Titanium Alloy Osseointegration in Cancellous and Cortical Bone of Ovariectomized Animals: Histomorphometric and Bone Hardness Measurements

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Purpose: Histomorphometry and microhardness measurements were performed after Ti6Al4V implantation in cancellous and cortical bone of healthy and ovariectomized animals to determine characterization of the bone-biomaterial interface in osteopenic bone. Materials and Methods: Nine ovariectomized and 9 sham-aged rats were used. Four months later, nails were implanted in the distal femurs, and the animals were sacrificed after 8 weeks. Moreover, 3 ovariectomized and 3 sham-aged sheep were used. Twenty-four months later, screws were implanted in the tibial diaphyses and the animals were sacrificed after 12 weeks. Results: Histomorphometry showed the development of osteopenia in both trabecular and cortical bone, and revealed a significant decrease in the osseointegration rate in osteopenic versus sham-aged animals for both trabecular (Affinity Index: –18.6%, P < .001) and cortical bone (Affinity Index: –23.5%, P < .005; Bone Ingrowth: –9%, P < .05). At the interfaces of the sham-aged animals in both trabecular and cortical bone, a decrease of bone microhardness was observed in comparison with preexisting bone (trabecular: –9.8%, P < .0005; cortical: –19.3%, P < .0005). In case of osteopenia, this decrease was even more extensive (trabecular: –15.5%, P < .0005; cortical: –24.7%, P < .0005). Discussion: The present data suggest that bone formation around Ti6Al4V was not associated with complete bone maturation, even in healthy animals. In case of osteopenia, both bone formation and maturation were delayed. Conclusion: These results apparently demonstrate the utility of investigating biomaterials in osteopenic bone and the importance of careful evaluation of the healing rate and bone maturation degree around implanted biomaterials. (INT J ORAL MAXILLOFAC IMPLANTS 2002;17:28–37)

Key words: bone-biomaterial interface, implant, microhardness, osseointegration, osteoporosis, titanium alloy

Osteoporosis is a health problem of clinical relevance that recognizes many different pathogenetic factors.1,2 Structural modifications related to osteoporosis mainly affect cancellous bone, but cortical bone also is compromised with a reduction of cross-sectional area because of endosteal resorption and medullary expansion.3,4 Osteoporotic patients develop pathologic fractures mainly affecting the hip, spine, and wrist, but the incidence of fractures also increases at many other sites,5,6 necessitating the greater use of plates, pins, screws, and arthroprostheses in osteoporotic bones. The extent of oral bone loss in conditions that cause skeletal osteopenia is still unclear.7 However, some experimental and clinical studies have shown jaw and mandible involvement in osteoporosis,8–11 and they illustrate the role played by the systemic bone mass decrease in the development of tooth loss in postmenopausal women.12,13 This suggests that the need for dental implants may be greater for osteoporotic patients.14

Clinicians recognize that the presence of osteoporosis could represent a risk factor for implanted
It is known that not only bone histologic and structural modifications typical of the osteoporotic state are responsible for poor bone ingrowth, but also a decrease of some cytokines or growth factors which are useful for osseointegration processes, associated with an increase in other damaging events, have additional negative consequences on the bonding of bone to biomaterials.

Many experimental studies in orthopedics and dentistry conducted on biomaterial osseointegration have focused attention on the clinical situation of osteoporosis because of the costs and severe effects on patients' physical and mental health. In previous experimental studies in rats, the current authors observed a significant decrease in the osseointegration rate of some metallic and ceramic materials when implanted in osteopenic cancellous bone. Histomorphometry was used to study the bone-biomaterial interface to yield quantifiable results.

The working hypothesis of the present study was that the osseointegration of Ti6Al4V can be delayed in trabecular and cortical osteopenic bone in comparison to healthy bone. Nails and screws made of Ti6Al4V were implanted in the cancellous and cortical bone of ovariectomized rats and sheep. First, the osteopenic state developed in animals was characterized, and then the bone-material interface was assessed. Histomorphometry was improved by microstructural data including microhardness measurements, thereby increasing the knowledge on bone maturity, mineralization rate, and mechanical resistance at the bone-biomaterial interface.

