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Biomaterials 24 (2003) 1583–1594

Biomaterials

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Mechanical and histomorphometric evaluations of titanium implants with different surface treatments inserted in sheep cortical bone

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Received 15 May 2002; accepted 28 October 2002

Abstract

Improvement of the implant–bone interface is still an open problem and the interest in chemical modification of implant surfaces for cementless fixation has grown steadily over the past decade. Mechanical and histomorphometric investigations were performed at different times on implants inserted into sheep femoral cortical bone to compare the in vivo osseointegration of titanium screws ($\varnothing$ 3.5×7 mm length) with different surface treatments. After 8 weeks of implantation, the push-out force of anodized and hydrothermally treated implants (ANODIC) was significantly higher than that of machined implants (MACH) (36%, $p<0.0005$), whereas a decrease of 39% was observed for acid-etched implants (HF) when compared to other surface treatments. After 12 weeks of implantation, the push-out force values of HF implants were still significantly lower than those observed for MACH (-19%, $p<0.01$) and hydroxyapatite vacuum plasma-sprayed implants (HAVPS, -25%, $p<0.0005$), and the highest push-out force was found in HAVPS ($p<0.001$) implants. After 8 and 12 weeks of implantation, the AI of HF implants was significantly ($p<0.05$) lower ($\sim -25\%$) than that of MACH, HAVPS and ANODIC implants. In conclusion, results appear to confirm that there are no specific differences between ANODIC and HAVPS implants in terms of behavior. Moreover, although MACH implants show some surface contaminating agents, they appear to ensure good osseointegration within 12 weeks both mechanically and histomorphometrically, as do ANODIC and HAVPS implants. However, further studies are required to investigate bone hardness and mineralization around implants.

Keywords: Titanium; Surface treatments; Sheep; Histomorphometry; Mechanical testing

1. Introduction

The improvement of the interface between bone and orthopaedic or dental implants is still an open problem which has been addressed to create a suitable environment where the natural biological potential for bone functional regeneration can be stimulated and maximized [1–4]. Of all the investigated factors, the surface structure, biomechanical factors and biological response have been demonstrated to have the greatest influence on implant osseointegration [5,6]. Nowadays, osseointegration is defined not only as the absence of a fibrous layer around the implant with an active response in terms of integration to host bone, but also as a chemical (bonding osteogenesis) or physico-chemical (connective tissue osteogenesis) bond between implant and bone [7–11]. Since the main target is to enhance the development of strong bonds, ceramic and biological coatings have been widely investigated as a means to improve metal osseointegration. In this regard sandblasting, plasma spraying and acid etching have become the three most common approaches used to alter the surface topography and increase the surface area of implants. The

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PII: S0142-9612(02)00548-3

definition of the healing time without load transfer to the implant is another important factor for osseointegration, since no consensus on load-bearing time has so far been obtained. A shortening of this delay is mandatory, in particular in dentistry, but too early loading of the implant may cause increased micromotions and result in the formation of fibrous tissue at the interface [12].

Interest in chemical modification of implant surfaces for cementless fixation has grown steadily over the last decade. These modifications are known to occur through the deposition of osteoconductive calcium phosphate materials such as hydroxyapatite (HA). Many authors have reported that a 20–50 μm HA-coating enhances implant performance at an early stage after implantation, because the interface attachment strength is higher in coated than in uncoated implants [13–17]. The best fixation of HA-coated implant is brought about by the chemical bonding between bone tissue and coating, but sometimes adhesion of the HA coating to the metal substrate is not sufficiently high compared to the bonding strength between bone and HA coating on account of the coating method. The poorer performance of long-term HA coatings has been related to problems in the adhesion of the deposits to the substrate and to an improperly controlled dissolution rate of the coatings depending on the level of HA crystallinity: the lower the crystallinity, the higher the potential for degradation [18]. Furthermore, it is difficult to obtain uniform and thin HA layers on implants with a complex surface geometry, such as screws and metal porous coatings, because widely applied technologies, such as the plasma-spray process, do not allow coatings thinner than about 40 μm to be obtained. Of the other coating methods [19–21], a particularly interesting process (anodization followed by a hydrothermal treatment) has been proposed by Ishizawa et al. [22–24]. They observed that this method was superior in terms of capability to form a thin (1–2 μm) and porous HA layer directly on metal substrates. In addition, mechanical strength and bone apposition were reported to be equivalent to those recorded on HA ceramics and much higher than those observed for titanium (Ti).

In a previous paper the current authors assessed the influence of anodization and anodization-hydrothermal treatment on Ti osseointegration in the cancellous bone of rat distal femoral epiphysis at 4 and 8 weeks [25]. Results in terms of bone apposition confirmed the surface osteconductive properties of non-anodized acid-etched Ti, demonstrated that anodized Ti showed the lowest osseointegration and indicated that anodized-hydrothermally treated Ti had the best performances because HA induced formation of a wider bone-to-implant contact area. Since higher bone apposition does not necessarily imply greater bone-bonding strengths [14], the present study was performed to assess the effect of implant surface treatments on bone response by means of mechanical and histomorphometric investigations. An in vivo comparison of osseointegration was made between anodized hydrothermally treated screws, and variously treated titanium screws implanted in sheep femurs, focusing the attention on the relationship between the histomorphometric and mechanical parameters in relation to surface roughness and implant design.

