Importance of Ca^{2+} Modifications for Osseointegration of Smooth and Moderately Rough Anodized Titanium Implants – A Removal Torque and Histological Evaluation in Rabbit

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ABSTRACT

Background: Incorporation of Ca^{2+} into the titania of anodized titanium surfaces has been found to enhance osseointegration. It provides a stable surface when the ions are incorporated into the oxide layer during the anodizing process. The Ca^{2+} may suggestively be prominent sites for mineral induction, attract proteins, and catalyze intracellular cascades.

Purpose: The aim of the present study was to evaluate the osseointegration of smooth (S_a < 0.5 μm) and moderately rough (S_a 1.0–2.0 μm) commercially pure titanium implants, with and without Ca^{2+}, in order to reflect on the importance of surface chemistry in relation to topography.

Materials and Methods: Anodized implants with (OxCa) or without Ca^{2+} (Ox), blasted implants (Bl), and blasted anodized implants, with (BlOxCa) or without Ca^{2+} (BlOx), were inserted in rabbit femur and tibia. The implant surfaces were characterized using interferometry, scanning electron microscopy, and X-ray photoelectron spectroscopy prior to implant installation. Removal torque (RTQ) measurements were executed on all implants after a healing period of 12 weeks. The implants were, thereafter, removed en bloc with surrounding tissues and prepared for histological evaluations.

Results: RTQ measurements of tibial implants revealed significantly higher values for BlOxCa implants (90.7 ± 23.3 Ncm) compared to OxCa (64.6 ± 18.2 Ncm) and BlOx implants (69.7 ± 17.5 Ncm) (p = 0.029). Ca^{2+} modification of smooth implants placed in the femur did not reveal any differences.

Conclusion: Ca^{2+} modification of smooth implants resulted in similar interfacial shear strength as moderately rough implants and Ca^{2+} modification of moderately rough implants demonstrated the significantly strongest interfacial shear strength when placed in rabbit tibia. This possibly demonstrated surface chemistry compensating for lesser roughness.

KEY WORDS: calcium, dental implants, implant surfaces

INTRODUCTION

Treatments with biomedical implants rely on the principles enabling osseointegration.[1] However, neither the principles nor the implant surface characteristics most important for a rapid and enhanced osseointegration is completely known today. Properties of micro- and nanotopography, as well as chemical composition of implant surfaces, are being extensively investigated.[2,3] Most likely, is it the combination of different surface factors, including microtopography, nanotopography, and chemistry, which provide optimal surfaces for osseointegration.

The term bioactivity is widely used but may have different meanings. It can be addressed as a surface that interacts and stimulates proteins and growth factors involved in the tissue healing, but originally it was suggested to be a surface that establishes a chemical bond with its surrounding tissues.[4] Although, no evidence of

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chemical bonding exists today. The required effects of bioactive surfaces, regardless of the definition of the term, are a quicker and enhanced integration of the implant. Suggested bioactive surfaces (eg, hydroxyapatite coated, alkali and heat treated, or ion incorporated) have shown enhanced bone response in comparison with nonbioactive surfaces in a number of both in vitro and in vivo studies.[5–8]

The mechanical interlocking of an implant gained by the tissue ingrowths into surface microstructures may decrease with a smoother surface. The optimal average roughness (S_a) of titanium implants for osseointegration has been suggested to be 1.5 μm.[9] Possibly, the optimal roughness differs between different types of processed surfaces as the experiments resulting in the suggestion of an optimal roughness were performed using blasted implants. Also, other surface parameters should be addressed when characterizing a surface, for example, developed surface area, summit density, and crystalline phase of the oxide layer, in order to increase the gathered knowledge of the effect of the surface on the tissue response. There may be an interest in smoother surfaces becoming properly osseointegrated when eventual future problems with bone resorption and biofilm formation on the implant surface increase with surface roughness.[10]

In previously published in vivo studies using anodized c.p. titanium surfaces with incorporated Ca^{2+}, calcium-modified surfaces have gained enhanced osseointegration.[11–13] The possible effect of calcium modifications may be that the calcium ions attract proteins and growth factors of importance for the bone cells and bone formation,[14,15] that they enhance bone cell growth,[16,17] or that they function as binding sites for bone mineral crystals.[18] Whether calcium modification could compensate for lesser roughness is not presented in the literature.

