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EPA-coated titanium implants promote osteoconduction in white New Zealand rabbits

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Key words: biomaterials, dental implant, fatty acid, implant coatings, surface chemistry, titanium

Abstract

Objectives: To investigate the effect of eicosapentaenoic acid (EPA)-coated Ti implants on osteoconduction in white New Zealand rabbit mandibles.

Material and methods: Sandblasted and cleansed planar titanium specimens with a size of $5 \times 5 \times 1$ mm were coated on one side with 0.25 vol% eicosapentaenoic acid (EPA). The other side of the specimens was kept highly polished (the control side). These specimens were inserted in rabbit mandibles. Twelve rabbits were randomly assigned into three study groups ($n = 4$). The rabbits were sacrificed at 4, 8, and 12 weeks. The harvested specimens with the implants were assessed for new bone formation on both sides of the implant using CBCT, conventional radiographs, and the biaxial pullout test. The results were statistically analyzed by a nonparametric Kruskal–Wallis test and Friedman's test as multiple comparisons and by Brunner–Langer nonparametric mixed model approach (R Software).

Results: A significant osteoconductive bone formation was found on the EPA-coated Ti implant surface ($P < 0.05$) at 8 weeks when compared to the polished surface (control). Biaxial pullout test results showed a significant difference ($P < 0.05$) after 8 and 12 weeks with a maximum force of 243.8 N, compared to 143.25 N after 4 week.

Conclusion: EPA implant coating promoted osteoconduction on the Ti implant surfaces, enhancing the anchorage of the implant to the surrounding bone in white New Zealand rabbits.

Contemporary dental implant treatment using titanium implants is considered to be reliable, showing over 90% survival rates after a long-term follow-up (Adell et al. 1990; Pietursson et al. 2007; Norowski & Bumgardner 2009). Titanium with various bioactive coatings (Choi et al. 2013) is considered an ideal subgingival implant material (Mallineni et al. 2012). Moreover, recent studies suggest that some novel silane coatings on Ti implants (Matinlinna et al. 2013) might also reduce biofilm formation (Villard et al. 2014). The use of organic carboxylic acid coatings, such as eicosapentaenoic acid (EPA), has the potential to promote osteoconduction. This might be interesting in clinical situations where some of the dental implant threads could rest without contact to bone. Such situations may be due to insufficient buccal bone or poorer bone quality and quantity.

In general, biocompatibility and adequate biomechanical properties are the main requirements in determining the clinical success for any type of dental implant material (Mallineni et al. 2012). The long-term success of dental implants is highly influenced by the quality and quantity of the host bone (Albrektsson et al. 1986). This said, it has been observed that areas with poor bone quality, such as the posterior maxilla, may exhibit high implant failure rates (Jaffin & Berman 1991).

Many strategies have been carried out to enhance the osseointegration rate and healing time at the bone–implant interface. In the past, some studies have focused to improve the mechanical fine structure of the implant fixtures (Wennerberg et al. 1998; Trisi et al. 1999), whereas other studies have been based on implant surface treatments and coatings

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(Khang et al. 2001; Cochran et al. 2002). Given this, some other studies have paid attention to dental implant coatings employing bioactive coating materials to enhance and ensure osseointegration along with reasonable healing times (Cheng et al. 2012; Zhao et al. 2013). To increase bone healing, different implant coating strategies were studied. Such approaches include deposition of bioactive surface coating layers by chemical vapor deposition, physical vapor deposition, electrochemical vapor deposition, metal-organic chemical vapor deposition, thermal or diffusion conversion, plasma and fusion coating, and various sol-gel techniques (Hanisch et al. 1997; Albrektsson 1998; Ben-Nissan et al. 2013; Choi et al. 2013). Ti has a natural affinity to bond with bone and tissues, but micromechanical interlocking based on surface roughening represents a more durable way for fixation. In addition, a customized surface roughness leads to an increased surface area with increased micromechanical interlocking. This said, the rationale for using implant coatings is based on the fact that faster healing and stable osseointegration could be achieved by increasing the implant surface roughness and changing the surface topography by depositing bioactive surface layers (Thomas et al. 1987; Wagner 1992; Choi et al. 2013). Osteoinduction process after dental implant surgery is recognized as a part of bone healing (Albrektsson & Johansson 2001). It contributes for the vast bulk of a newly formed bone. Some implant materials such as titanium may be osteoinductive which is not a requirement for bone induction *per se* (Barra-das et al. 2011; Guo et al. 2012).