MATERIALS AND METHODS

Experimental Design

The specimens used in this project were obtained from a larger study currently in progress. The study was performed in compliance with the European and Italian Laws on animal experimentation and with the Animal Welfare Assurance No #A5424-01 of the National Institute of Health (NIH-Rockville, MD). The animal research protocol was approved by the responsible public authorities as requested by the Italian Law according to the EC rules (Law by Decree, 27 January, 1992, No. 116).

Intact rats and sheep served as control groups (sham-aged), while the ovariectomized animals comprised the experimental groups. Grade 5 Ti6Al4V (ASTM F-136), sterilized via gamma radiation (2.5 megarad), was implanted in animals. Osseointegration in trabecular bone was tested in rats where cylindrical nails, 2 mm in diameter and 5 mm in length, were used, while osseointegration in cortical bone was tested in sheep in which cylindrical tapered screws, 3.5 mm in outer diameter and 12 mm in length, were implanted (Fig 1).

Bone implants were placed 4 and 24 months after ovariectomy in rats and sheep, respectively. The postovariectomy time intervals selected had already shown the development of an osteopenic state in previous tests by the current authors. Therefore, they proved to be useful for the present investigation in both animal species. Rats were housed in metal cages for the entire study at 20°C ± 0.5°C and relative humidity of 55%; food (Mucedola Srl, Settimo Milanese, Milan, Italy, Ca 0.93%, P 0.64%) and tap water were allowed ad libitum.

Sheep were maintained in a stall until postoperative day 20 (1°C and relative humidity of 55%; food (Mucedola Srl, Settimo Milanese, Milan, Italy, 0.85% Ca and 0.51% P) and tap water were allowed ad libitum. About 20 days after surgery, sheep were moved to external housing conditions.

All surgical procedures were performed in aseptic conditions and under general anesthesia. Animals were euthanized under general anesthesia with intravenous administration of Tanax (Hoechst, Frankfurt am Main, Germany) at selected experimental times.

Rats: Trabecular Bone Implants. Eighteen rats, weighing between 450 ± 30 g and aged 10 months at the beginning of the study were used; 9 animals were bilaterally ovariectomized through a lumbar approach and 9 nonoperated sham-aged rats were used as controls. General anesthesia was obtained...
Sheep: Cortical Bone Implants. Six mongrel sheep, body weight 70 ± 5 kg, aged 5 years at the beginning of the study, were used. General anesthesia was obtained by premedication with 10 mg/kg bw ketamine, 0.3 mg/kg bw xylazine im and 0.0125 mg/kg bw atropine sulphate sc. It was induced with 6 mg/kg bw sodium thiopentone iv (2.5% solution, Pentothal, Hoechst AG, Germany), and maintained with O₂, N₂O and 1% to 2.5% halothane under assisted ventilation (Servo Ventilator 900 D, Siemens, Germany). Three sheep were submitted to bilateral ovariectomy while the remaining animals served as sham-aged control group. Twenty-four months later, a total of 18 screws were implanted in the left tibia of all sheep (3 per tibia). After incision of the skin and dissection of the fasciae, the left tibial diaphyses were exposed. A 2.7-mm-diameter drill was used to predrill 3 holes. They were tapped with a 3.5-mm device and 3 screws were then placed into the diaphyseal cortex of each bony segment, with the same final insertion torque of about 1.8Nm. Antibiotics and analgesics were administered for 5 and 2 days, respectively (1 g/day cephalosporin, Totacetf, Bristol Myers Squibb SpA, Italy; and 500 mg/day ketoprofen, Orudis, Rhone-Poulenc Rorer SpA, Milan Italy). All the animals were euthanized after 12 weeks, and each implant site was isolated using a saw and harvested.

