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Osteopontin functionalization of hydroxyapatite nanoparticles in a PDLLA matrix promotes bone formation

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Abstract

We studied the osteoconductive tissue response of hydroxyapatite (HA) nanoparticles functionalized with osteopontin (OPN) in a matrix of poly-D,L-lactic-acid (PDLLA). In a canine endosseous 0.75-mm gap implant model, we tested the osteointegrative impact of the OPN functionalized composite as an implant coating, and a non-functionalized composite was used as reference control. During the four weeks of observation, the OPN functionalized composite coating significantly increased the formation of new bone in the porosities of the implant, but no differences were observed in the gap. The study provides evidence of its potential use either alone or in combination with other osteoconductive compounds.

Keywords

biocompatibility; osteopontin; hydroxyapatite; composite; osteointegration

INTRODUCTION

Even though orthopedic joint replacements are highly successful, the longevity of orthopedic joint replacements is still limited, and revision surgery of loosened implants constitutes a substantial fraction of total surgeries.¹ Therefore, improving the longevity of orthopedic joint replacements would have a large socioeconomical impact. One strategy would be to design osteoconductive implants that improve early fixation by enhancing the formation of anchoring bone tissue on the implant surface. It is generally accepted that early stable fixation is a prerequisite for favorable long-term implant performance.^{2,3}

Osteopontin (OPN) is an extracellular bone protein discovered in 1985.⁴ It is part of the SIBLING (small integrin-binding ligand *N*-linked glycoprotein) family of proteins and is found abundantly in the mineral/tissue interface of bone.⁵ It contains a hydroxyapatite (HA) binding sequence and a cell binding domain with an Arginine-Glycine-Aspartic acid (RGD) sequence. OPN plays a key role in the bone remodeling process by directing the activity of

bone resorbing osteoclasts and bone forming osteoblasts.^{6–9} Furthermore, OPN has been shown to promote angiogenesis^{6,10} and to reduce foreign body reaction to implants.^{11,12} Thus, by anchoring OPN to the surface of orthopedic joint replacements, it may be possible to improve their osteoconductivity.

The *in vitro* cell interaction of OPN depends on the chemistry of the surface to which the protein is adsorbed.^{13–16} Previous *in vivo* studies have focused on the 2D surface functionalization of single component biomaterials requiring only small amounts of OPN, e.g., by spontaneous surface adsorption on HA¹⁷ or by covalent immobilization on poly-HEMA.¹¹ An alternative way of incorporating OPN in biomaterials is to functionalize the surface of particles with OPN and disperse the particles in a polymer matrix. This approach incorporates a larger, effective functionalization area and 3D distribution of the protein throughout the material. Accordingly, it requires a larger amount of OPN. Bovine milk contains large amounts of OPN, and extraction of OPN from this particular source is very cost efficient. In a recent study, it was further shown that OPN from bovine milk enhanced spreading and acted chemo-tactically on human mesenchymal stem cells when adsorbed on hydroxyapatite (HA).¹⁸

Biodegradable two-component composites of HA particles and poly-D,L-lactic-acid (PDLLA) have consistently shown excellent osteoconductivity,^{19–21} and it appears to be correlated to the availability of HA on the composite surface.^{21,22} The osteoconductivity of HA is partly ascribed to the selective adsorption of proteins effective in attracting bone-forming cells.²³ Preadsorbing the HA particles of a PDLLA/HA composite with particular proteins could therefore predetermine the functionality of the composite specifically. OPN preadsorption may guide a bone-directed remodeling process by attracting and activating bone resorbing osteoclasts and bone forming osteoblasts.