Cis-5,8,11,14,17-eicosapentaenoic acid (EPA) is a carboxylic acid with a terminal –COOH group, and it is of an omega-3 type acid, also known as timnodonic acid. It is a highly polyunsaturated fatty acid (abbr. n-3-PUFA) that consists of five conjugated unsaturated C=C bonds (Nettleton 1995; Calder 1997). It has been shown that EPA has provided anti-inflammatory effects (Sethi et al. 1996). EPA also was found to be advantageous for bone remodeling processes (Petzold et al. 2008). Ultimately, some types of n-3-poly-unsaturated fatty acids, which included EPA, were verified to positively influence bone health and skeletal biology (Watkins et al. 2001; Petzold et al. 2011). Due to its positive effects on biological membranes, EPA regulates significantly the development and the physiology of the body (Nettleton 1995). Interestingly, there is even some evidence of EPA's antineoplastic capacity

(Babcock et al. 2000; Simopoulos 2002; Murphy et al. 2011). A positive influence of EPA onto bone mineral content and bone formation was found on bone biology in growing animals (Watkins et al. 2001; Kruger & Schollum 2005). However, most of these studies have used EPA as an oral supplement and not as a coating material of titanium implants. Current knowledge related to bone growth on EPA-coated implants as well as how EPA influences osseointegration is still somewhat fragmentary.

This current *in vivo* study investigated the effects of EPA coating on sandblasted implants and their osseointegration using an animal model. It was hypothesized that the EPA coating might (i) increase osteoconduction, (ii) increase the rate of bone formation on the coated Ti surface compared to the polished side of the Ti implant, (iii) form a direct anchorage between bone and the implant surface.

Material and methods

The ethical approval for this animal study was obtained from the ethical committee of the International Islamic University Malaysia (IIUM 314/G/14/11/2), according to the guidelines for Laboratory Experiments of the Institutional Animal Care and Use Committee (IACUC) by The National Research Council (2011).

The flip sides of the polished Ti surfaces were sandblasted with silica-coated alumina powder to increase the surface roughness. After rinsing, EPA coating was applied on the specimens. The whole process is summarized in Fig. 1. The details of surface treatments of titanium specimens are described below.

Polishing of Ti specimens

Twenty-four planar c.p. titanium specimen plates (Permascand, Ljungaverk, Sweden) with the dimensions of $5 \times 5 \times 1$ mm were polished with 400-grit silicon carbide polishing paper under running water. All Ti specimens were next cleansed ultrasonically for 10 min in 70% ethanol and then rinsed with 70% ethanol. They were allowed to dry at room temperature for 30 min.

Surface treatment of Ti by sandblasting

The flip sides of these planar titanium plates were next sandblasted using 110 μ m silica-coated alumina powder (3M ESPE, Seefeld, Germany) at a constant pressure of 300 kPa for 15 s per an area of 1 cm² using even, rotating motions. The nozzle was kept at a

perpendicular distance of 10 mm from the specimen surface (Matinlinna et al. 2006). The specimens were then cleansed in an ultrasonic bath (Decon Ultrasonics, Hove Sussex, UK), immersed in 70% ethanol for 10 min before being rinsed with 70% ethanol. They were next allowed to air-dry at room temperature for 30 min.

Preparation of EPA solution

A fatty acid (EPA) solution was prepared of 0.25 vol% of *cis-5,8,11,14,17-eicosapentaenoic acid*, with a purity of 99%, and it was not redistilled before use (Sigma Aldrich, St. Louis, MI, USA). EPA was diluted in a solvent mixture of 95 vol% absolute ethanol (99.8%) (Riedel-de Haën, Seelze, Germany) and 5 vol% deionized water. The pH of the solvent mixture was adjusted to 4.0 with 1 M acetic acid (CH₃COOH).

EPA coating of titanium specimens

The fatty acid coating solution was applied onto the sandblasted and cleansed titanium specimen surfaces, using for each Ti specimen a new fine application brush. The coating then was allowed to dry for 5 min at ambient room temperature.