Histomorphometry

The bone specimens containing the implants were fixed in 4% buffered paraformaldehyde for 24 hours, dehydrated in graded series of alcohols until the absolute was reached (for 24 hours at each concentration), and then included in polymethylmethacrylate resin until solidification, which usually occurred after 7 days. All the processing was carried out at a temperature of 22°C ± 1°C and humidity rate of 48%. Blocks were sectioned along a plane perpendicular (nails in rats) or parallel (screws in sheep) to the long axis of the implants by using a Leica 1600 diamond saw microtome (Ernst Leitz, Wetzlar, Germany) and yielding undecalcified sections of 60 to 80 µm in thickness. The sections were ground and polished (Struers Dap-7, Strues Tech A/S, Rodovre/Copenhagen, Denmark) and stained with Fast Green. Histomorphometric analyses were performed on 3 consecutive sections using an optic microscope (Zeiss Axioskop, Carl Zeiss, Jena, Germany) connected to an image analyzer system (Kontron KS300 v.2, Kontron Elektronik, Munchen, Germany). The nomenclature approved by the American Society of Bone and Mineral Research (ASBMR) was used for cancellous and cortical bone calculations and the following static measurements were calculated at 1.25x:

1. In cancellous bone (bone bed around the implants): trabecular bone volume (BV/TV, %), the whole spongy bone area, expressed as a percentage of the total tissue area in the sampling site and converted to a volume; trabecular thickness (Tb.Th, µm), trabecular separation (Tb.Sp, µm), trabecular number (Tb.N, /mm) and index of connectivity (number of nodes/number of trees, N.Nd/N.Tm) were calculated from BV/TV and the perimeter of trabeculae (BS/TV) according to Parfitt’s formulas

2. In cortical bone (mid-diaphysis): cross-sectional area (Se.Ar, mm²), area of bone and marrow cavity within the periosteal surface; medullary area (Me.Ar, mm²), area delineated by the endocortical surface of the cross section; cortical bone area (Ct.Ar, mm²), Se.Ar-Me.Ar; endocortical perimeter (Ec.Pm, mm), length of bone perimeter on the endocortical surface; and periosteal perimeter (Ps.Pm, mm), length of bone perimeter at the periosteal surface.

Affinity Index (the ratio of the length of the region in which bone is directly apposed to the implant without the presence of fibrous membrane divided by the total length of the bone-implant interface multiplied by 100) was calculated for nails implanted in rat trabecular bone. Affinity Index and Bone Ingrowth (amount of bone grown into the screw grooves, expressed as a percentage of the groove area) was calculated for screws implanted in sheep cortical bone.

Each measurement was made semiautomatically by an experienced blinded investigator.

Microhardness. After the histologic analysis, the resin-embedded blocks containing the remnant of the implanted material were ground and polished following the methodology described by Huja and associates, and then used to measure the level of...
bone hardness by means of an indentation test (Microhardness VMHT 30, Leica, Wien, Austria). Microhardness measurements were taken with a Vickers indenter (4-sided pyramid with square base and an apex angle between opposite sides of 136 ± 15 degrees) applied to the bone at a load of 0.05 kgf and dwell time of 5 seconds. The Vickers hardness degree (HV) was calculated by dividing the indentation force by the surface of the imprint (4 pyramid surfaces) observed at the microscope. The resulting formula was where \( F \) is the weight applied to the pyramid expressed in kg, \( \beta \) is half of the pyramid angle, and \( d \) is the average diagonal length of the imprint expressed in \( \mu \)m. The average value for each sample was calculated on 10 measurements for each examined area, eg, at the interface and at 1,000 \( \mu \)m from it. By allowing a minimum distance of about 3\( d \) between the imprints, their mutual influence was avoided, too.

**Statistical Analysis**

Statistical analysis was performed by using a SPSS version 7.5 software (SPSS/PC, Chicago, IL). Data were reported as mean ± SD at a significance level of \( P < .05 \). After having verified normal distribution and homogeneity of variances with Levene’s test, Student \( t \) test was used to compare data between groups. Paired Student \( t \) test was used in case of dependent samples.