The aim of the present study was to evaluate the osseointegration of smooth ($S_a < 0.5$ μm) and moderately rough (S_a 1.0–2.0 μm) commercially pure anodized titanium implants with and without incorporated Ca^{2+}. The implants used had a similar topography but altered chemistry, as well as similar chemistry but altered topography, in order to reflect the importance of surface chemistry in relation to surface roughness. A blasted implant with surface roughness favorable for osseointegration,[9] frequently used by the authors, was used as a control.

Ca^{2+} modification of smooth implants installed in rabbit femur was expected to result in greater interfacial shear strength. Ca^{2+} modification of moderately rough implants was expected to result in the greatest interfacial shear strength when installed in the rabbit tibia. Additionally, it was hypothesized that smooth Ca^{2+} modified implants would have similar interfacial shear strength to the moderately rough implants without Ca^{2+} modification.

MATERIALS AND METHODS

Implant and Surface Preparation

A total of 60 turned, threaded grade 4 c.p. titanium implants (Ospol AB, Malmö, Sweden), with the length 8 mm and diameter 3.5 mm, were used in this study. The implants were divided into five groups and processed with different surface modifications: Ox, anodized with an electrolyte that consisted of sodium glycerophosphate hydrate $C_3H_6(OH)_2PO_4Na_2 \times xH_2O$; OxCa, anodized with an electrolyte that consisted of: sodium glycerophosphate hydrate $C_3H_6(OH)_2PO_4Na_2 \times xH_2O$ and calcium acetate $Ca(CH_3COO)_2$; Bl blasted with 100 μm-sized Al_2O_3 particles; BlOx blasted and then anodized; or BlOxCa blasted, anodized, and calcium modified.

The anodic oxidation process was performed in accordance to the prescription of Sul and colleagues.[13,19] All implants were rinsed and cleaned in an ultrasonic bath with diluted Extran MA01â (Merck, Darmstadt, Germany), rinsed in absolute alcohol, and then sterilized in an autoclave before insertion into rabbit bone.

Surface Analyses

Three implants of each surface treatment were topographically characterized using an optical interferometer (MicroXam™, PhaseShift, Tucson, AZ, USA) after the sterilization process. Each implant was measured on nine sites of the threaded area (three tops, three valleys, and three flanks).[9] Each measurement was performed over a 200 × 260 μm area. A high pass Gaussian filter sized 50 × 50 μm was used to separate roughness from errors of form and waviness as recommended by Wennerberg and Albrektsson.[20] The surface parameters' S_a, summit density (S_{ds}), developed interfacial area ratio (S_{dr}), and core fluid retention index (S_{ci}) were calculated with the Surfascan software (Somicronic Instrument, Lyon, France).

The implants were depicted with a scanning electron microscope (SEM) and chemically analyzed with an X-ray photoelectron spectroscopy (XPS). The XPS (PHI 5000 ESCA system, Perkin Elmer, Wellesley, MA, USA) analysis was made with an operating angle of 45° at 150 W using an Al excitation source.

Animals and Surgical Technique

A total of 10 female New Zealand White rabbits were used in the experiment, approved by the local animal ethics committee at the University of Gothenburg. The animal model using 10 rabbits with three implants in each leg offers a sample size large enough to perform proper statistical analysis of intraspecimen comparisons and nonpaired comparisons between implant groups. The animals were adult (9 months of age) and weighed between 3.5 and 4.5 kg. The rabbits received one implant in each distal femoral metaphysis and two in each proximal tibial metaphysis. Smooth implants were placed in the femur to have a paired intraspecimen test of the effect of Ca^{2+} incorporation. In tibia, the implants were distributed in a randomized manner and the smooth OxCa implants were compared with the moderately rough implants in order to investigate its potential of being interlocked by the bone in relation to the rougher implants. The distribution of the implants is given in Table 1.