Animal experiment and surgical procedures

Twelve New Zealand white male rabbits were used in this study. The animals were 6.8 ± 2.2 months old, with an average weight range of 3.175–3.628 kg. The rabbits were kept in a ventilated animal house in which the day and night rhythm was controlled and simulated. The room temperature was adjusted to $20 \pm 2^\circ\text{C}$, and the average ambient humidity was $55 \pm 10\%$. The rabbits were given an adaptation period of 10 days for acclimatization before surgery.

The rabbits were randomly assigned into three study groups ($n = 4$). A total of 24 prepared Ti implants were inserted, two per a rabbit: one implant in the left and the other in the right mandible. Before insertion, each sandblasted implant surface was EPA coated on one side and the flip side was left highly polished, as described above. Implants were notched at midway on both sides before they were inserted halfway into the bone for subsequent radiographic and cone-beam computer tomography (CBCT) analyses.

A long-acting benzathine benzylpenicillin, 2,400,000 IU/UI (Retarpen, Sandoz, Austria), was given intramuscular in the thigh as a single dose. A dissociative anesthetic 100 mg/ml Ketamine™ as a hydrochloride (Troy Laboratories, Glendenning, NSW, Australia) was administered intramuscularly as 0.2 ml/kg.

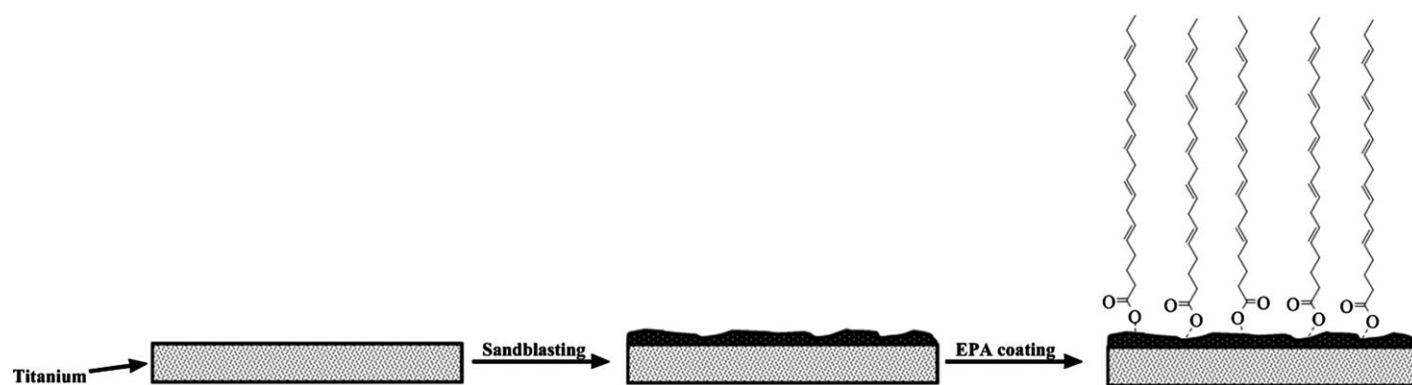


Fig. 1. The process of preparing an eicosapentaenoic acid (EPA) coating on a titanium surface.

Anesthesia was followed by a muscle relaxant 10 mg in 2 ml diazepam (a benzodiazepine derivative tranquilizer; Diapine Atlantic; Atlantic Laboratories, Bangkok, Thailand) to provide anxiolytic, sedative, and anticonvulsant effects as well as the central muscle relaxation.

After validating the anesthesia, the hair at the mandible was shaved. The animal was placed in a supine position on the operation table, controlling the anesthetic maintenance during the surgery. All the surgical procedures were performed by the same surgeon. The surgical protocol was based on a previous study model for functional attachment of implants *in situ* (Rønold & Ellingsen 2002). Local anesthesia was given intraorally at the surgical site prior to incision. A 2-cm incision was made on the lateral part of the mandible. The incision penetrated the fascial layers. Lateral reflection of these layers exposed the underlying periosteum. When the mandible bone of the rabbit was used, 2 implants could be placed in each mandible, one on each side. A standardized positioning was followed according to a published protocol (Rønold & Ellingsen 2002). The implants were fixed into the bone area anterior-to-posterior teeth to avoid damaging the roots. Four guide holes were prepared using a 1.0-mm-diameter twist drill (Edenta, Switzerland). Then, the holes were connected by passing the bur in a straight move with profuse physiological saline solution irrigation. A socket was prepared for the planar implants to the depth of 2.5 mm. The implants were then gently hammered into the correct depth and position.