**RESULTS**

**Rats: Trabecular Bone Implants**

Percent variations in static histomorphometric parameters of the cancellous bone between experimental and control groups are reported in Fig 2. These data confirmed the development of a significant osteopenic state in the bone bed where Ti6Al4V was implanted at 4 months after ovariectomy.

Figures 3a and 3b show the histologic appearance of healthy and osteopenic trabecular bone implanted with Ti6Al4V. When Ti6Al4V is implanted in osteopenic bone, some areas without any apparent bone-to-implant contact can be observed.

Morphometric data also confirmed the histologic appearance: the Affinity Index decreased significantly (\( P < .001 \)) in osteopenic versus healthy bone (experimental group: 54.63% ± 8.88%; control group: 67.08% ± 10.51%).

Bone microhardness results obtained around implants at the interface and at 1,000 \( \mu \)m from it are reported in Table 1. Percent decrease in microhardness data observed at the interface and compared to that obtained at a 1,000-\( \mu \)m distance revealed a significant difference (\( P < .0005 \)) in experimental (–15.51% ± 5.05%) and control (–9.79% ± 9.95%) groups.

**Sheep: Cortical Bone Implants**

The percent variations in cortical static histomorphometric parameters measured in tibia and reported in Fig 4 demonstrated the development of osteopenia in cortical bone.

Figures 5a and 5b show less bone-to-implant contact in osteopenic cortical bone versus healthy bone.

Morphometric data are consistent with histologic observations: Significant decreases in the Affinity Index (\( P < .005 \): experimental group: 57.99% ± 6.17%; control group: 75.79% ± 12.17%) and Bone Ingrowth (\( P < .05 \): experimental group: 79.38% ± 6.12%; control group: 87.24% ± 8.57%) were observed in osteopenic cortical bone.

Figure 6 shows an image depicting microhardness rhomboidal imprints after an indentation test at the bone-biomaterial interface. The microhardness values measured at the Ti6Al4V-bone interface and at a 1,000-\( \mu \)m distance in both the experimental and control groups are shown in Table 2. Percent decreases in microhardness data observed at the interface and compared to those obtained at 1,000 \( \mu \)m, revealed a significant difference (\( P < .001 \)) in experimental (–24.70% ± 2.95%) and control (–19.33% ± 1.61%) groups. A significant decrease...
Fig 3a  Rat trabecular normal bone implanted with a Ti6Al4V nail 8 weeks after surgery. Bone is directly apposed to the implant surface (Fast Green, 4×).

Fig 3b  Rat trabecular osteopenic bone implanted with a Ti6Al4V nail 8 weeks after surgery. Some areas without bone-to-implant contact are visible. The rarefaction of bone trabeculae in the implanted bone bed caused by osteopenia is also observable (Fast Green, 4×).

Table 1  Bone Microhardness Around Ti6Al4V Nails Implanted in Rats Expressed in Vickers Degrees

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<th>Control group</th>
<th>Experimental group</th>
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<tr>
<td></td>
<td>Interface 1000 µm</td>
<td>Interface 1000 µm</td>
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<tr>
<td>Ti6Al4V</td>
<td>60.15 ± 5.33</td>
<td>57.35 ± 4.08 a</td>
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<td></td>
<td>66.87 ± 2.97</td>
<td>67.89 ± 2.95</td>
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Paired Student t test: aComparison between interface and 1000-µm data within each group (P < .0005).
Average of 10 determinations; mean ± SD, n = 9.

Fig 4  Percent variation in histomorphometric parameters of cortical bone bed where Ti6Al4V was implanted between experimental and control groups. The significant increase of endocortical perimeter and medullary area together with the increase of periosteal perimeter, cross-sectional area and the decrease of cortical area indicate the development of an osteopenic state in sheep 24 months after ovariectomy (a = P < .05).
**Fig 5a** Sheep cortical normal bone implanted with a Ti6Al4V screw 12 weeks after surgery. Bone is visible in the screw threads directly apposed to the biomaterial surface (Fast Green, 4×).

