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Tensile force testing of optimized coin-shaped titanium implant attachment kinetics in the rabbit tibiae

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In the present study, the bone response of titanium implants at early bone healing stages, was evaluated using a tensile test. Test surface of coin-shaped cp. titanium implants were standardized by grit blasting with TiO_2, grain size 180–220 μm. The surface topography of the implant specimens was examined by SEM, and by a confocal laser scanner for evaluation of S_a, S_t and S_{dr}. The implants were placed onto the leveled site on the tibia of 12 New Zealand White rabbits, 4 implants in each animal. The rabbits were divided into three groups with different observation times i.e. 2, 4 and 6 weeks. The retention of 12 implants were tested by measuring the pull-out force needed to detach the implant from the bone. There was a significant increase in implant retention from 2 to 4 and to 6 weeks healing time ($p < 0.05$). Four implants from each time point were randomly chosen for histological evaluation. The histological appearance of the implant–bone interface at the different healing times showed noticeable differences in the degree of bone healing and maturation, suggesting that, in rabbits, 6 weeks healing time is a suitable observation point for tensile testing of surface optimized osseointegrating implants.

1. Introduction

Biomaterials are materials for application in biological systems [1]. The term is mostly used for materials for implantation into the human body. Generally, it is the surface of the implanted biomaterial that comes in direct contact with the biological system, and thus encodes the biological properties of the implant. Because of superior mechanical and biocompatible characteristics, titanium is the material of choice for bone-anchored devices. Lately, much research has been aimed at characterizing and optimizing the surface properties of this metal when used for bone-anchored implants. Modern orthopedic and dental implant treatment aims at rapid strong and long-lasting attachment between implant and bone for optimal performance. Shortening the time from implantation to load has been a priority in recent implant development. Today, surface roughness is recognized as one major factor for improving the bone attachment of the biomaterial [2–5]. The surface structure and topography of titanium implants can be modified in several ways, with effects on the chemical-, topochemical-, physical-, and mechanical properties of the metal surface [6].

Results from several *in vitro* studies have suggested a positive correlation between surface roughness and cellular activity [7–10]. These observations are supported by *in vivo* studies, where attachment between bone and implant is directly assessed by mechanical testing [3, 4, 11].

A variety of different surface structures have been tested with respect to healing times. Biomechanical properties are most often evaluated by measurements of shear forces, either by pull-out/push-out tests [12–19], with removal torque tests [20–22], or with tensile tests [23–25]. Results from these studies all indicates that mechanical attachment increases with the healing time.

In a study in rabbits where the effect of increasing implant surface roughness on functional attachment in bone at 10 weeks [26] was tested, we found that the strongest bone attachment was observed with implant surfaces that had been blasted with TiO_2 particles of 180–220 μm, producing a surface with a S_a value of 3.62 μm. Micro-roughness finer or more coarse than this value was shown to be less favorable at this time-point, 10-week healing time. The aim of the present study was two sided; (i) to investigate the bone attachment kinetics for this particular titanium surface [26] at shorter healing times, and (ii) to evaluate the tensile-test method [27]

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when used at early implant healing stages, that is before and during early bone maturation.

2. Materials and methods

2.1. Implants

Commercial pure (cp) titanium, grade 2 (ASTM B 348), was the basis for the test implants used in the present study. The 48 implants were made from a 10 mm cylindrical bar, machined down to coin shape appearance with a diameter of 6.25 mm and a height of 1.95 mm. The flat test surfaces of all discs were grit blasted with TiO_2 particles with a grain size of 180–220 μm. On the reverse face of the coin-shaped implants a threaded non-penetrating hole for the connector for the tensile test, was made [27].

The blasting distance from the implants to the jets was approximately 20 mm, and the TiO_2 particle stream hit the surface at an angle of 90°. The air pressure used for blasting was set to 0.5 MPa. Blasting of each implant was performed with repeated horizontal and vertical movements during an 8-s period. During the blasting procedure the test implants was placed in a pre-holed silicon plate to protect the vertical parts of the implants from the blasting particles. To prevent any further uncontroled wear the test implants were stored separately in glass containers after blasting.

Prior to implantation, all implants were then separately treated with trichloroethylene in an ultrasonic bath and rinsed with ethanol before the final rinsing with deionized water. The implants were thereafter autoclaved for 7 min (2 ATM, 137 °C) and stored separately in glass containers.