In this study, a novel implant coating of HA nanoparticles (20–70 nm diameter) preadsorbed with bovine OPN and mixed with PDLLA at 50/50 vol% was tested in a canine 0.75-mm gap model. To evaluate the osseointegration of the implant, the OPN-functionalized implant coating was compared with a similar coating without OPN. Osseointegration was evaluated by examining the mechanical implant fixation and the relative amounts of bone, fibrous tissue, and marrow tissue formed in the peri-implant region. It has been hypothesized that OPN preadsorption on the HA particles increases bone formation and reduces fibrous tissue formation, thereby improving the mechanical fixation of the implant. The obtained results indeed show increased bone formation, but no differences in the fibrous tissue formation and mechanical fixation of the implant.

MATERIALS AND METHODS

Experimental design

Nine dogs were included and each animal received two implants in the proximal tibiae. The implants (6 mm diameter) were surrounded by a 0.75-mm coaxial defect (Fig. 1). The implants were coated with either pure PDLLA/HA composite (COMP) coating as control or OPN functionalized composite (OPN-COMP) as intervention. The study was conducted in a

paired design where each dog received both treatments. The implantation site was alternated systematically with random start. The observation time was 4 weeks.

Implants

Custom-made cylindrical titanium alloy core implants (Ti-6Al-4V) with commercially available pure titanium porous coating (Porocoat) manufactured by Depuy Inc., Warsaw, IN, were used. The dimensions of the coated implants were 10 mm in length and 6 mm in diameter. The porosity was provided by multiple layers of titanium beads sintered onto the core implant, giving an average porosity of 40% by volume and an average pore size of 250 μm with lowest porosity and pore size (20%, 100 μm) at the core implant and highest at the outer boundary (90%, 500 μm).²⁴ Endcaps (7.5 mm diameter) were attached to implant ends. When inserted into a 7.5-mm drill hole, the endcaps centered the implant and provided a uniform 0.75-mm gap around it. The implants were autoclaved before the coating procedure.

Coating procedure

The coating procedure including characterization was a slight modification of a previously reported method²⁵; 20–70-nm HA particles in ethanol (Berkeley Advanced Biomaterials, CA) with a surface area of 110 m²/g were used as specified by the manufacturer. The HA particles were dried 24 h in an evacuated exicator with silica gel. Two 1-g portions of dried particles were probe sonicated for 1 min in milliQ water at 50 mg/mL (20 mL) in 50-mL test tubes in ice cooled water. The pH value was adjusted to 7.4 with 1M phosphoric acid (Sigma-Aldrich, Brøndby, Denmark); 52 mg of OPN (26 mg/mL in milliQ water) was added to the first particle dispersion and 2 mL of pure milliQ was added to the second dispersion. Both dispersions were hand stirred. After 1 min both dispersions were probe sonicated for 10–15 s and immersed in liquid nitrogen to limit aggregation. The frozen dispersions were freeze dried for 5 days at –30°C and 100 mTorr.

A small sample from each portion was taken for surface elemental analysis using an x-ray photoelectron spectroscope (XPS; Kratos Axis Ultra DLD spectrometer, Kratos Analytical, UK) equipped with a monochromatized aluminum x-ray source (AlK α , $h\nu = 1486.6$ eV) operating at 15 mA and 15 kV (225 W). A hybrid lens (electrostatic and magnetic) mode was employed along with an analysis area of approximately 300 $\times$ 700 μm . Survey spectra were recorded over the range of 0–1100 eV binding energy with an analyzer pass energy of 160 eV. XPS data were processed with CasaXPS software (Casa Software Ltd., UK).

Henceforth, all handling of components was done under sterile conditions. Two solutions of PDLLA (50 mg; Resomer 203, Boehringer-Ingelheim, Germany) in 1.5 mL in ethylacetate (Sigma-Aldrich, Brøndby, Denmark) were mixed in Eppendorf-tubes and stirred regularly for 1 h; 125 mg of freeze-dried HA particles with or without OPN were added to the PDLLA solutions. Both solutions were probe sonicated for 30 s prior to implant coating. The autoclaved implants were dipped twice in the sonicated solution/dispersion with a 10-s interval, placed in a sterile exicator, vacuum dried for 48 h, and double packed in sterile bags for surgery. The resulting coating characteristics were reported previously.²⁵

Animals and surgical procedure

Nine skeletally mature mongrel dogs with a mean weight of 25.1 kg (range 23.5–26.5) were included in the study. The dogs were bred for scientific purposes, and the experiment was approved by IACUC of the Minneapolis Medical Research Foundation, performed in their AAALAC approved facility, and conformed to NIH guidelines for use of animals in research.