**Fig 5b** Sheep cortical osteopenic bone implanted with a Ti6Al4V screw 12 weeks after surgery. In some areas bone is not directly apposed to the screw surface (Fast Green, 4×).

**Fig 6** Microhardness rhomboidal imprints after indentation test at the bone-Ti6Al4V screw interface. S = screw; B = Bone; rhomboidal imprints after indentation test.

**Table 2** Bone Microhardness Around Ti6Al4V Screws Implanted in Sheep Expressed in Vickers Degrees

<table>
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<th>Control group</th>
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<th>Experimental group</th>
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<tr>
<td></td>
<td>Interface</td>
<td>1000 µm</td>
<td>Interface</td>
<td>1000 µm</td>
</tr>
<tr>
<td>Ti6Al4V</td>
<td>63.97 ± 7.67a</td>
<td>79.30 ± 5.15</td>
<td>59.29 ± 9.71b, 9.71b</td>
<td>78.77 ± 5.77</td>
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Paired Student t test: aComparison between interface and 1000-µm data within each group (P < .0005) (paired); bComparison between experimental group and control group at interface (P < .05) (unpaired).

Average of 10 determinations; mean ± SD, n = 9.
in bone hardness (P < .05) was also observed at the interface of the experimental group when compared to the control group (−16.7%).

**DISCUSSION**

Longer life expectancy, at least in western countries, is associated with an increased interest in the behavior of devices implanted in pathologic bone. The common metabolic bone disorders affecting potentially implanted patients are different and include renal osteodystrophy, osteomalacia, and Paget disease. However, aged osteopenic and osteoporotic bone seems to represent a significant concern. Symptomatic osteoporosis, related to aging, and secondary osteoporosis, subsequent to estrogen deficiency and to many systemic disorders or therapies, affect a great part of the population, depending on age, race, gender, and lifestyle.1,2,11,19

In the present study, histomorphometry of the implanted bone bed in ovariectomized animals showed the development of osteopenia both in cancellous and in cortical bone. Ti6Al4V highlighted a significant osseointegration decrease, when implanted in trabecular and cortical osteopenic bone and compared to control animals. Microhardness measurements made distant to the implant, showed no difference in trabecular and cortical bone hardness between experimental and control animals. In fact, according to the definition of osteoporosis (reduction of bone mass without alterations in the composition of bone, leading to fractures), the microhardness value, which mainly depends on bone mineralization, is expected to be similar for healthy and osteoporotic bone. Such a hypothesis was confirmed by present results, where measurements were taken at 1,000 µm from the implant in preexisting bone.

In healthy and in osteopenic bone a significant decrease in bone hardness was observed at the Ti6Al4V-bone interface compared to the preexisting bone located distant to the implant. Such a result obtained for both groups—even in the absence of pathologic conditions—confirmed that bone around the material was still immature and not well mineralized at 8 and 12 weeks for trabecular and cortical bone, respectively. Histomorphometric investigation had, in fact, shown better osseointegration of Ti6Al4V in trabecular and cortical bone of control animals. However, the newly formed bone around the materials was less resistant to the indentation test as compared to the distant bone also found in healthy bone of control animals. A significant decrease in bone hardness at the Ti6Al4V-bone interface was observed in osteopenic versus normal bone, but only in the cortical diaphyseal implants and not in the trabecular bone. These data, which exceed the hypothesis, require some pathophysiologic considerations that are based on clinical experience and, regretfully, on limited basic science evidence of the established osteoporosis effects on fracture healing.40 Bone osseointegration could be considered as a reparative process similar to fracture healing.41,42 In this regard, fractures of osteoporotic patients that usually occur in the trabecular bone are commonly observed in clinical practice and appear to heal with the formation of a normal callus and bone repair as in healthy subjects. On the contrary, when studying fracture healing in diaphyseal cortical bone, some authors have failed to find differences,40 but most of them observed abnormalities in osteoporotic bone43-46 both in the early and late phases of bone healing.