2.2. Animals

Twelve New Zealand White female rabbits, 6 months old and having a weight between 3.0–3.5 kg, were used in this study. The animals were kept in separate cages during the experimental period. Room temperature and humidity were standardized at 19 ± 1 °C and $55 \pm 10\%$, respectively, according to local regulations.

The experiment had been approved and registered by the Norwegian Animal Research Authority (NARA). All procedures were conducted in accordance with the national Animal Welfare Act of 20 December 1974, No. 73, chapter VI sections 20–22 and the Regulation on Animal Experimentation of 15 January 1996.

2.3. Sedation

The rabbits were sedated by injection with fluanisone/fentanyl (Hypnorm®, Janssen, Belgium) 0.05–0.1 ml/kg s.c. and further anasthetized with midazolam (Dormicum®, Roche, Switzerland) 2 mg/kg bw i.v. The sedation was maintained with diluted Hypnorm® (1 ml Hypnorm and 9 ml sterile water). Lidocain/adrenalin (Xylocain/Adrenalin®, Astra, Sweden) 1.8 ml s.p. was used for infiltration analgesia at the site of surgery.

2.4. Surgical procedures

Prior to surgery, the operation sites were depilated and washed with soft soap and disinfected with colored chlorhexidingluconat 5 mg/ml (Klorhexidin, Galderma Nordic AB, Sweden). Animals were placed on their back on the operation table, covered with sterile cloths and the operating sites were disinfected once more with chlorhexidingluconat 5 mg/ml. An incision was made on the proximal-anterior part of tibiae, penetrating all soft tissue layers. The periosteum was elevated, and four small guide holes penetrating into the bone marrow, were made with a twist drill (Medicon®, Germany) using a drill guide to ensure standardized positioning. The outermost two holes were used for fixation of the bone plate. The two holes in the center were used for stabilization of the custom-made stainless steel bur in levelling the two platforms for the implants in the cortical bone, as shown in Fig. 1(a). All manipulation with bone tissue was executed with copious physiological saline solution irrigation. The implants were then placed onto the cortical preparations with only the grit blasted face in bone contact. The outer faces were covered with the polytetrafluoroethylene (PTFE) caps, placed to inhibit bone growth on the vertical parts of the implant as well as bone overgrowth [27]. The PTFE-covered implants were then fixed to the bone with a pre-shaped bone plate (Medicon® CMS, Germany), retained with two titanium screws (Medicon® CMS, Germany), as seen in Fig. 1(b). After the implant procedures, the soft tissue layers were repositioned and the wound closed using a polyglycolic acid suture. Following surgery, each animal received a s.c. injection with buprenorphin (Temgesic®, Reckitt & Colman, England) s.c. A second injection of Temgesic, was given 3 h post-surgery. The animals health condition was monitored throughout the study period and the operation sites were examined daily until wound healing was considered complete or the animals were sacrificed for the tensile test. A total of 48 implants were placed in 12 rabbits ($n = 48$, 4 in each animal).

2.5. Tensile test

Bone–implant attachments were tested 2, 4 and 6 weeks after implantation. Functional attachments of 12 implants from each time-point were evaluated ($n = 12$ for each time point). The nine rabbits were randomly chosen for the tensile test that is, 12 test implants of each time point. At the end of the planned observation period, the rabbits were euthanized using an i.v. injection of 1.0 ml fluanison/fentanyl (Hypnorm®, Janssen, Belgium) followed by 1 ml/kg bodyweight pentobarbital (Mebumal®, Rikshospitalets Apotek, Norway) i.v. Immediately after euthanization, the titanium plate covering the implants was exposed and removed. A hole was made in the center of the PTFE cap with a small injection needle, and pressurized air was applied using a syringe to remove the caps and expose the reverse part of the implant without applying any retractive forces on the implant. The tibial bone was then fixed in a specially designed device that stabilized the bone during the tensile test procedure. Subsequently, the implants were connected to the load-cell by a threaded pin with a ball-head connected to a 300-mm long wire. This set-up allowed for perpendicular alignment of the load force with respect to the test