The animals were kept in separate cages for 1 week after the implant installation and kept together thereafter. There was free access to tap water and standard diet at all times. Antibiotics (Borgal® vet, Intervet, Sollentuna, Sweden) were administered prophylactically at the time of surgery and 3 days later. At surgery, general anesthesia was induced by intramuscular injections of fentanyl 0.3 mg/mL and fluanisone 10 mg/mL (Hypnorm Vet, Janssen Pharmaceutica, Beerse, Belgium) at an initial dose of 0.5 mL per kg body weight and intraperitoneal injections of diazepam (Stesolid® novum, Actavis AB, Stockholm, Sweden) at a dose of 2.5 mg per animal. Additional doses of Hypnorm at a dose of 0.1 mL per kg body weight were given every 20 minutes during the surgical procedure. The hind legs were shaved and cleaned with chlorhexidine. Local anesthetic lidocain (Xylocain®, Astra Zeneca, Södertälje, Sweden) at a dose of 1 mL was injected into each insertion site in connection with the surgery. The skin and fascial layers were opened and closed separately. Both layers were sutured with resorbable sutures. The implant sites were drilled at a low rotary speed and profuse saline cooling was used. The same person inserted all implants. The animals were allowed to bear their full body weight immediately after surgery. Twelve weeks after implant installation, the animals were sacrificed with Pentobarbital vet (Apoteket AB, Uppsala, Sweden) after sedation with 1.0-mL Hypnorm Vet.

Removal Torque Evaluation

The peak loosening torque was evaluated with removal torque (RTQ) measurements. The instrument can be considered a three-dimensional test as it reflects the interfacial shear strength between the bone tissue and the implant.[21] A static torque was applied to the implant at a linear rate of 9.5 Ncm/s and the device core ensured

TABLE 1 Implant Placement Schedule

	Femur Left	Right	Tibia Left Prox	Left Dist	Right Prox	Right Dist
1	Ox	OxCa	BlOxCa	BlOx	Bl	OxCa
2	Ox	OxCa	BlOxCa	OxCa	Bl	BlOx
3	Ox	OxCa	BlOxCa	Bl	OxCa	BlOx
4	Ox	OxCa	BlOx	OxCa	Bl	BlOxCa
5	Ox	OxCa	BlOx	Bl	BlOxCa	OxCa
6	OxCa	Ox	BlOx	BlOxCa	OxCa	Bl
7	OxCa	Ox	OxCa	BlOxCa	BlOx	Bl
8	OxCa	Ox	OxCa	Bl	BlOxCa	BlOx
9	OxCa	Ox	Bl	BlOx	OxCa	BlOxCa
10	OxCa	Ox	Bl	OxCa	BlOxCa	BlOx

Dist = distally positioned; Prox = proximally positioned.

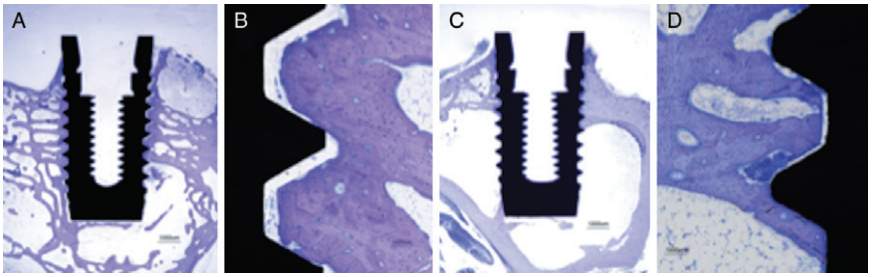

Figure 1 Undecalcified ground sections of (A) an implant placed in femur, (B) the implant shown in Figure 1A in higher magnification, (C) an implant placed in tibia, and (D) the implant shown in Figure 1C in higher magnification. There were no differences in qualitative observations and no implants showed signs of acute inflammation. The implant–bone interface borders were not intact because of the loosening of the implants during removal torque measurements.

that it was fixed. The removal torque was stopped when the implants started to turn in order to keep the implant site as intact as possible for histological sampling.