With the dogs under general anesthesia and in sterile conditions, a skin incision was made with cautery on the medial proximal tibia leaving the medial collateral ligament intact. The periosteum was removed only at the implantation site. A guide wire was inserted anteromedially approximately 3.4 cm distal to the joint line oriented perpendicular to the surface. Over the guide wire, a cannulated drill ($\varnothing$ 7.5 mm) was used to drill cylindrical cavities at a speed of maximum two rotations per second. The edge of the hole was trimmed with a scalpel to remove excessive periosteum, and the cavities were irrigated with 10 mL of saline for removal of loose bone chips. In each drill hole, the implants with endcaps were inserted in a uniform central placement and the soft tissues closed in layers. The procedure was repeated for the opposite side. All 18 implants were inserted by the same surgeon. The dogs were given 1 g of ceftriaxone administered immediately before each surgery and 3 days postoperatively. Buprenorphine hydrochloride at a dosage of 0.3 mg/mL 0.0075 mg/kg per day intramuscularly was given as postoperative analgesic treatment. All animals were allowed unlimited activity. After an observation time of four weeks, the dogs were sedated and euthanized with an overdose of hypersaturated barbiturate.

Specimen preparation

The proximal tibiae were harvested and stored at -20°C prior to preparation. Two transverse bone-implant specimens were cut on an Accutom-50 precision cut-off machine (Struers, Ballerup, DK). The outermost specimen of 3 mm was stored at -20°C prior to mechanical testing (Fig. 2). The innermost section was prepared for histomorphometry (Fig. 2). These specimens were dehydrated in graded ethanol (70–100%) containing basic fuchsin and embedded in methylmethacrylate (Technovit 7200 VCL, Heraeus-Kulzer, Hanau, Germany). Using the vertical sectioning technique,²⁶ each specimen was cut into four 30- μm thick histological sections with a microtome (KDG-95, MeProTech, Heerhugowaard, Holland) (Fig. 2). Finally, these were surface counter-stained with 2% light green for 2 min, rinsed, and mounted on glass. This preparation provided red staining for noncalcified tissue and green staining for calcified tissue.

Mechanical testing

Thawed specimens were tested to failure by the axial push-out test on an MTS 858 Mini Bionix test machine (MTS Systems Corporation, Eden Prairie, MN) with a 2.5-kN axial load cell. Testing was performed blinded and in one session. The specimens were placed with the cortical side facing up on a metal support jig with the implant centered over a 7.4-mm opening and under a cylindrical test probe of 5 mm diameter (Fig. 2). A preload of 2 N defined the contact position for the start of the test. The implants were then pushed out of the surrounding tissue in the direction of the implant axis at a velocity of 5 mm/min. Load vs implant displacement data were continuously recorded. From these data, the mechanical

implant fixation parameters such as ultimate shear strength, apparent shear stiffness, and total energy absorption were calculated as described earlier.²⁷

Histological testing

Histomorphometry was performed blinded using an Olympus BX-50 light microscope and a stereological toolbox (C.A.S.T-Grid; Olympus, Denmark), which includes both software and a motorized stage. With the aid of the software, two regions of interest were defined: zone 1 from the innermost part of the core implant surface and 400 μm of the porosity of the Porocoat implant, and zone 2 from the outer limit of zone 1 to the outer limit of the drilled bone defect except for the outermost 10% (see Fig. 1). In zone 1 the area fractions of new bone, fibrous tissue, and marrow space on the implant surfaces were quantified by the line-interception technique.²⁶ In this zone, the point-counting technique for sectional tissue area fractions for estimating volume fractions was not suitable. Given the narrow crevices of the Porocoat implant, the area fractions estimated with the line-interception technique are assumed as representative estimates of the tissue volume presentations in the porous space. In zone 2, area fractions and volume fractions of the same tissues were estimated by the line-interception technique and point-counting technique, respectively.²⁸ These techniques provide highly reliable results with negligible bias.²⁷