2. Materials and methods

2.1. Implant material preparation

Self-tapping screw implants made of commercial grade 2 Ti (chemical composition: N = 0.009%, C = 0.021%, H = 0.0017%, Fe = 0.06%, O = 0.10%, Ti balance) were used (Plan 1 Health Srl, Villanova di S.Daniele del Friuli, Italy). The titanium machined (MACH) implants resulting from the machining process and prepared on a turning lathe were used as controls and substrates for other surface treatments. The screws of each set had the same thread geometry: the major and minor diameters of the screws were 3.75 and 3.15 mm, respectively, the length was 7 mm and the thread pitch 0.625 mm. The following surface treatments were considered:

- *Chemical etching with hydrofluoric acid (HF)*. The HF implants were obtained by chemical etching of the MACH implants using a 25% in volume solution of HF for 20 s at room temperature. After chemical HF etching, the implants were passivated in a 25% in volume solution of HNO_3 for 1 h.
- *Hydroxyapatite vacuum plasma-sprayed coating (HAVPS)*. The vacuum plasma spray was used to coat the outer surface of MACH implants with HA. This treatment was commercially performed (Biocoatings S.p.A, Fornovo di Taro—Parma, Italy). The coating thickness was 70 ± 10 μm and thus complied with the standard AFNOR HA with crystalline HA purity > 97%, crystallinity index > 70%, amorphous phase < 36%, Ca/P equal to 1.667 ± 0.002, adhesion to the total coating substrate > 30 MPa, 4% porosity.
- *Anodization treatment (ANODIC)*. The ANODIC implants were obtained by means of a two-step procedure performed on the MACH implants as previously described [25]. Briefly, the anodization treatment of the dental implant was performed at 275 V in an aqueous electrolytic solution of 0.06 mol/l β-glycerophosphate and 0.3 mol/l calcium acetate with a current density of 50 mA/cm^2. The anodic Ti oxide coating thickness obtained was about 5 μm. After anodization the implants were washed with

distilled water and then dried. Afterwards, they were hydro-thermally treated in autoclave at 300°C for 2 h, resulting in a submicrometric layer of HA crystals of about 1 μm in thickness on the anodic coating.

All implants were washed with distilled water, dried and sterilized by γ-rays with a dose of 25 kGy.

2.2. Implant surface analyses

Surface morphology characterizations were examined by scanning electron microscopy (SEM JEOL, J840A, Japan) and 3D laser profilometer (UBM-Microfocus Compact, NanoFocus AG, Germany). Roughness was measured on the lateral surface of 3 mm-diameter cylindrical rods specifically prepared with the same surface treatment of the implant in question; five rods were considered for each of the four materials. The roughness parameters were calculated on five 1.75 mm-long profiles obtained parallel to the axis. Analysis was performed using six surface profile parameters: R_a, R_{max}, S_m, K, Sk, and Mr1. Both R_a and R_{max} are roughness amplitude parameters generally used for the purpose of comparison (R_a is the arithmetic mean of the area between the roughness profile and its mean line; R_{max} is the maximum peak to lowest valley vertical distance within a single sample length), S_m is a roughness spacing parameter measuring the mean spacing between peaks, whereas K, Sk and Mr1 are statistical parameters describing the amplitude distribution function. K (kurtosis) evaluates the distribution of the profile height around an ideal average line, and such comparison is related to the Gauss distribution (Fig. 1a)

$$K = \sigma^{-4} \int_{-\infty}^{+\infty} z^4 p(z),$$

Sk (skewness) provides information on the symmetry of the distribution of the profile height around the ideal average line (Fig. 1b)

$$\mathrm{Sk} = \sigma^{-3} \int_{-\infty}^{+\infty} z^3 p(z)\,\mathrm{d}z,$$

while Mr1 is the fraction of the surface which consists of small peaks above the main plateau.

2.3. Study design

This study was performed in compliance with the European and Italian Laws on animal experimentation, the principles stated in the “Guide for the Care and Use of Laboratory Animals” and the Animal Welfare Assurance No. A5424-01 by the National Institute of Health (NIH-Rockville Maryland USA): the animal research protocol was approved by the responsible public authorities as requested by the Italian Law in accordance with EU regulations.