Preparation of Specimens and Histomorphometrical Measuring

The implants and their surrounding tissues were removed en bloc and immersed in 4% neutral buffered formaldehyde. The specimens were dehydrated in graded series of ethanol and embedded in light-curing resin (Technovit 7200 VLC, Kültzer and Co. GmbH, Friedrichsdorf, Germany). Undecalcified ground sections were ground ad modum Donath (1988) to a thickness of about two cell layers (15–20 μm) and stained with Toluidine blue mixed with pyronin G. A computer-connected microscope was used to measure the estimated bone-to-implant contact and bone area surrounding the implants. The following histological measurements were performed: bone lengths (length of bone estimated to have been in contact with the implants), surrounding bone area (percentage of bone within the threads with average depth of 330 μm), surrounding bone area within the three best consecutive threads (the distance of three threads are usually in accordance with the height of the cortical bone plate which is the region where an implant may be strongest interlocked), and shear force (RTQ values correlated with the histomorphometrical bone length measurements to calculate the mean shear strength by using the equation $F = T/[d \times \pi \times l \times r_m]$, where T = removal torque [Nmm], d = mean diameter of the implant [3.25 mm], r_m = lever arm/radius [1.625 mm], and l = the entire bone length along the implant surface [mm][22]).

All measurements were performed with a 4× objective and 10× eyepiece in a blinded manner. Because of the loosening of the implants, the histological measurements were estimations of bone in close vicinity to the implants and bone area within the threads. All sections were qualitatively analyzed in terms of inflammatory cells or discrepancy appearance. Figure 1, A and B represents a ground section of an implant placed in femur in two different magnifications and Figure 1, C and D represents an implant placed in tibia, from which the histological analyses were performed.

Statistics

Values of the surface parameters were treated with one-way analysis of variance (ANOVA) followed by the post hoc test Games–Howell. Data from the histomorphometrical evaluations were treated with the nonparametric Wilcoxon matched-pair signed-rank test for the femur group and the nonparametric Kruskal–Wallis one-way ANOVA test for the tibia group. If significant difference was found with Kruskal–Wallis, further investigations were performed with the nonparametric Mann–Whitney *U* test to clarify between groups. p Values <.05 were considered statistically significant.

RESULTS

Surface Topography

Figure 2 displays the topographical characteristics of the surfaces in a diagram of the surface parameter values and SEM images of all surfaces with two magnifications. The Ox and the OxCa implants were smooth implants as they had a mean deviation from a mean plane (S_a) of 0.41 ± 0.06 and 0.30 ± 0.13 μm, respectively. There was, though, a statistical difference between the Ox and OxCa implants. The BlOx (1.35 ± 0.18 μm), the BlOxCa (1.40 ± 0.28 μm), and the Bl implants (1.25 ± 0.12 μm)