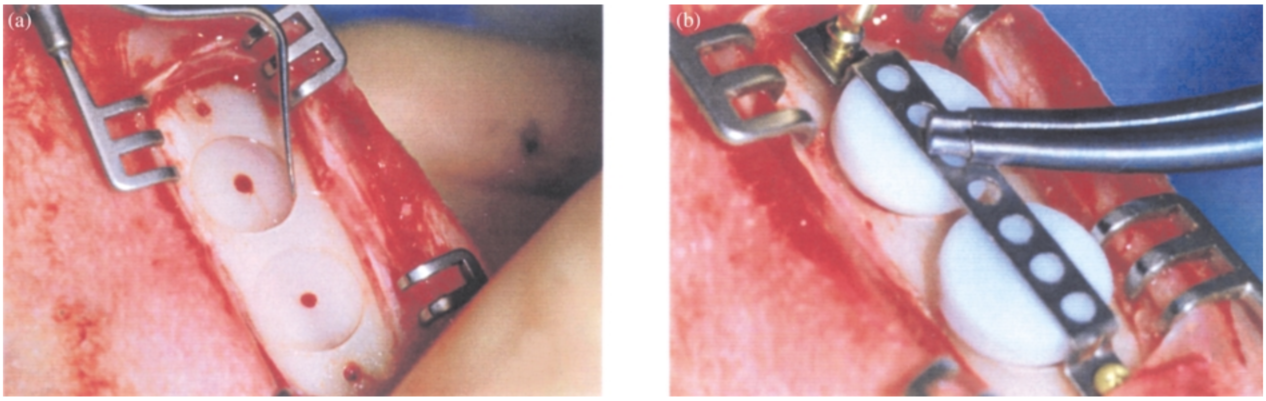

Figure 1 The surgical procedure: (a) The leveled site for the implants. (b) The implants with PTFE caps placed and retained with the pre-formed titanium band.

surface of the implants, minimizing the influence of shear forces during the tensile test procedures.

The tensile test was performed with a Lloyds LRX Materials testing machine fitted with a calibrated load-cell of 100 N. Cross-head speed range was set to 1.0 mm/min. Force measuring accuracy was ± 1% (Certificate of calibration: NAMAS Calibration No. 0019 issued by Instron Calibration Laboratory No. 1000356). Load was applied until the implants detached from the bone and recorded on a load versus time plot.

2.6. Surface characterization

Before implantation details of the implant test surfaces were recorded using scanning electron microscopy (SEM, a Philips XL 30 ESEM, Philips Electron Optics, The Netherlands) for morphological control, and confocal laser scanning microscope (CLSM, a Leica TCS 4D, Leica, Germany). For CLSM, the area of measurement was set to $1000 \times 1000\,\mu\text{m}^2$, and the average height deviation value (S_a), the maximum peak-to-valley roughness (S_t) and the developed surface area ratio (S_{dr}) within this region was mapped.

2.7. Histology

In addition to surface analysis and tensile tests, histology was performed on implants from three rabbits, four implants from each group. They were investigated with regard to the formation of bone in close contact with the implant surface. The bones with the implants were fixed in 4% neutral buffered formaldehyde and dehydrated before being embedded in resin (Technovit 7200 VLC®, Kultzer & Co, Germany). Two of the implants at each time point sectioned perpendicular to the long axis and two was sectioned parallel to the long axis of the bone. The sections, approximately 1 mm thick, were polished with fine grit carborundum paper to approximately 20 μm. These sections were stained with Toluidine Blue and histological evaluation was performed in a light microscope.

2.8. Statistical analyses

A statistical analysis of the results was accomplished by using the Pearson's correlation coefficient. Analyses for determination of differences in the measured properties between groups and confidence intervals were done using Student *t*-test in a pairwise comparison.

3. Results

3.1. Surface analyzes

Blasting of the titanium implants with 180–220 μm TiO_2 particles resulted, as shown in Fig. 2, in a relative homogenous, rough surface structure as observed by SEM and CLSM. The test surface had a structure with