Wound closure was carried out using the single suture technique from inside-to-outside. The muscles and their fasciae were sutured with a plain catgut (DemeTech, Miami Lake, FL, USA), whereas Vycril Plus™ 3/0 (Ethicon, Johnson & Johnson, Hong Kong,

China) was used to seal the skin. Postoperatively, during the healing period, the rabbits were kept paired per cage. They were fed daily with 150 mg of feeding pellets with an average protein content of around 15–16% (Alam Jitu Enterprise, Pahang, Malaysia).

All animals were sacrificed on the proposed study date by administering an overdose of pentobarbital sodium (150 mg/kg; Alfasan International, Woerden, Holland). Osteoconduction was assessed on the specimens using conventional radiographs, CBCT, and a biaxial pullout test analysis at 4, 8, and 12 weeks postoperatively.

Cone-beam computed tomography (CBCT)

CBCT (Planmeca ProMax 3D; Planmeca, Helsinki, Finland) was used to investigate the bone–implant interface. Bone density of newly formed bone was also assessed. At the same time, CBCT was used to examine new bone formation on the exposed part of the Ti implant. The specimens were positioned on a special specimen holder to fit the CBCT cylinder. The longitudinal axis of the cylinder was oriented parallel to the long axis of the specimens. The density of bone was examined at 90 kV and 10 mA, using special digital software (Planmeca Romexis 2.8.0.R; Planmeca).

Radiographic assessment

The specimens were analyzed further by taking X-ray images (Planmeca Intra) of the implants and the adjacent bone structure in different angulations to analyze the formation of newly formed bone at the coated side and to compare it with the bone at the control side (i.e. uncoated Ti). Planmeca Romexis digital software was used to visualize the radiographs.

In addition to the CBCT and radiographic analysis, osteoconductivity was assessed clin-

ically by measuring the amount of new bone covering the exposed surfaces of implants during the study period. After completion of the imaging assessment, the implant surfaces were further examined under the image magnifier lens (Sony NEX-3; Sony, Tokyo, Japan). For the current study, a new design of indexing (in a scale from 0 to 3) was created to examine the new bone growth on the implant surfaces. The following indices were used: “0” = “no bone on the surface,” “1” = “bone growth on less than half of the surface,” “2” = “bone growth on more than half of the surface,” and “3” = “bone growth on the entire surface.”

Biaxial pullout test

Biaxial pullout test of the specimens was performed according to literature (Hong et al. 1992) and appropriately modified to accommodate the planar shape of the tested Ti implants (Fig. 2). The bone specimens were embedded into acrylic molds to fit the jig of a universal testing machine (Tabletop AGS-X; Shimadzu, Tokyo, Japan). The lateral notches of the specimens were used for fixing the pulling hooks from the testing machine. A constant load of 500 ± 50 N was applied to the bone–implant specimens perpendicularly to the interface using a crosshead speed of 2.5 mm/min. A failure was recorded at the point when the complete implant detached from the bone.

Statistical analysis

With respect to the sample size, a nonparametric Kruskal–Wallis test and multiple time series comparisons, such as Friedman’s test, were employed. Both analyses were carried out at a significance level of $P < 0.05$. The analyses were performed to compare differences in the amount of Ti implant covered by new bone formation between the EPA-coated and the noncoated (polished only)