Bone was surface-stained green, and therefore, easy to distinguish from the other tissues. Fibrous tissue was identified by the presence of clearly visible fiber complexes and low cell density. The fibrous tissue largely appeared not only as a oriented, dense, and well-organized network, but also as a loosely, not clearly oriented, interconnected fibrous network. Marrow space consisted of fat vacuoles and surrounding blood cells.

Statistics

Statistical analysis was performed using STATA Intercooled 9.0 software (STATAcorp, College Station, TX). Normal distribution was assumed on all the data. Therefore, a parametric paired analysis was performed with Student's paired *t*-test (two-tailed). P values less than 0.05 were considered statistically significant.

RESULTS

XPS of HA particles with and without OPN

The XPS results of the elemental composition of the particle surfaces is depicted in Table I. On both types of HA particles (with and without OPN), calcium (Ca), phosphorus (P), oxygen (O), and carbon (C) were present, whereas nitrogen (N) was found exclusively on particles with adsorbed OPN. The Ca/P ratio was 1.69 ± 0.05 and 1.71 ± 0.03 (mean $\pm$ SEM) for pure HA particles and OPN functionalized HA particles, respectively, which is in good agreement with the theoretical Ca/P value of 1.67 for stoichiometric HA. Although carbon is not present in pure HA, it was detected in relatively large amounts on both samples, most likely due to hydrocarbon adsorption from the ambient. On HA with OPN, the carbon presence was slightly larger.

Observations on animals

All nine dogs were fully weight bearing within three days after surgery and completed the four-week observation period without signs of infection or other complications.

Mechanical test

Implants with OPN-COMP did not perform better compared with COMP. In all parameters, however, a tendency toward stronger mechanical fixation in the OPN-COMP group was observed, although not with statistical significance. The results are listed in Table II. The total energy absorption of the bone/implant binding was on average 38% higher when using the OPN-COMP coating. The ultimate shear strength and apparent shear stiffness of the same implants were moderately larger (14% and 3%, respectively) as compared to the COMP coating.

Histological observations

Overall no signs of infection or severe foreign body reaction were observed. In Figure 3, representative sections of the implant/tissue interfaces for both coatings are depicted at low and high magnification. With both coatings newly formed bone trabeculae were observed in the gap, and surface associated bone was observed on the inner pore surfaces in the Porocoat implant. No apparent differences in the gaps were observed, whereas the porosities with OPNCOMP coating clearly contained a higher degree of newly formed bone. The remains of the porosities were dominated by fibrous tissue, whereas the gaps were dominated by marrow tissue.

Histomorphometry

The results from the histomorphometry analysis are listed in Table III. The pore zone 1 of implants coated with OPNCOMP were covered 30% on average with newly formed bone, and equal amounts of fibrous and marrow tissue covered the remaining region. Zone 1 of implants with COMP coating were covered with significantly lower (15%) newly formed bone and the remaining region with primarily fibrous tissue (51%).

The outermost surfaces of the titanium Porocoat implant corresponding to the innermost boundary of zone 2 were covered in both groups primarily with marrow tissue. On OPN-COMP coated implants, a tendency toward higher ongrowth of bone and lower ongrowth of fibrous tissue was seen.

No obvious differences in volume fractions were observed in the marrow tissue-dominated gaps.