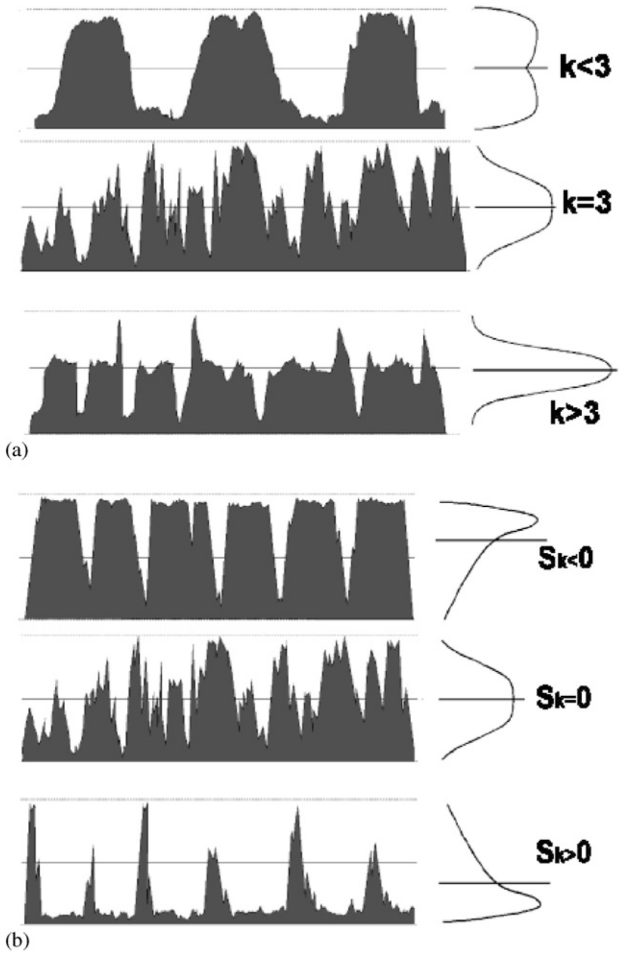

Fig. 1. Peak distribution shape parameters: (a) K, when $K = 3$ the profile has the same dispersion of the Gauss distribution, when $K < 3$ the dispersion of the profile height is higher than in Gauss distribution, when $K > 3$ the height is less spread out then in Gauss distribution; and (b) Sk, when Sk is about zero the profile distribution is symmetric around the average line, when $\mathrm{Sk} > 0$ the density distribution is asymmetric on the left, and when $\mathrm{Sk} < 0$ the distribution is asymmetric to the right.

Eight crossbred (Bergamasca—Massese) sheep, 3.5±0.5 years old and 80±5 kg b.w., were submitted to bilaterally screw implantation in femurs under general anesthesia following a standardized protocol [26]. After performing an incision along the lateral surface of femoral diaphysis, the muscles were retracted to expose the femur which was skeletonized to $\frac{3}{4}$ of the diaphysis. A 2.7 mm-diameter drill was used to pre-drill four holes in each diaphysis at low speed under sterile 0.9% NaCl. The holes were then flushed and cooled with sterile 0.9% NaCl to remove bone debris. Afterwards, the screws were randomly placed and then tightened.

Antibiotics and analgesics (cephalosporin 1 g/day for 5 days and ketoprofen 500 mg/day for 3 days) were administered postoperatively. At the end of experiment, 4 animals were pharmacologically euthanized under