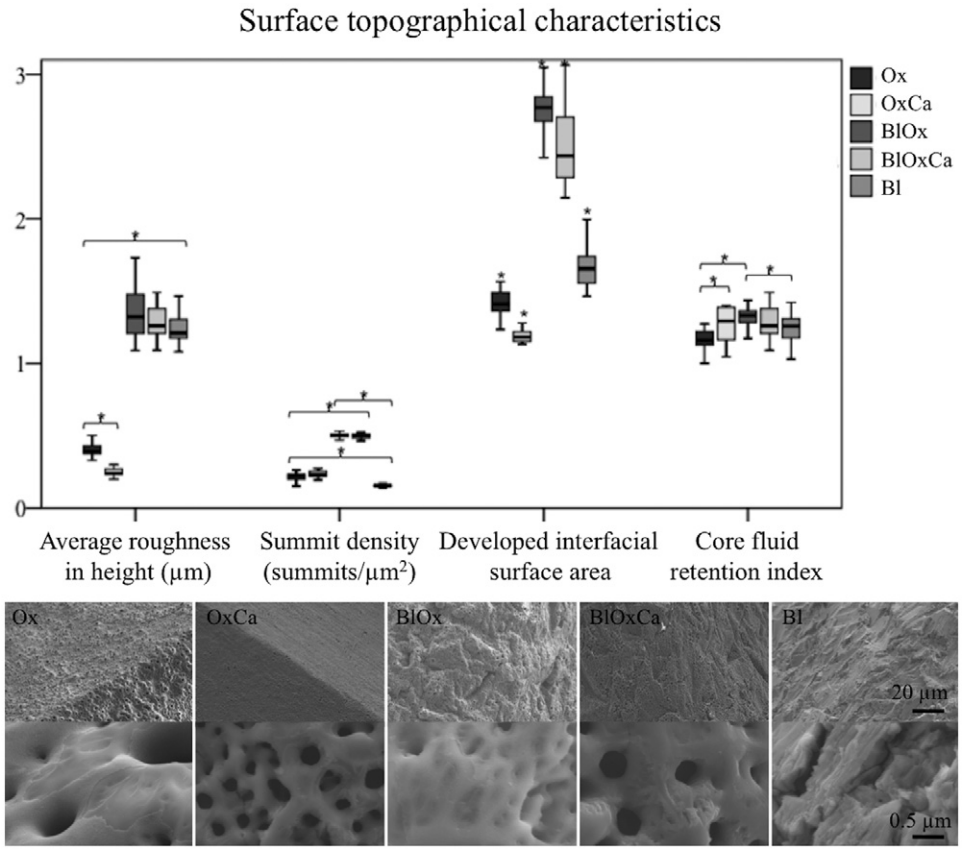

Figure 2 Diagram and SEM images with two magnifications presenting the topographical characteristics of the implant surfaces. Statistical differences marked with *. Ox = anodized; OxCa = anodized and Ca^{2+}-modified; BlOx = blasted with 100 μm-sized Al_2O_3 particles then anodized; BlOxCa = blasted with 100 μm-sized Al_2O_3 particles then anodized and Ca^{2+} modified; Bl = blasted with 100 μm-sized Al_2O_3.

were moderately rough and all had significantly higher S_a values than the smooth implants.

The Bl implants were not as densely peaked (S_{ds}) as any of the anodized implants with 156 ± 6 summits/μm². The Ox implants had a summit density of 215 ± 27 summits/μm² and the OxCa implants had 233 ± 24 summits/μm². The BlOxCa (496 ± 17 summits/μm²) and the BlOx implants (500 ± 14 summits/μm²) had the significantly highest summit densities.

There were significant differences between all implant groups regarding developed interfacial area ratio. The OxCa implants had the smallest developed interfacial area ratio of 19 ± 4%, followed by Ox implants with 44 ± 14%, Bl implants with 67 ± 13%, BlOxCa implants with 149 ± 27%, and BlOx implants with 173 ± 16%.

The Ox implants had significantly lower S_{ci} (1.17 ± 0.12) compared with the BlOx (1.31 ± 0.08) and the OxCa implants (1.36 ± 0.28). Also, the Bl implants had a significantly lower index compared with the BlOx implants. The index for the BlOxCa implants was 1.27 ± 0.16, and not significantly different to any of the other surfaces.

By visual evaluations from SEM images, it was noted that the Ca^{2+}-incorporated anodized implants generally had smaller and more densely positioned porous structures compared with the anodized implants that did not contain Ca^{2+}. This accorded to both smooth and moderately rough implants. The diameter of the pores was estimated to be 0.2 to 0.5 μm for the Ca^{2+}-incorporated implants and 0.3 to 1.2 μm for the other anodized implants.

Surface Chemical Composition

The atomic percentage of the various surface elements is presented in Table 2. Titanium and oxygen constituted more than 92 atomic% of all surfaces. Sodium was detected on the three surfaces that were not Ca^{2+} modified; calcium was only found on the two surfaces that were Ca^{2+} modified; alumina was only found on the

TABLE 2 Atomic Percentage of Surface Elements, Evaluated with X-ray Photoelectron Spectroscopy

Atomic%	Sodium	Alumina	Titanium	Oxygen	Phosphor	Calcium
Ox	0.1		26.3	68.9	4.6	
OxCa			31.0	67.6	0.3	1.2
BlOx	0.1	2.2	23.8	68.5	5.3	
BlOxCa		2.3	28.9	66.7	0.3	1.8
Bl	0.1	5.6	28.6	65.7		

The main composer of the outer layer was TiO_2.

blasted surfaces; and phosphor was found on all anodized surfaces.

Animals and Surgical Technique

All animals remained throughout the study. One Ox implant placed in femur was not stable at insertion and the same was not integrated at the time for retrieval. Also, one BlOxCa implant placed in tibia could not be histomorphometrically analyzed because of problems with the preparation of the ground sections.

Evaluations

For the Ox and OxCa implants inserted in femur, there were no significant differences in terms of RTQ, histomorphometrical evaluations, or grade of inflammation. The mean RTQ peak value with standard deviation was 109.9 ± 20.6 Ncm for the Ox implants and 85.3 ± 25.6 Ncm for the OxCa implants. The mean length of bone in close vicinity to the implants was 8.18 ± 1.80 mm for the Ox implants and 7.66 ± 2.23 mm for the OxCa implants. Mean percentage of bone surrounding the implant was 50.8 ± 11.0% for the Ox implants and 38.6 ± 9.6 % for the OxCa implants. Mean shear force was 8.34 ± 1.90 N/mm^2 for the Ox implants and 6.75 ± 1.25 N/mm^2 for the OxCa implants.