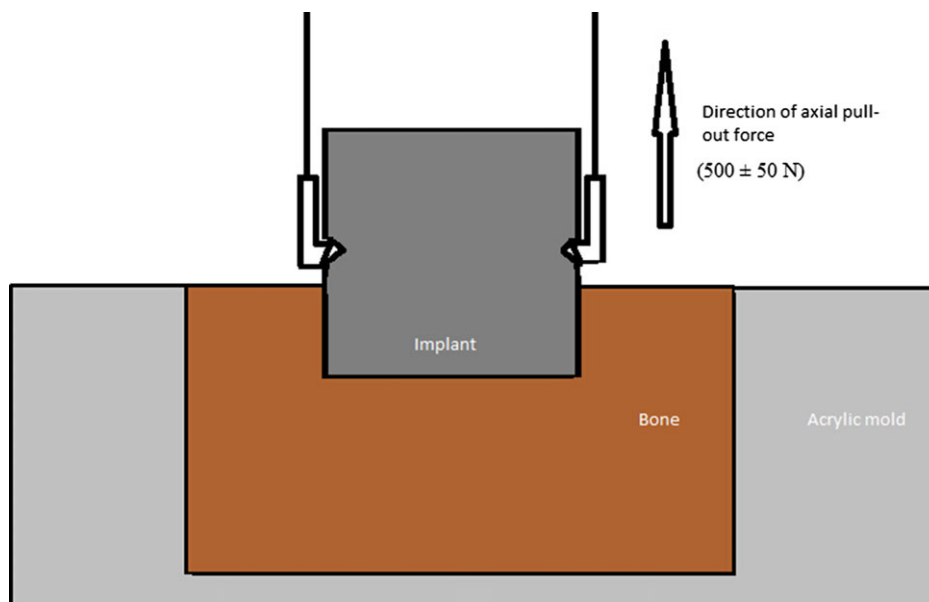


Fig. 2. The implant was subjected to biaxial pulling force (500 ± 50 N) perpendicular to the bone-implant interface.

implant surfaces in different time intervals. Osseointegration was analyzed by comparing the amount of force needed in the biaxial pullout test, after the periods of 4, 8, and 12 weeks, respectively. All data were analyzed using the SPSS 16 software (SPSS, Chicago, IL, USA). In addition to the preceding analyses, Brunner-Langer nonparametric mixed model approach using R software was used to confirm the significance level of the obtained results.

Results

The animals were checked daily related to any general health issues, wound healing, appetite, discomfort, and weight by the research team. All rabbits showed normal daily activities and nutritional behavior 2 days postoperatively. No weight loss was observed in the first postoperative week, except for one animal, which presented a weight loss of 250 g. The same animal further showed loss of appetite within the first few days after surgery. Otherwise uneventful postoperative course and wound healing occurred in all animals until they were sacrificed according to the study protocol.

CBCT and radiographic assessment

Figure 3 highlights new bone formation with an increased density on the coated Ti specimen side after 12 weeks. New bone growth due to osteoconduction could be detected both on the EPA-coated Ti as well as on the



Fig. 3. CBCT image showing osteoconductive bone formation after 12 weeks (black arrow) covering the entire EPA-coated surface of the implant (red arrow).

polished Ti implant surface, however, with more growth on the coated side.

X-ray findings revealed and suggested osteoconductive new bone formation on the EPA-coated Ti, indicating new bone growth. Figure 4 shows the EPA-coated surface with adjacent new bone formation after 4 weeks postoperatively. Figure 5 shows osteoconductive bone formation on both sides of the specimen after 8 weeks of surgery. All specimens showed osteoconductive activity on the Ti implant side coated with EPA. Both CBCT and radiographic findings were defined in terms of osteoconductive bone formation to indicate the new bone formation of the exposed part of the implant. Using the nonparametric Kruskal-Wallis test, statistical analyses revealed a significant difference after 12 weeks ($P = 0.05$) on all the EPA-coated sides compared to the noncoated sides. There were significant differences after 8 weeks



Fig. 4. X-ray of the mandible showing osteoconductive bone formation after 4 weeks of surgery on the EPA-coated implant side (arrow).



Fig. 5. Radiograph image showing complete osteoconductive bone formation at both sides after 12 weeks of surgery (arrows).

($P = 0.05$) on coated, compared to noncoated Ti surfaces. There was a significant difference after 12 weeks ($P = 0.05$) on all surfaces when comparing to surfaces at 4 weeks. No significant differences were observed between the two surfaces at 4 weeks ($P < 0.05$).

Figures 6–8 are highlighting new bone formations covering the exposed surfaces of the EPA-coated Ti implants after 4, 8, and 12 weeks. Table 1 visualizes the indexing for bone growth on the implant surfaces at 4, 8, and 12 weeks. Statistical analyses using the nonparametric Friedman's test showed a significant difference ($P = 0.025$) between the bone growth on coated surface at 12 weeks, compared to 4 weeks. No significant difference ($P = 0.083$) between the bone growth on the EPA-coated surface (B) at 12 weeks com-

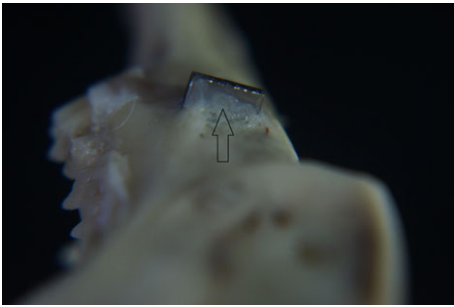


Fig. 6. Osteoconductive bone formation at the EPA-coated surface after 4 weeks.