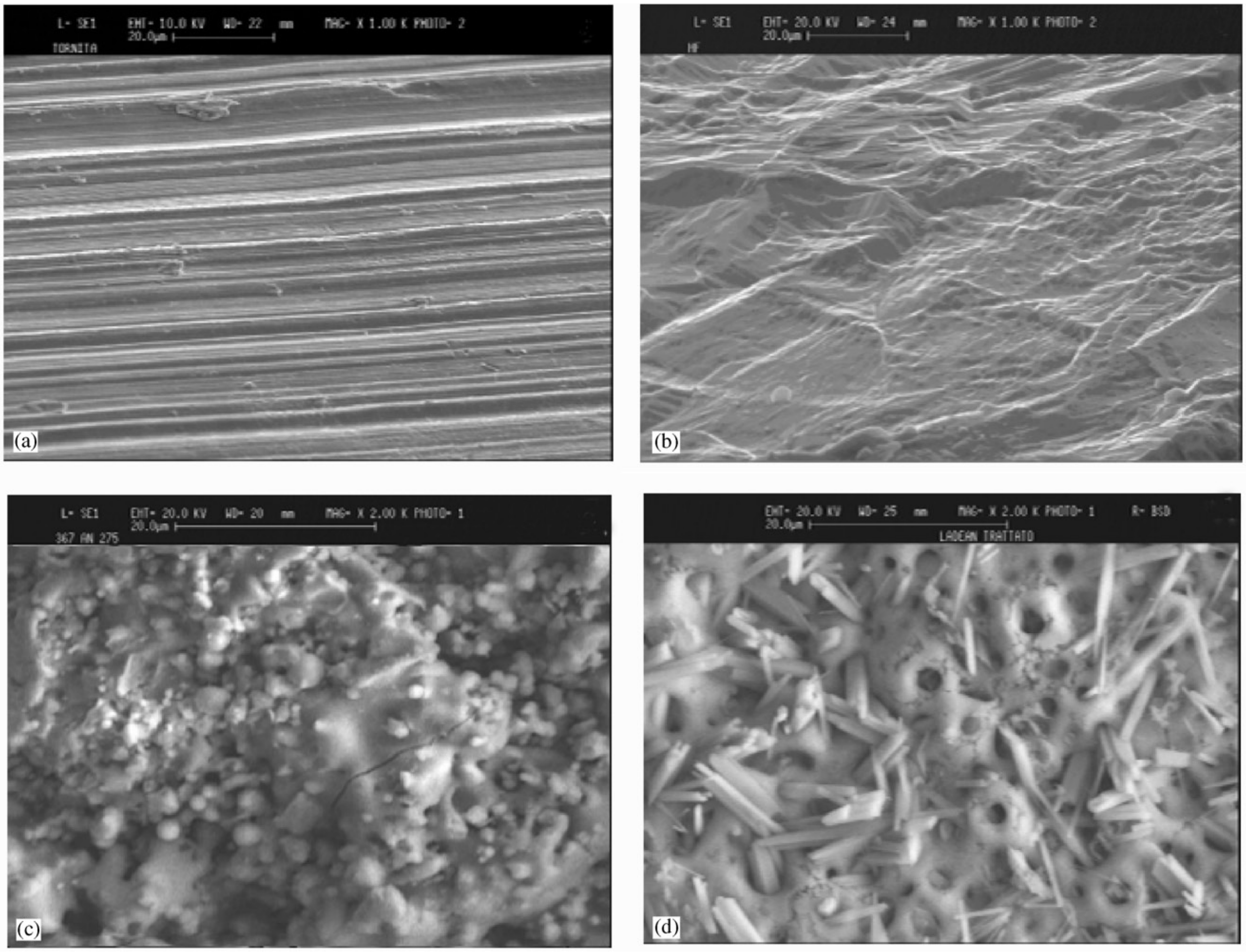

Fig. 2. Scanning electron micrograph: (a) MACH, original magnification ×1000: grooves and metallic particles secondary to the turning lathe process can be observed; (b) HF, original magnification ×1000: remnants of the turning lathe process cannot be detected any more; (c) HAVPS, original magnification ×2000: thin HA coating without cracks; (d) ANODIC, original magnification ×2000: columnar HA crystals precipitate discontinuously on the amorphous TiO_2 layer of ANODIC implants.

general anesthesia at each experimental times (8 and 12 weeks), and femurs were explanted, excised and cleaned of soft tissue. Cylindrical segments, each one containing an implant, were obtained from the femoral diaphysis using the cutting system EXAKT B System 300 CL (EXAKT Apparatus GmbH, Norderstedt, Germany). The left femoral segments were fixed in 4% paraformaldeyde for histomorphometric evaluation. The right femoral segments were further reduced to a quarter of the circumference containing the implant by the EXAKT cutting system. They were then embedded in 0.9% NaCl-soaked gauze and stored at −20°C before mechanical testing.

All measurements for all techniques were carried out by blinded operators.

2.4. Mechanical testing

Before conducting the test, the femoral segments were taken out of the freezer and maintained wet in normal saline at 4°C for 12 h. They were then thawed in 0.9% NaCl at room temperature for at least 3 h and finally conditioned in 0.9% NaCl at 37°C during the subsequent stages of the mechanical testing performed within 1 h [27]. Periosteal overgrowth was removed by grinding to obtain proper alignment of the specimen in the testing apparatus. The push-out test was carried out by placing the femoral segments on a support jig (∅ 4.9 mm) using a MTS apparatus (Sintech-1/M, MTS Adamel Lhomargy, Ivry sur Seine, France). The force applied to the implant from the medullary side at a constant cross-head speed of 1 mm/min pushed it out of its bony bed. The following parameters were measured and calculated:

- Maximum push-out force (F_{max}): the peak force resulting in the detachment of the bone-(coating)–implant system and corresponding to screw holding power.

- Ultimate shear strength (σ_u) defined as follows:

  $\sigma_u = F_{max}/((\pi \text{ OD}\bar{t})\text{TSF})$,

  where OD is the outer diameter of the screw (mm), $\bar{t}$ is the cortical bone thickness (mm) and TSF is a factor that accounts for screw thread geometry defined by the D732-90 ASTM standard [28] as follows:

  $\text{TSF} = (0.5 + 0.57735 d_m/p)$,

  where d_m is the thread depth (mm) = (OD−RD)/2, RD the root diameter (mm), and p the thread pitch (mm).

After the push-out tests, the samples collected for each surface treatment at 12 weeks were randomly fixed in 2% glutaraldehyde/0.1 M phosphate buffer, dehydrated in ethanol series, dried at CO_2 top critical point in a bomb (Top critical Point 30, W. Pabish, Pero-MI, Italy), mounted on aluminum stub using a carbon tape, and coated with 20 nm Au/Pd layer using a coating unit (Coating unit E5100 Polaron, Polaron Equipment Ltd., Watford Hertfordshire, United Kingdom). The specimens were then observed with SEM (J840A, Jeol Tokyo, Japan) in secondary electrons mode in order to examine the failure mode at the bone-(coating)–implant interface [29].

2.5. Histomorphometry

The left femoral cylinders, that had been previously fixed in 4% paraformaldeyde for 48 h, were dehydrated in ethanol series and embedded in polymethylmetacrylate. Bone–implant sections of 60 μm in thickness were obtained parallel to the long axis of the screw by means of the EXAKT cutting system and were then further ground to 15 ± 5 μm with the EXAKT 400CS Micro-Grinding System (EXAKT Apparatus GmbH, Germany). The sections of each screw were stained with solochromocyanine.

Histomorphometric analyses were performed on three consecutive sections using an optic microscope (BX41, Olympus Optical Co. Europa GmbH, Germany) connected to an image analyzer system (Qwin, Leica Imaging Systems Ltd., United Kingdom). Each measurement was taken semi-automatically at an original magnification of × 4. The following histomorphometric parameters were measured bilaterally to the implants in a 2000 μm-long and 1400 μm-wide reference area calculated from the base of the threads starting from the endosteal side:

- BV/TV (%): the bone volume per tissue volume around the implant.
- BI (bone ingrowth, %): the amount of bone growth inside screw threads measured in an area located between the bottom and the top of the threads and expressed as percentage.
- AI (affinity index, %): the interface contact between bone and implant calculated on the three consecutive best threads and considered as the length of the bone profile directly opposed to the implant with respect to the length of bone–implant interface [5,30,31].