Among the tibial implants, there were significant differences between various groups of implants for the conducted evaluations (Figure 3). The BlOxCa implants had a significantly greater mean peak RTQ value (90.7 ± 23.3 Ncm) compared with the OxCa implants (64.6 ± 18.2 Ncm) and the BlOx implants (69.7 ± 17.5 Ncm) ($p = .029$). The Bl implants had a mean value of 79.9 ± 17.4 Ncm. The BlOx implants (8.39 ± 2.10 mm) had significantly more bone in

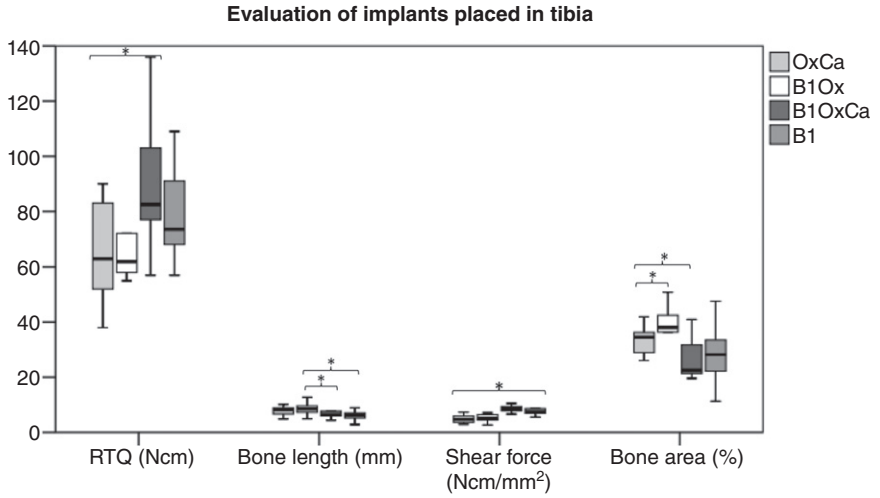

Figure 3 Results from RTQ and histomorphometrical evaluations of implants placed in tibia. BlOxCa implants had significantly greater (*) mean RTQ peak value compared with OxCa and BlOx implants ($p = .029$). BlOx implants had significantly more bone in close vicinity to the implant compared with Bl and BlOxCa implants ($p = .026$). OxCa and BlOx implants had significantly higher percentage of bone surrounding the implant compared with BlOxCa implants ($p = .047$). Both BlOxCa and Bl implants had significantly higher shear force values compared with OxCa and BlOx implants ($p < .001$).

close vicinity to the implant compared with the Bl (6.26 ± 1.64 mm) and the BlOxCa implants (6.47 ± 1.26 mm) ($p = .026$). The OxCa implants had a mean value of 7.81 ± 1.59 mm. The OxCa (33.5 ± 4.8%) and the BlOx implants (37.0 ± 9.4%) had a significantly higher percentage of bone surrounding the implant compared with the BlOxCa implants (26.8 ± 8.1%) ($p = .047$). There were no significant differences between the implant groups when only the three best threads were evaluated. The OxCa had a mean percentage of 70.9 ± 9.5%, the BlOx had 70.8 ± 7.4%, the BlOxCa had 64.2 ± 16.2%, and the Bl had 63.1 ± 13.5%. However, both the BlOxCa and the Bl implants had significantly higher shear force values (mean values 8.55 ± 1.40 and 8.09 ± 2.41 N/mm^2, respectively) compared with the OxCa and the BlOx implants with mean values of 5.23 ± 2.17 and 5.21 ± 1.38 N/mm^2, respectively ($p < .001$).

DISCUSSION

Ca^{2+} incorporation resulted in stronger interlocking of moderately rough implants and similar interlocking of smooth implants, as compared with moderately rough anodized and blasted implants when placed in rabbit tibia. However, calcium modifications of smooth implants did not improve the osseointegration when placed in rabbit femur.