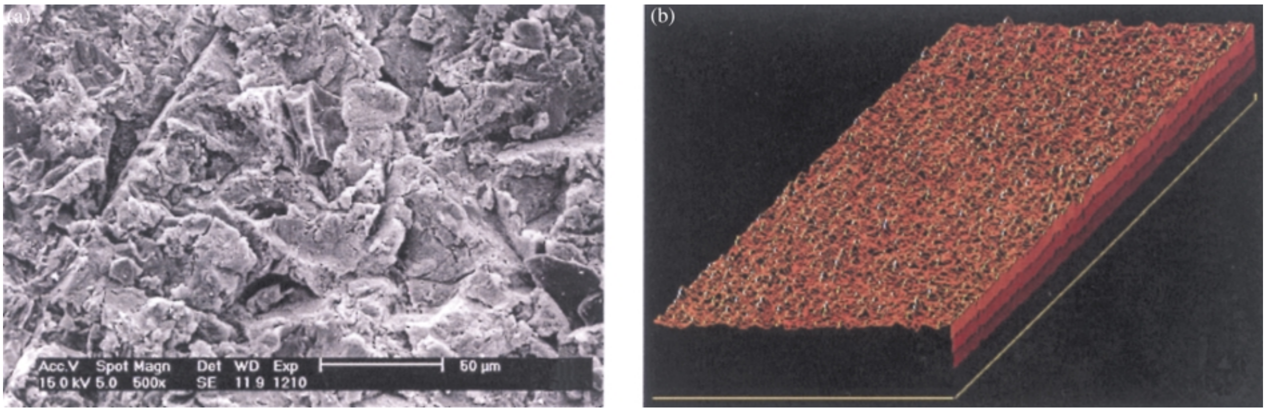

Figure 2 Scanning electron micrographs (a) (× 500) and confocal laser scannings (b) of the prepared test surfaces. Surface blasted with 180–220 μm particles of TiO_2.

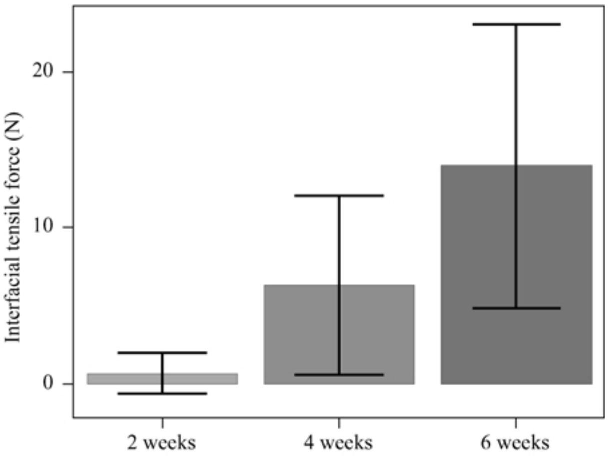

Figure 3 Results of the tensile test measurements of the three different time periods. Error bars represent mean values ± SD. Statistical evaluation revealed $p < 0.022$ between all groups.

variation in the geometry of the peaks and valleys, and appeared relatively coarse at 500 × . Several flat facets up to 50 μm in size, could be observed. The facets also had small irregularities appearing as pits and stripes. The numerically description of the surface gave an average height deviation value of 3.37, the maximum peak-to-valley roughness 75.88 and the developed surface area ratio of 2.02.

3.2. Retention analyses

Generally, implant retention increases with time. As illustrated in Fig. 3, the weakest functional attachment was observed at 2 weeks (0.6 N). At this early time, 25% of the implants had attached to the bone $(n = 3)$. At 4 weeks healing time, the mean tensile force binding the implant to bone increases with a factor of 10 (6.3 N) compared with the 2 weeks results. At this time point, 83% of the test implants had attached to the bone $(n = 10)$. After 6 weeks of healing, the mean tensile force necessary to detach the implants had significantly increased to 14.0 N, and 93% of the implants were attached to the underlying cortical bone $(n = 11)$. A statistical significant increase in the tensile strength needed to detach the implants from bone could be demonstrated when the implants were tested after 2 weeks and after 6 weeks $(p = 0.00035)$ and after 4 and 6 weeks $(p = 0.022)$ and between 2 and 4 weeks healing period $(p = 0.006)$.

3.3. Histology

At 2 weeks after implantation, the tissue adjacent to the implants did not show any sign of advanced bone formation as shown in Fig. 4(a). In these specimens the implant surface facing the cortical bone was lined by a non-mineralized collagenous tissue filling the space between the implant and the planed bone. The underlying cortical bone appears normal with osteocytes present. The situation resembles early bone healing with reparative processes ongoing, but with bone matrix secretion yet to start.

At 4 weeks, the tissue filling the space between the implant and bone had started to mineralize as shown in Fig. 4(b), but the outline of the newly formed bone is still evident. The deeper parts of the cortical bone have a normal appearance.