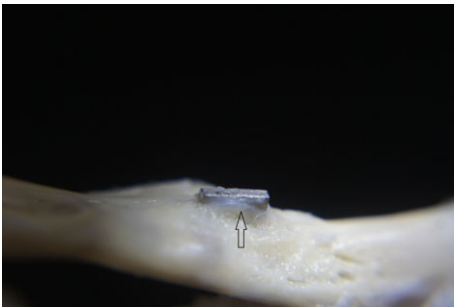


Fig. 7. Osteoconductive bone formation at the EPA-coated side after 8 weeks from surgery.

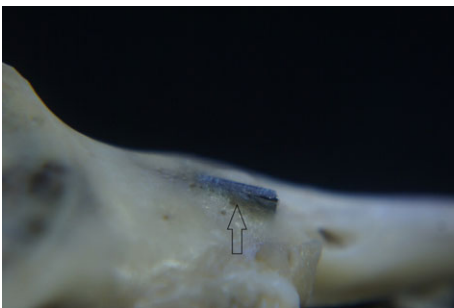


Fig. 8. Complete osteoconductive bone formation at the EPA-coated side after 12 weeks from surgery.

pared to 4 weeks could be found. There was a significant difference ($P = 0.001$) between the bone growth on the noncoated side (A) at 12 weeks compared to 4 weeks and between the bone growth for noncoated surface at 12 weeks compared to 4 weeks ($P = 0.002$).

Biaxial pullout test

Nonparametric Friedman's test (Table 2) revealed a significant difference ($P = 0.01$) between the amount of biaxial pullout force needed after 8 and 12 weeks compared to 4 weeks.

Brunner–Langer nonparametric mixed model approach (ANOVA-type statistic, R Software)

ANOVA of Factorial Model (Table 3) shows significant effect among the factors of site, time, and groups (Noncoated Ti, EPA-coated Ti). Moreover, it shows clear interaction among the factors.

Discussion

The current study investigated the effect of EPA coating on osseointegration and osteoconduction. CBCT results illustrate the quantitative increase in new bone formation and in bone density on EPA-coated Ti specimens when compared to the control surfaces. The radiographic investigations of this study found bone growth along EPA-coated surfaces, as highlighted in Figures 3 and 4. However, it proved to be somewhat difficult to estimate the direct effect of EPA, or its layer thickness, on bone growth (Damsgaard et al. 2012), something which could be addressed in the future research.

Radiographic and CBCT results suggest that early bone growth could be achieved on EPA-coated titanium surfaces in rabbit mandibles already within 4 weeks after implantation. Eight weeks postoperatively, new bone formation was found on both Ti implant surfaces, that is, EPA coated and not coated. Osteoconduction was detected on both surfaces of the tested titanium specimens. Imaging analyses 8 weeks postoperatively showed more new bone formation on the EPA coated than on the polished only implant surfaces. Such findings of osteoconduction on the control side were in line with some other findings suggesting that titanium surfaces might induce osteoconduc-

tion processes (Jaatinen et al. 2011). Twelve weeks postoperatively, both titanium surfaces became covered with new bone, confirming osteoconduction. It was stated (Barradas et al. 2012) that conclusive evidence for osteoconduction only would be given if new bone growth occurred in locations where naturally bone does not grow. It is noteworthy that on titanium surfaces, a thin titanium oxide layer forms spontaneously (Jaatinen et al. 2011; Guo et al. 2012). Various steps during the insertion procedure might damage such a protective titanium oxide layer, which under normal circumstances, recovers within nanoseconds by repassivation. It may be assumed that due to surface stabilization EPA coatings might provide faster bone formation than the titanium oxide layer only.