2.6. Statistical analysis

Statistical analysis was performed using SPSS v.10.1 software (SPSS Inc., Chicago, Illinois, USA). Data are reported as mean ± SD at a significant level of $p < 0.05$. After testing data for normal distribution and homogeneity of the variance, a multiple way ANOVA was used to assess significant interactions between selected factors (sheep id., implant sites, surface treatments and experimental times) and mechanical and histomorphometric data. When such interactions were found, a univariate ANOVA was done to investigate the effects of the factors on the data by means of hypotheses expressed as linear matrix according to the SPSS syntax. When no interaction was found and in the case of roughness results, a one-way ANOVA followed by Bonferroni multiple comparison test were done to assess for the presence of significant differences between the results obtained from the different surface treatments. The unpaired Student's t test was performed to compare data between experimental times within the same surface treatment. Finally, the linear regression analysis was used to detect the associations between those variables yielding correlation coefficients.

3. Results

3.1. Implant surface analyses

The microstructures observed by SEM and resulting from the different surface treatment are shown in Fig. 2. The one-way ANOVA showed significant differences between surface treatments in terms of surface roughness (R_a: $F = 54.66$, $p < 0.0005$; R_{max}: $F = 46.34$, $p < 0.0005$; Sk: $F = 7.53$, $p < 0.005$; S_m: $F = 19.05$, $p < 0.0005$; Mr1: $F = 5.26$, $p < 0.05$), as reported in Table 1. Both R_a and R_{max} highlighted the same significant differences among surface treatments when Bonferroni test was used. In accordance with expectations, the MACH implant showed the lowest roughness value ($p < 0.0005$), while the ANODIC implant was significantly the ($p < 0.0005$) roughest when compared to the other surface treatments. The HAVPS implant showed Sk positive results which significantly differed from those obtained with the ANODIC implant ($p < 0.001$). The significantly lower S_m value of the HF implants confirmed a considerable decrease in the

Table 1
Surface roughness for the four different surface treatments (Mean $\pm$ SD; $n = 5$)

Parameter	Unit	MACH	HF	HAVPS	ANODIC
R_a	μm	0.20 ± 0.01	0.56 ± 0.06[a]	1.06 ± 0.21[b,c]	1.97 ± 0.64[b,c,d]
$R_{\max}$	μm	1.46 ± 0.15	3.68 ± 0.39[c]	5.84 ± 1.02[b,c]	14.02 ± 4.86[b,c,d]
S_m	μm	0.530 ± 0.410	0.106 ± 0.014[c]	0.288 ± 0.034[b]	0.225 ± 0.039[e,f]
K	—	2.89 ± 0.23	3.04 ± 0.31	3.19 ± 0.50	3.03 ± 0.45
Sk	—	0.08 ± 0.12	0.06 ± 0.49	0.60 ± 0.16	-0.14 ± 0.28[g]
Mr1	%	10.2 ± 1.3	9.7 ± 1.8	14.3 ± 2.12[h]	9.3 ± 3.4[i]

MACH: machined titanium; HF: titanium etched with hydrofluoric acid; HAVPS: titanium with hydroxyapatite vacuum plasma-sprayed coating; ANODIC: anodized and hydrothermally treated titanium; R_a: mean of the departures of the roughness profile from the mean line; $R_{\max}$: maximum profile valley depth; S_m: roughness spacing parameters measuring the mean spacing between peaks; K: distribution of the profile height around an ideal average line; Sk: distribution symmetry of the profile height around an ideal average line; Mr1: fraction of the surface which consists of small peaks above the main plateau.

Bonferroni multiple comparison test:

[a] versus MACH, $p<0.001$.
[b] versus HF, $p<0.0005$.
[c] versus MACH, $p<0.0005$.
[d] versus HAVPS, $p<0.0005$.
[e] versus HF, $p<0.01$.
[f] versus MACH, $p<0.05$.
[g] versus HAVPS, $p<0.001$.
[h] versus HF, $p<0.05$.
[i] versus HAVPS, $p<0.05$.

distance between the various points of roughness as compared to the other surface treatments. A significant ($p<0.05$) difference was also found between ANODIC and MACH implants in terms of S_m. Finally, the Mr1 of HAVPS implants was significantly ($p<0.05$) higher than the Mr1 of HF and ANODIC implants, thus confirming an increase in the fraction of the surface which consists of small peaks above the main plateau.

3.2. *Clinical observations*

All animals tolerated surgery well and survived the post-surgical period without any infective complications. When dissecting the femurs, the screw implants were macroscopically checked, and neither malpositioning nor signs of inflammation or tissue reaction around the implant sites were observed.

3.3. *Mechanical testing*

Results of the push-out test are reported in Table 2. The four-way (sheep id., implant sites, surface treatments and experimental times) and three-way (implant sites, surface treatments and experimental times) ANOVAs detected no significant interactions of all factors on mechanical results. When a two-way (surface treatments and experimental times) ANOVA was done, a significant interaction of selected factors was noted for the maximum push-out force ($F_{\max}$, $p<0.005$) and ultimate shear strength (σ_u, $p<0.05$), as well as significant effects of the single factors on the $F_{\max}$ (surface treatments, $p<0.005$; experimental times, $p<0.0005$) and a significant ($p<0.005$) effect of 'experimental times' factor on the σ_u.