DISCUSSION

The purpose of the study was to investigate the osseointegrative effect of OPN functionalization of a HA/PDLLA composite coating on a commercially available titanium implant surface. Mechanical implant fixation and histomorphometrical analysis of tissue in contact with implant surface and peri-implant tissue formation were used as parameters. The implants with the OPN functionalized coating showed a significantly larger amount of bone

formed in the implant porosities (zone 1). These implants also showed a tendency toward better anchorage in the surrounding bone; however, no statistically difference was observed.

Canine species is a commonly used large animal model in orthopedic implant research. This is due to the resemblance of bone structure with human bone structure. The proximal part of tibia was chosen because of the large amount of trabecular bone, which is representative of the bony fixation regions of clinically used joint prostheses. In clinical settings, erratic gaps between the implant and surrounding bone bed are present and gap models are considered relevant in experimental implant research. Even with well-fitted uncemented implants, gaps of 0.75 mm can be expected.^{29,30}

Contralateral implants allow a paired study design, allowing OPN functionalized and non-functionalized composite coatings to be compared within each animal, thereby reducing the effect of biological difference between individuals. Therefore, the design allows a reduction in the number of individuals needed to be included in the study. The implant model is nonarticulating and thereby limited as the effects of direct weight-bearing conditions are not addressed.

The choice of using large amounts of OPN from a different species is debatable for an implant material intended for human clinical application. Potential transfer of disease and acute or chronic immunological reactions to the protein must be carefully considered and evaluated. In the conceptual frame of this study, no such occurrence was observed, and further research, however, should address this question. The use of milk OPN also raises a question regarding post-translational modifications. It is well known that posttranslational modifications of OPN may vary in different tissues. Phosphorylations in particular are considered important in OPN function. Milk-derived OPN is known to be highly phosphorylated. In bone OPN, the phosphorylation level fluctuates with the functional status of the tissue.³¹ The high phosphorylation level of OPN in general and bovine milk-derived OPN in particular have been shown to promote osteoclastic resorption activity,³² which is the starting point of the bone remodeling process. The application of milk osteopontin in a biomaterials context should aim at utilizing this effect. This is attempted in the present study by functionalizing a synthetic material, thereby making it susceptible to the osteoclastic remodeling process.

The choice of OPN amount for functionalization of HA particles corresponds to a particle surface coverage of 50%, assuming full availability of the HA particle surface area.¹⁸ This amount was chosen to avoid free nonadsorbed OPN. It is recognized that preadsorbing the particles in the control coating with an inactive control protein, e.g. albumin, would be a more suitable control. This study, however, is an extension of a previous study in our group.¹⁸ Here OPN was characterized and tested in an *in vitro* cell study, where OPN was adsorbed on HA and was compared with HA adsorbed with proteins from serum-containing media. To maintain deductive continuity, the HA particles in the control group of this study were not preadsorbed with proteins and instead allowed for random adsorption of serum proteins.

XPS survey spectroscopy was used to quantify the relative surface elemental composition of HA particles with and without protein. The Ca/P ratio of pure HA particles (1.69 ± 0.05) is not statistically different from stoichiometric HA (1.67). The Ca/P ratio of OPN functionalized HA particles (1.71 ± 0.03) is statistically different from stoichiometric HA. This small difference most likely is a direct effect of the OPN functionalization or an indirect effect that changes the calcium and/or phosphate detection. The carbon present on HA particles without OPN is most likely contamination, which is difficult to avoid with XPS of non-carbonaceous particles.³³ With addition of OPN, the analysis identifies an increase in carbon and the appearance of nitrogen. The relative amount of nitrogen detected is in agreement with a previous study of full coverage protein adsorption on HA microparticles.³³

The 50/50 vol% mixture of PDLLA and HA was chosen to allow for particle aggregation and the void between particles to be filled with polymer. The coating technique is identical to that used in Ref. 25, which includes a detailed description of the resulting coating including surface characterization with atomic force microscopy and thickness estimation with scanning electron microscopy. The coating thickness resulting from a single dip on a flat surface was approximately 12 μm . It is recognized that a uniform thickness on the implants cannot be anticipated due to accumulation in concavities. Furthermore, a completely homogeneous composite surface cannot be anticipated due to formation of large HA-particle aggregates before evaporation of the organic solvent. This may result in voids within the bulk material of low HA presence and in HA-aggregate protrusions on the surface.