In a published histomorphometrical study, anodized implants with incorporated Ca^{2+} did show significantly higher rate of bone-to-implant contact compared with anodically oxidized and blasted implants.[11] The Ca^{2+} may not have been the only reason for the enhanced bone response when the surface topography varied somewhat and, moreover, the nanotopography was not evaluated. The Ca^{2+}-incorporated implants were, however, the smoothest and should not have been a positive factor for bone response when there a number of studies that indicate that a rougher surface results in enhanced bone contact.[23] In the present study, moderately rough blasted anodized implants with incorporated Ca^{2+} showed significantly higher torque strengths compared with smooth surfaces with similar chemistry. This possibly indicates the importance of the surface roughness for a strong interlocking of implants in bone tissue. Nevertheless, calcium-modified moderately rough anodized implants also had significantly higher torque strength compared with the moderately rough anodized implants. Solely blasted implants had a lower mean value but the difference was not statistically significant. The topography of the BlOxCa implants was similar to the BlOx implants and in the same range as the Bl implants. That may indicate an additional effect of the surface chemistry/the incorporated Ca^{2+} on the bone response, which is in accordance with other published studies.[12,13]

The BlOxCa and the BlOx implants in the present study had greater surface area and were also more densely peaked compared with the other surfaces. Increased surface area may enable greater possibility for interactions between the surface and its surrounding tissues. The summit density could possibly affect the bone response as the summits may act as binding sites for the initial mineral crystal growth. Similarly, the summit density and the distribution pattern may influence surrounding cells and proteins. Arvidsson and colleagues found a low core fluid retention index to be favorable for biological outcome.[24] That was not clearly the case in the present study. However, implants with significantly lower core fluid retention index (Ox and Bl) had somewhat higher RTQ values compared with implants with higher indexes (OxCa and BlOx), but the differences were not statistically different. The results may leave room for other factors or surface parameters, which may increase our knowledge and understanding of the importance of surface topography on the bone response.

There were no significant differences considering the bone response between the Ox and the OxCa implants placed in femur. Even if both implants were smooth, the Ox implants had a greater surface roughness, a larger developed surface area, and lower core fluid retention index. Although the outcome was not as hypothesized, it could be explained by the previously discussed advantages. Possibly, there may have been differences at earlier time points. After 12 weeks of healing in a rabbit, newly formed bone has matured.[25] A suggested advantage with altered surface chemistry could be a faster and increased bone response around implants. Shortened healing periods have been discussed in the literature.[26]

When comparing the RTQ results between implants placed in femur and implants placed in tibia, the femoral implants generally showed higher interfacial shear strength though being only smooth implants. The same tendency with higher RTQ values for femoral implants can be found elsewhere in the literature.[27,28]

Histomorphometrical evaluations of tibial implants were somewhat contradictory to the RTQ results; the implants with highest quantity of bone in close vicinity to the implant had the lowest RTQ strength. The amount of bone in close vicinity to an implant and the RTQ strength has been reported to be correlated in other studies.[22,29,30] There may have been a stronger interlocking of the BlOxCa compared with the BlOx implants, perhaps because of surface chemistry, resulting in a significantly higher RTQ although with less estimated bone to have been in contact. The strength of the interlocking may even affect the tearing of the border, possibly somewhat affecting the histological view and, thereby, the evaluation of the implant–bone border. Shear forces have in some cases been calculated with the height of the cortical layer as a factor.[31] It is an important region for the stability of an implant when it is interlocked strongly by the dense bone in the cortical layer. The three best threads, from which the results did not differ between the surfaces, often correlates to the region of the cortical layer. However, it is common that surrounding bone area does not correspond to the rate of osseointegration.

The possible effect of calcium modifications to compensate for lesser surface roughness should be further evaluated with clinical and biomolecular in vivo and in vitro studies to achieve a better understanding of the integration process as well as to strengthen the hypothesis further.

CONCLUSION

Moderately rough Ca^{2+}-modified anodized implants had significantly higher torque strength compared with smooth Ca^{2+}-modified anodized implants and moderately rough anodized implants when placed in rabbit tibia. At the same time, smooth Ca^{2+}-modified anodized implants had similar torque strength to moderately rough anodized and blasted implants; possibly demonstrating surface chemistry to compensate for a lesser roughness.

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