At 6 weeks, the space between the implant and the planed bone surface has filled completely with a highly mineralized, bone-like tissue as shown in Fig. 4(c). The new tissue is rich in osteocytes and the demarcation line between the newly formed tissue and the old cortical bone is barely visible. At this stage, osteoblasts activity is not present, indicating that bone maturation has started.

4. Dicussion

The design of the implant and the wider preparation of the bone allowed identification for the tensile force needed to detach the implant from the cortical bone [27].

Only a few studies have evaluated attachment after 2 weeks of healing and then only as pullout and pushout studies measuring shear forces. In a tensile strength evaluation of glass–ceramic and alumina–ceramics implants, no bone–implant attachment was found at 2-week healing time [23]. In that study, shear forces measured on the same implants were 1.9 and 0.2 kg/cm^3, respectively. Using plasma-sprayed hydroxyapatite (HA) coating on titanium implants [25], bone attachment was measured to 0.66 MPa in a tensile test performed after 2 weeks of healing. Comparing turned and acid-etched titanium implant surfaces mean values of approximately 10 and 20 N respectively was found using implants in rat femur [13]. In the rabbit tibia model, mean pull-out forces of 39.7 N and 58.8 N was measured in machined and dual etched titanium implants, respectively [12]. Removal torque values (RTV) on machined cp titanium implants are reported to be 19.9 N m in a rat tibia model [20]. All these studies suggest that very little bone attachment can be found for titanium implants at this early stage of bone healing when bone matrix secretion has just started, even when different implant surfaces are tested and a variety of mechanical evaluation methods are used. The present study confirms this trend by tensile force testing. Moreover, histological evaluation clearly show that at this early stage mineralized tissue has yet to form at the bone–implant interface. At this stage only a non-mineralized collagenous interface is present, offering little resistance against forces imposed to the implant.

Other tensile test results have demonstrated a 30–45% increase from 2 to 4 weeks of healing when biomineralization of the matrix have started [25]. These findings were however, not consistent, but varied with the type of surface. In all shear studies, no attachment was found at 4 weeks of healing evaluating alumina–ceramic implants, but with glass–ceramic implants a mean strength of 8.4 kg/cm^3 was observed [23]. Interestingly, the differences from 2 to 4 weeks of healing in the measured shear forces in pull-out and push-out models was ranging from 16% decrease (machined surfaces) to 50% increase (acid-etched surfaces). RTV measured with machined surfaces revealed no changes from 2 to 4 weeks. This suggests that the period between 2 and 4 weeks is a critical period in bone healing where surface structure and chemistry strongly influence processes of bone healing and the strength of implant–bone attachment.