The dip coating step under sterile conditions ensures sterility of the implants, as all parts are directly exposed to organic solvents. No microbial culture testing, however, was performed. In a larger production scale for clinical application, additional sterilization may be needed. As autoclaving will denature the protein, gamma radiation could be considered. Further research, however, on the optimization of coating procedure and the effect thereof is necessary.

The OPN-COMP coating did not significantly improve mechanical fixation. It is recognized that the observation time of four weeks evaluates fixation before the mechanical properties of newly formed tissue are fully matured.³⁴ A longer observation time and consequently a more mature tissue response might enlarge the observed difference. The fairly short observation time, however, has previously proven to be sensitive to the impact of bone active surface coatings in a gap model.³⁵ The results for both implant coatings are similar to that of a pure HA coating as tested with the same *in vivo* model and observation time.³⁶ It should be noted that the bone tissue around the implant part was not examined histologically following the mechanical test. By visual inspection, the failure generally occurred in the inner part of zone 2 corresponding to the outer limit of the Porocoat implant. Although the implants are completely implanted in trabecular bone, the mechanical fixation may be weakened by resorption of the cortical bone surrounding the implant site. Another potential bias is weakening of the bone tissue during freezing and thawing before analysis.³⁷ This effect may also weaken the bonding strength of the bone/implant interface. These potential biases cannot be accounted for; they are, however, not expected to impact the relative results.

In the inner zone 1 of the Porocoat implant, a significantly larger amount of newly formed bone covered the surface of the implants in the OPN-COMP group. Furthermore, a nonsignificant tendency toward lower fibrous tissue formation was observed with OPN-COMP. While both coating types are resorbed during the observation time, it appears that OPN functionalization improves the ability to guide formation of bone during degradation of the coating. The improved osteoconductivity is most likely a result of induced activity of bone forming cells, since studies evaluating prefunctionalization of 2D surfaces have shown that it is possible to control the cellular response to a surface *in vitro*^{13–16,18} and *in vivo*¹¹ by coating the surface with OPN, whereas releasable proteins in PDLLA coatings have proved to provide the opportunity for sustained protein activity *in vivo*.^{38–40} In the present study, both modes of protein actions were considered. The improved activation of bone forming cells can be speculated to be either an effect of OPN acting as a surface protein facilitating the cell/material interaction, which was the intention with immobilization on the HA particles, or as an effect of OPN being released and acting paracrine. The positive impact of OPN on new bone formation was observed directly on the inner surface of the porosities where the coatings were present preoperatively. This suggests that the impact was directly in the cell–material interaction or, in the case of a protein release effect, a localized effect.

On the outer limit of the Porocoat implant corresponding to the inner part of zone 2, a nonsignificant tendency toward higher ongrowth of bone and lower ongrowth of fibrous tissue was observed on the OPN-COMP coated implants. Compared to zone 1, the OPN-COMP coated implants had a lower bone ongrowth in zone 2. The control had approximately the same ongrowth in zone 1 and 2. Apparently, the OPN-COMP coating had a lower impact in zone 2. Evidently, a larger amount of effective coating material was found in zone 1 caused by the larger area relative to space in the porosity. It could, furthermore, be an effect of coating accumulation in the concavities of the implant during the dip-coating procedure, which leaves a relatively thinner and less effective coating on the outer beads of the Porocoat implant.