After 8 weeks of implantation, the multiple comparison between surface treatments showed that the $F_{\max}$ results (Table 2) were significantly higher for ANODIC than for MACH (36%, $p<0.0005$) and lower for HF than for MACH (−29%, $p<0.05$), ANODIC (−47%, $p<0.0005$) and HAVPS (−41%, $p<0.001$) implants. Also the σ_u (Table 2) was significantly lower for HF than for ANODIC (−47%, $p<0.001$) and HAVPS (−44%, $p<0.05$) implants. After 12 weeks of implantation, the $F_{\max}$ results for HF remained significantly lower than for MACH (−19%, $p<0.01$) and HAVPS (−25%, $p<0.0005$) implants, while the highest $F_{\max}$ was observed in HAVPS ($p<0.001$) implants (Table 2). Significant increases in $F_{\max}$ and σ_u were found when comparing mechanical results at the different experimental times for each surface treatment, and they are reported below in a decreasing order:

- $F_{\max}$: HF (110%; $p<0.005$); MACH (86%; $p<0.005$); HAVPS (65%, $p<0.0005$); ANODIC (64%, $p<0.05$).
- σ_u: HF (129%, $p<0.005$); MACH (88%, $p<0.005$); HAVPS (43%, $p<0.0005$); ANODIC (24%, $p<0.05$).

SEM observations revealed that bone was separated from the implant surface by the push-out test at 12 weeks (Fig. 3); fractures were seen at the bone–implant interface and bone micro-fractures were observed at the base of the threads. Bone separation was almost always complete at the level of the thread tips and more evident

Table 2
Push-out test results for the four differently coated implants. Mean ± SD ($n = 4$)

Parameter	Unit	Week	MACH	HF	HAVPS	ANODIC
F_{max}	kN	8	1.4 ± 0.2	$1.0 \pm 0.2^{a,b,c}$	1.7 ± 0.2	1.9 ± 0.1^{d}
		12	2.6 ± 0.3**	$2.1 \pm 0.3^{e,f,}$**	$2.8 \pm 0.3^{g,}$*	2.3 ± 0.2***
σ_u	MPa	8	33.1 ± 8.5	$23.4 \pm 5.2^{g,h}$	41.3 ± 1.7	44.0 ± 8.9
		12	62.5 ± 14.3**	53.8 ± 13.1**	59.8 ± 8.8**	54.5 ± 4.1***

MACH: titanium machined; HF: titanium etched with hydrofluoric acid; HAVPS: titanium coated with hydroxyapatite vacuum plasma spray; ANODIC: titanium anodised and hydrothermal treated. F_{max}: push-out force; σ_u: ultimate Shear Strength.

Univariate ANOVA test among surface treatments within each experimental time.

8 weeks:
[a] versus ANODIC, $p < 0.0005$.
[b] versus HAVPS, $p < 0.001$.
[c] versus MACH, $p < 0.05$.
[d] versus MACH, $p < 0.0005$.

12 weeks:
[e] versus HAVPS, $p < 0.0005$.
[f] versus MACH, $p < 0.01$.
[g] versus ANODIC, $p < 0.001$.
[h] versus HAVPS, $p < 0.05$.

Univariate ANOVA test between experimental times within each surface treatment:
*$p < 0.005$.
**$p < 0.005$.
***$p < 0.05$.