The present data seem to suggest that modeling and remodeling processes in cortical bone are differently affected by osteoporosis than in trabecular bone, but further histodynamic investigations concerning bone quality around implants are necessary.47

The rat was selected because the characterization of such a model for studies on osteoporosis is excellent.48,49 Moreover, it is the only osteopenic animal that has been used for research on osseointegration, except for a few studies in which dogs and rabbits were employed30,31,50 However, placement of screws or implants in the diaphyseal cortical bone of this small-size animal for the evaluation of biomaterials cannot be recommended, according to the Standards of the International Organization for Standardization.51 Moreover, the rat is reported to be a poor model for the study of the effects of ovariectomy in cortical bone because of the lack of Haversian systems.52 Hence, the sheep was used for the study of cortical bone implants, and this animal seems to be a good model for postmenopausal osteoporosis, even though it is less standardized than the rat.35,53-55

As far as the experimental times are concerned, the interval of 8 weeks after implantation was chosen for the rats because the main significant differences in biomaterial osseointegration between osteopenic and normal animals are first observed at that time and persist until week 24, according to the present authors’ previous experiences.24,32 In sheep, 12 weeks postsurgery represent the shorter experimental time for evaluating bone-implant osseointegration in cortical bone following ISO 10993-6 rules.31 As far as the present authors know, this study is the first to report on bone implants and
osteopenia in diaphyseal cortical bone of long-term ovariectomized sheep.

Microhardness tests have also been used by other researchers to assess the bone-material interface. They have emphasized that bone hardness measurements in the proximity of an implant can provide important information on many qualitative bone characteristics, such as calcification degree, arrangement and number of collagen fibers, ratio between collagen fibers and ground substance, mineral quantity per volume unit, and elastic modulus. However, as far as the current authors know, the microstructural technique has never been used in studies investigating osseointegration in pathologic osteopenic bone. Most of these studies concentrated mainly on morphologic tests such as histomorphometry, although the importance of characterizing the newly formed bone around biomaterials in osteopenic animals is well recognized both in orthopedics and dentistry.

Moreover, microhardness measurements enable the study of bone hardness at different distances from the implant, obviating the need for additional specimens since the same specimens can also be used for undecalcified bone histomorphometry. Bone fixation and infiltration processing may always be considered and standardized to achieve reliable measurements and results.

Finally, these data suggest that even in healthy animals, bone formation around Ti6Al4V was not associated with complete bone maturation; when an osteopenic state was present, both bone formation and maturation were delayed.

Present histomorphometric data are consistent with other results achieved on pure Ti and Ti6Al4V implants in osteopenic bone, which demonstrated the superiority of ceramic materials, especially in trabecular bone, although their osseointegration was affected by the presence of osteoporosis in some cases.

The osteopenic state delayed bone formation around endosseous implants both in trabecular and cortical bone. Osseointegration of Ti6Al4V was obtained, but bone remodeling processes leading to maturation took longer both in trabecular and cortical bone in healthy bone.

SUMMARY

These results cannot be automatically transferred into clinical practice where implants are differently loaded and further studies focusing more on clinical experience are mandatory. The current findings would seem to suggest that biomaterials should be preclinically tested in pathologic bone and, in addition, the bone-to-implant interface may be characterized with both morphologic and microstructural investigations to acquire a better knowledge of the relation between bone ingrowth and bone status. In fact, although it is still unclear what size of implant-bone contact area or degree of bone maturation is required to ensure adequate anchorage for successful implantation of a load bearing device, it may be useful to take into account the various different healing responses of osteopenic bone, thus providing clinicians with suitable macro- or micromorphology of implants, healing period, and loading modalities.

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REFERENCES


19. Celiker R, Arslan S. IFG-I levels were found to be lower in women with osteoporosis. Rheumatol Int 2000;19:205–208.