No significant differences were observed in the volumetric tissue fractions of zone 2. In a previous *in vivo* study in our laboratory, a coating identical with the composite coating without OPN used in this study was investigated, and it was correspondingly found that the osteoconductive effect was limited to the surface of the coating.²⁵

OPN addition appears to have only a local effect, which indicates, that the protein activity is in the direct cell/material interaction. Nonetheless, with the preparation technique used, it is not possible to further elucidate the osteoconductive mechanism as only tissue type can be quantified. It may be that OPN enhances the activity of osteoclasts and osteoblasts directly in the material–tissue interface during material degradation. This was the intended mode of material/tissue interaction, but other scenarios are possible. For instance, it is also possible that the relatively large presence of protein on the particle surfaces changes the degradation profile of the composite, thereby changing the presentation of HA to the implant exterior. The study encourages further research in this new approach for functionalizing composites and particularly in the potential role of OPN from bovine milk in osteoconductive materials.

CONCLUSION

We have shown that preadsorption of osteopontin from bovine milk on the HA particles of a degradable PDLA/HA composite enhances the composite osteoconductive properties when used as a coating on a commercial titanium implant. The bulk functionalization approach stimulates further research in composite materials and their versatility as biocompatible materials with controllable properties at the nanoscale. The clinical use of proteins derived from another species, however, should be carefully considered in further research. A cost-efficient device fabrication and wide possibilities for advancement in mechanical properties and functionalization might lead to replacement of pure HA in orthopedic application areas with HA-containing composites.

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FIGURE 1.

Implant model. End-caps with same diameter as the drill hole (7.5 mm) are applied to maintain a uniform gap (0.75 mm) around the implant (diameter 6 mm). Zone 1 is the porous 400 μm of the implant, zone 2 is the area from outer limit of zone 1 to the outermost 10% of the gap.

FIGURE 2.

Specimen preparation. From the central part of the inner 7 mm of implant four sections are cut for histomorphometry. The outer 3 mm of the implant is exerted through the pushout-test to evaluate mechanical fixation.

FIGURE 3.

Representative pictures of the implant surfaces. COMP coated implants in the upper row (–OPN) and OPN-COMP coated implants in the lower row (+OPN). IMP: implant, NB: new bone, FT: Fibrous tissue, MT: marrow tissue. The porosities of the OPN-COMP coated implant clearly contained a higher degree of newly formed bone. No differences are observed in the marrow-dominated gaps. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE I

X-Ray Photoelectron Spectroscopy Surface Elemental Analysis of HA Particles with and without OPN

	Ca%	P%	N%	O%	C%
HA – OPN	14.7 ± 1.0	8.7 ± 0.5	0	52.0 ± 1.0	24.6 ± 0.8
HA + OPN	13.3 ± 0.4	7.8 ± 0.3	1.3 ± 0.4	49.3 ± 0.5	28.2 ± 0.5

TABLE II**Mechanical Push-Out Test**

Implant/ Parameter	Total energy absorption (J/m²)	Ultimate shear strength (MPa)	Apparent shear stiffness (MPa/mm)
–OPN	462 ± 306	2.5 ± 1.5	16 ± 9
+OPN	635 ± 450	3 ± 2	17 ± 13

No significant differences were observed.

TABLE III

Histomorphometrical Quantification of Tissue Area Fractions on Implant Surface in Zone 1, Tissue Area Fractions on Implant Surface in Zone 2 and Volume Fractions in Gap Zone 2

Implant/ Parameter	New Bone (%)	Fibrous Tissue (%)	Marrow Tissue (%)
Ongrowth zone 1			
–OPN	15 ± 8	51 ± 27	35 ± 21
+OPN	30 ± 7*	35 ± 16	35 ± 11
Ongrowth zone 2			
–OPN	15 ± 7	23 ± 18	62 ± 19
+OPN	18 ± 7	17 ± 16	65 ± 15
Volume zone 2			
–OPN	19 ± 12	10 – 8	71 ± 17
+OPN	21 ± 12	8 ± 7	71 ± 15

All data is presented as mean ± SD;

* denotes P value below 0.05 with Student's paired T-test