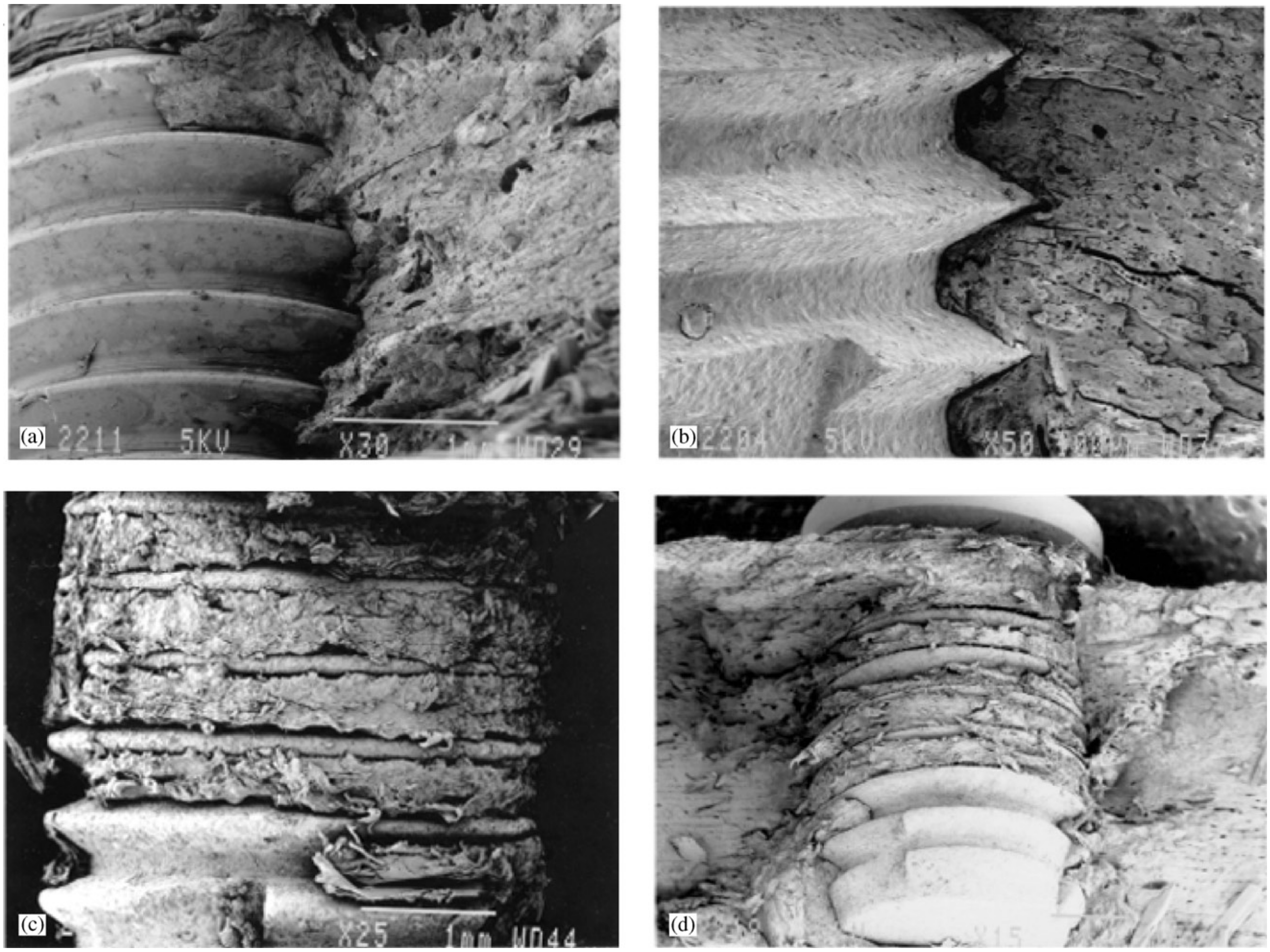

Fig. 3. Scanning electron micrograph of implants after the push-out test: (a) MACH, original magnification 30 ×; (b) HF, original magnification 50 ×; (c) HAVPS, original magnification 25 ×; and (d) ANODIC, original magnification 15 ×.

in MACH and HF implants. In contrast, HAVPS and ANODIC implants more frequently showed evidence of bone entrapped between the threads.

3.4. Histomorphometry

The histological findings demonstrated that all samples were correctly implanted in the femoral diaphyseal cortical bone, and no interference in bone apposition to the implant surface was observed for any of the four surface treatments (Fig. 4). Neither inflammatory cell infiltrate nor signs of infection were seen.

The histomorphometric analysis of BV/TV showed that no significant differences existed for perimplant bone tissue among surface treatments both at 8 (BV/TV: 83.67±6.03%) and 12 (BV/TV: 85.48±7.38%) weeks. In contrast to the mechanical testing, no significant interactions of selected factors on BI and AI were highlighted by two-way ANOVA (surface treatments, experimental times), whereas significant effects of 'surface treatments' (BI, $p<0.01$; AI, $p<0.05$) and 'experimental times' (BI, $p<0.0005$; AI, $p<0.005$) factors were observed. When the histomorphometric results at 8 and 12 weeks were analyzed separately (Table 3), the one-way ANOVA showed significant differences for AI at