The osseointegration rate of titanium dental implants highly depends on their surface composition and roughness which influence bone anchoring based on biomechanical fixation (Le Guéhennec et al. 2007). On the other hand, there is some evidence that appropriate titanium roughness improves the result in primary implant stability, thereby enhancing persistent mechanical fixation (Junker et al. 2009). In the current study, the surface roughness was maintained after sandblasting even followed with EPA coating. This may have contributed to increased mechanical interlocking between the bone and the coated Ti surface.

Some investigated implant coatings have been related to addition of growth factors with a consecutive bone healing with cementless prosthetic components (Lind 1998). Animal studies have indicated that EPA might function as a bone growth factor, and hence, it might increase bone formation. It was concluded that certain types of n-3-PUFA may have the potential to enhance Ca absorption in animals (Kelly et al. 2003). Moreover, EPA could indirectly affect bone growth by inhibiting bone resorption stimulators (Raisz et al. 1989).

Table 1. Result of index of the bone remnants on the implant surfaces after periods of 4, 8, and 12 weeks

Subject	4 week				8 week				12 week			
	Noncoated Ti		EPA-coated Ti		Noncoated Ti		EPA-coated Ti		Noncoated Ti		EPA-coated Ti	
	Side A	Side B	Side A	Side B	Side A	Side B	Side A	Side B	Side A	Side B	Side A	Side B
1	0	0	1	1	1	1	2	2	1	1	3	3
2	0	1	1	2	0	1	2	2	1	2	3	3
3	0	1	1	1	1	1	3	2	1	1	3	3
4	0	0	1	1	0	0	3	2	1	1	3	3

Key: "0" = "no bone on the surface," "1" = "bone growth on less than half of the surface," "2" = "bone growth on more than half of the surface," and "3" = "bone growth on the whole surface."

Table 2. Mean and standard deviation of biaxial pullout strength (N) of all test groups

Subject (animal)	4 weeks			8 weeks			12 weeks		
	t1*	t2	Mean ± SD	t1	t2	Mean ± SD	t1	t2	Mean ± SD
1	109.73	112.81	111.27 ± 2.18	153.12	167.87	160.5 ± 10.43	212.99	220.17	216.58 ± 5.08
2	133.12	125.72	129.42 ± 5.23	133.65	138.25	135.96 ± 3.25	198.53	175.32	186.93 ± 6.41
3	121.06	116.95	119.0 ± 2.91	161.82	145.48	153.65 ± 11.55	243.82	231.19	237.51 ± 8.93
4	143.25	136.17	139.71 ± 5.01	150.75	154.15	152.45 ± 2.40	207.12	197.25	202.19 ± 6.98

* [t1 = right side, t2 = left side of mandible].

Table 3. ANOVA of Factorial Model shows that sites (A and B), weeks (4, 8, 12), and groups (Non-coated Ti, EPA-coated Ti) are not only given the significant effect on index of bone, but also have interaction with each other

Brunner–Langer nonparametric mixed model approach (ANOVA-type statistic, R Software)			
	Statistics	df	P-value
Site	1.0652174	1.000000	3.020282e-01
Week	59.0115782	1.467265	4.608179e-20
Coating	169.3828062	1.000000	1.009181e-38
Site: week	1.7430154	1.467265	1.836214e-01
Coating: week	5.2830140	1.961430	5.367045e-03
Site: coating	3.2189781	1.000000	7.278906e-02
Site: week: coating	0.3318008	1.961430	7.134072e-01

This animal study was designed to investigate the outcome of omega-3 fatty acid (EPA) coated dental implants on osteoconduction in mandibles of white male New Zealand rabbits. Significant osteoconduction became obvious 4 weeks postoperatively. Therefore, it may be suggested that EPA affected osteoconduction and functions as a growth factor *in vivo*. Future research should focus on

EPA's direct effect and its interactions with some other bone growth factors. EPA-coated titanium surfaces in rabbits were found to enhance osteoconduction at the bone–implant interface, a finding which might be considered to point at an increased osseointegration capacity of EPA-coated Ti implants.

The hypotheses were met. In other words, this study suggests and concludes that EPA

coatings on sandblasted Ti implants might (i) increase the osteoconduction process, (ii) increase the rate of new bone formation on the EPA-coated implant surface, (iii) ensure a direct anchorage of bone to implant surface, (iv) increase protein absorption, and (v) also enhance chemical bonding of protein molecules.

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