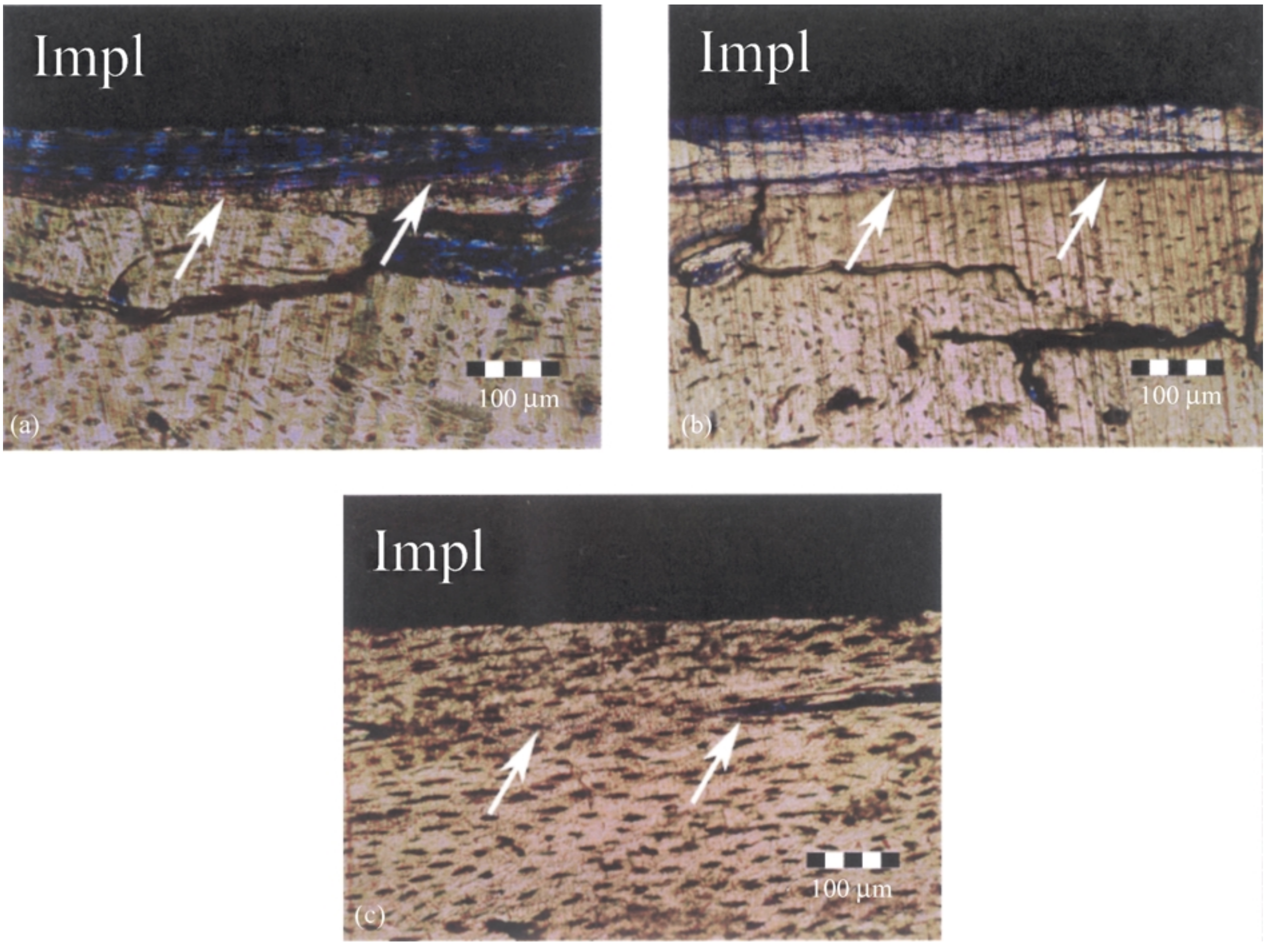

Figure 4 Histologic appearance of the implant–bone interface after 2 weeks, 4 weeks and 6 weeks of implantation for cp titanium implants (Ti) grit blasted with TiO_2, grain size 180–220 µm. (a) 2 weeks after implantation, shows a non-mineralized collagenous tissue between the implant surface and the cortical bone. The outline of the defect is clearly visible (arrows). (b) 4 weeks after implantation, the collagenous bone has been replaced by immature bone with a high organic content. The demarcation line is still prominent (arrows). (c) 6 weeks after implantation, shows that at this stage the gap between implant surface and the cortical bone is filled with mineralized tissue. The new bone is maturing and the demarcation line is now barely visible (arrows). All sections are stained with Toluidine Blue.

This is also supported by the histological evaluation showing that at 4 weeks the bone–implant interface had transformed from a purely non-mineralized collagenous structure into a mineralized tissue resembling immature bone. This newly formed bony tissue offer much better resistance against implant loading, which is reflected in the increase in the tensile-test values.

To our knowledge no tensile force studies have performed bone attachment measurements on pure titanium surfaces after 6 weeks of observation. A study comparing attachment of untreated titanium implants at four and 8 weeks after implantation showed no increase in the measured values. [24]. This suggests that the initially poor contact between bone and implant with such surfaces fills with a fibrous tissue during wound healing that is not replaced by mineralized bone matrix, and thus the implants are not fully integrated in the bone. HA-coated implants showed a slight decrease in attachment for the same time period [25]. The author explains this phenomenon by bone being progressively bonded to the implant with time, reaching maximum bonding strength at 4 weeks. At 8 weeks, a gradual degradation of the HA coating, as confirmed by the failure mode analyses, was the reason for the observed decrease in the tensile strength. Machined implant surfaces in a removal torque study increased with 50% [20] from four to 8 weeks, suggesting that maturating bone in the threads provides friction enough to increase RTV. Another study revealed a numerical decrease although not of statistical significance [21]. In this study, the author related this observation to a relatively high standard deviation (SD) for both implant groups and suggested that the variation of RTV was primarily due to the variation in local bone density, since the position of the implants had a significant influence on the measured RTV. When interpreting the results in the latter study, however, one should also be aware of the relatively small numbers of implants tested $(n = 54)$ within the three groups per time period $(n = 6)$ and the assumption of normal distribution in the statistical analysis.

