and some of the new bone which had united with implanted OCPcol, indicated a mosaic pattern In the β-TCPcolTPTDf 1.0, β-TCPcolTPTDf 0.1, and β-TCPcol groups, part of the implanted β-TCP granules was surrounded and replaced by the newly formed bone. In addition, the implanted β-TCP granules were surrounded by abundant fibrous connective tissue is in the bone defect. Hematoxylin eosin stain, Asterisks: Implanted OCP or β–TCP, Bars: 200 μm

Figure 6: Quantitative analysis of newly formed bone in defects:

The percentage of newly formed bone in defects (n-Bone%) ± standard deviation (SD) is shown for the OCPcolTPTDf 1.0, OCPcolTPTDf 0.1, OCPcol, β-TCPcolTPTDf 1.0, β-TCPcolTPTDf 0.1, and β-TCPcol groups. A significant difference was observed in mean values between the OCPcolTPTDf 1.0 group and the other four groups (OCPcol, β-TCPcolTPTDf 1.0, β-TCPcolTPTDf 0.1, and β-TCPcol), between the OCPcolTPTDf 0.1 group and the other four groups (OCPcol, β-TCPcolTPTDf 1.0, β-TCPcolTPTDf 0.1, and β-TCPcol), and between the OCPcol and β-TCPcolTPTDf 0.1 groups. Data are shown as the means ± SD of five specimens. Asterisks: $p < 0.05$