If one compares the present study with previous results using the same implant surfaces and evaluated by the same test method, but after 8 and 10 weeks healing time [26, 27], the attachment between the implants and the bone seems to be developing most rapidly between weeks 8 and 10 as illustrated in Fig. 5. The observed increase in retention of implants between 8 and 10 weeks is, probably a result of advanced bone maturation as observed.

Based on these findings, a 6 weeks observation time could be the best (shortest) set point for assessing improved bone–implant attachment kinetics using tensile

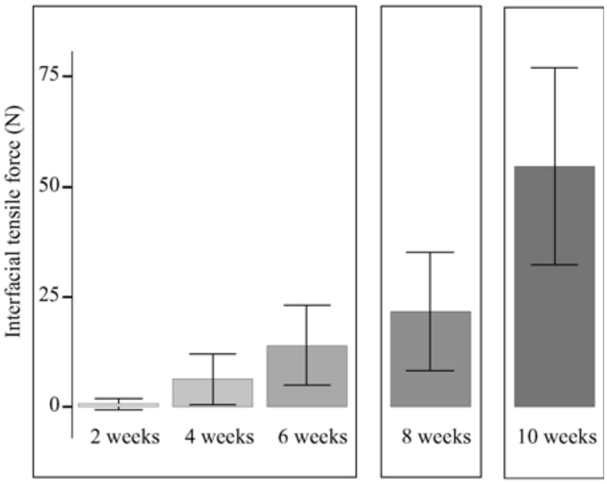

Figure 5 The results from the present study compared with 8- and 10-week data compiled from earlier studies [26, 27]. A statistically significant increase in attachment could be observed from 6–10 weeks ($p = 0.013$), but not six weeks to 8 weeks ($p = 0.084$). Bars represent mean values ± SD.

testing methods in studies on bone integration of improved titanium surfaces. This is supported by findings of Roberts *et al.* [29] and Sennerby *et al.* [30] who concluded that the bone around an implant was mature after 6 weeks based on biomechanical testing and histology. Results from studies revealing implant retention versus time show a clear connection between the implant materials surface and the gradient of the mechanical forces needed to detach the implants from the tissue.

However, in the study presented here, the number of unattached implants at the time of testing was much higher with shorter healing times, in particular 2 and 4 weeks, compared to 6 weeks and the 8 week observation. At 4 weeks, 17% of the implants were unattached. This number of unattached implants decreased to 9% at 6 weeks. This is probably due to the need of maturation and mineralization of the bone before a firm implant tissue attachment can be established that can resist relatively high load forces. It can not be ruled out that the removal of the teflon caps prior to the pull-out procedures produced loads strong enough to detach implants that are only loosely attached. However, the technique used only introduces very small loads to the implants and do not exert any ''pull-out force''. The loose implants in this study thus most probably represent implants that are not bound to the underlying tissue at the time of tensile test procedure.

The experiment demonstrated that the applied test procedure is capable of measuring increases in the bone-to-implant attachment over time. The method is also, to a very little extent biased by factors that give false positive results that is interlocking and friction. This is clearly visualized by the number of implants that still not had attached to bone before the tensile test procedure was conducted. This number was reduced with longer healing times, and particularly after 6 weeks when bone maturation has started. Several authors have stressed the importance of analyzing time-zero implants in order to distinguish mechanical impaction of bone from the biological process of bone ingrowth [30] This is irrelevant to the approach presented here using coin-shaped implants and a tensile test set-up, because implant retention in this model totally rely on new bone in-growth and subsequent implant integration into bone.

5. Conclusions

The presented results suggest that functional bone attachment of titanium dioxide blasted implants increases slowly but steadily over the first 6 weeks of healing in rabbits, during which wound healing and bone matrix secretion take place. Thereafter, remodeling and maturation of bone mass occurs with a more rapid, additive effect on implant–bone attachment. This suggests that, in rabbits, 6 weeks healing time is a suitable observation point for tensile testing of surface optimized osseointegrating implants. At this time point, the tensile test results will reflect both the extent of new bone secretion and the onset of bone maturation. Thus, analyses at this stage allow identification of small changes in the onset of maturation and hence the speed of implant integration. Knowing such critical time points is important if new implant surfaces are to be developed for rapid bone healing and improved implant performance.

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