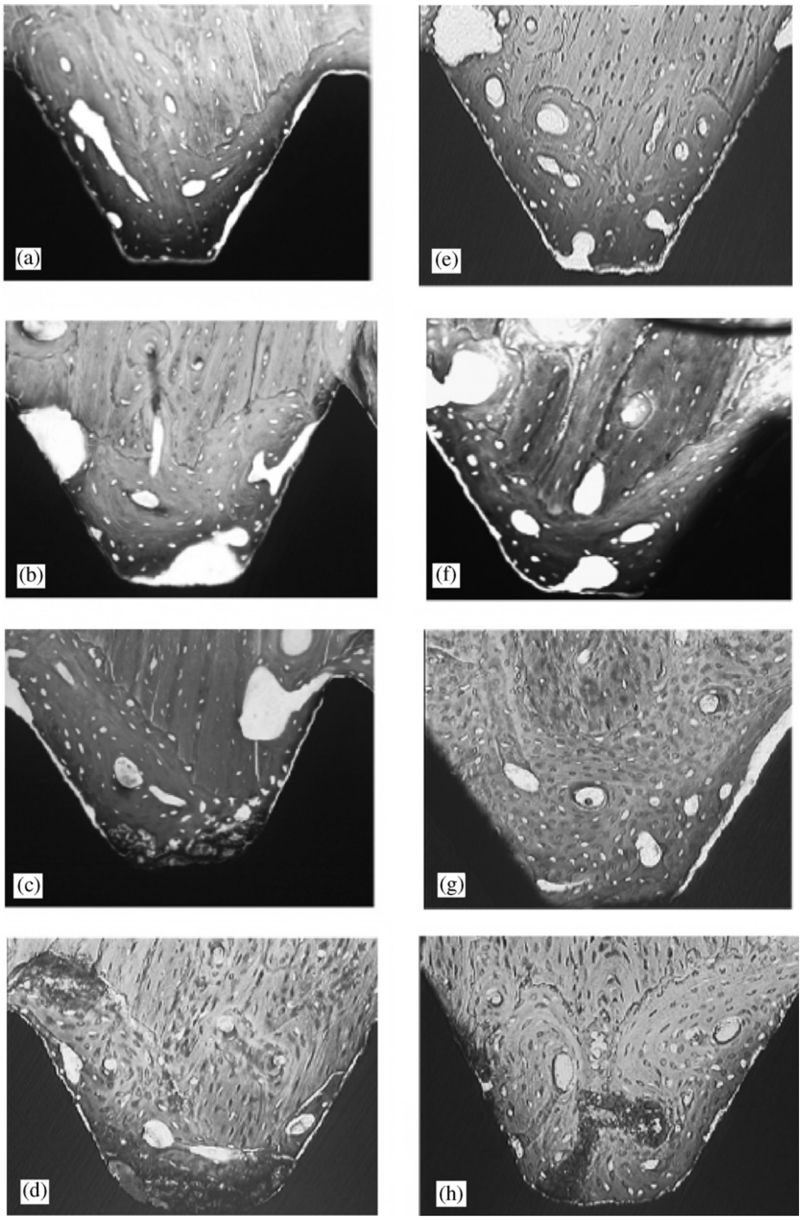

Fig. 4. Histology of implants in sheep bone showing bone apposition and ingrowth inside a screw thread: the new bone can clearly be identified interposed between the original bone and the implant. 8 weeks: (a) MACH; (b) HF; (c) HAVPS; (d) ANODIC; 12 weeks (e) MACH; (f) HF; (g) HAVPS; (h) ANODIC (solochromocyanine, original magnification 100 ×).

Table 3
Histomorphometric results for each surface treatment at 8 and 12 weeks (Mean±SD, $n = 4$)

Parameter	Unit	Weeks	MACH	HF	HAVPS	ANODIC
BI	%	8	77.9±8.0	65.9±10.6	69.2±3.8	66.0±2.5
		12	88.3±2.9**	76.7±8.7	80.8±7.0**	81.2±5.3*
AI	%	8	59.8±8.9	49.1±8.6[a]	61.5±6.4	63.6±7.4
		12	75.3±7.8**	50.9±7.8[a]	71.6±3.8	72.5±7.2

MACH: titanium machined; HF: titanium etched with hydrofluoric acid; HAVPS: titanium coated with hydroxyapatite vacuum plasma spray; ANODIC: titanium anodised and hydrothermed. BI: bone ingrowth; AI: affinity index.
Unpaired Student's t test between experimental times within each surface treatment:
* $p<0.0005$.
** $p<0.05$.
Bonferroni multiple comparison test among surface treatments within each experimental time
[a] versus other surface treatments ($p<0.05$).

both experimental times (8 weeks, $F = 7.62$, $p<0.01$; 12 weeks, $F = 6.46$, $p<0.05$). After 8 and 12 weeks of implantation, the AI of HF implants was significantly ($p<0.05$) lower than that of MACH (8 weeks: −17.9%; 12 weeks: −32.4%), HAVPS (8 weeks: −20.1%; 12 weeks: −28.9%) and ANODIC (8 weeks: −22.7%; 12 weeks: −29.7%) implants. When the histomorphometric results obtained at the different experimental times for each surface treatments were compared, increases in BI and AI were seen and were found to be statistically significant for MACH (BI and AI, $p<0.05$), HAVPS (BI, $p<0.05$) and ANODIC (BI, $p<0.0005$) implants, as shown in Table 3.

4. Discussion

The aim of the present study was to assess the positive effect of anodized and hydrothermally treated titanium screws on bone response in terms of architectonic features and mechanical resistance of perimplant bone, and to compare these surface treatments with others.

Surface profile measurements showed that ANODIC and HAVPS implants presented a higher fraction of the surface which consists of small peaks above the main plateau. If compared to other surface treatments, ANODIC implants presented the highest height descriptive parameters of roughness but the lowest peak density of the profiles, thus showing a surface with more valleys than peaks. However, if the S_m parameter was taken into account, the only difference appeared to be the one encountered for the HF material, where a considerable reduction of the distance between the various roughness points could be observed. Finally, no correlations were found between surface profile and histomorphometric results ($r = 0.3$), nor between surface profile and mechanical results ($r = 0.2$), and this finding contrasts with a previous report by the current authors [25].

Regarding mechanical results, F_{max} at 8 weeks confirmed that the ANODIC surface accelerated the process of bone-material mechanical bonding to the implant as compared to HF and MACH implants ($p<0.0005$). Such a finding is considered of some value because of the general need, in dentistry in particular, to accelerate the process of bone-material bonding mechanically, thus reducing post-implantation times without load transfer [5]. After 12 weeks of implantation, all of the materials showed significantly improved mechanical behavior, but HF was still performed as the worst surface in terms of mechanical bonding to the surrounding bone. The ultimate shear strength generally paralleled the F_{max}. The highest σ_u value observed for ANODIC implants was similar to that of HAVPS implants and was significantly better than that of HF implants. An improvement in σ_u, was also seen for each surface treatment at 12 weeks, but no significant differences were found among the tested surfaces. Finally, significant ($p<0.01$) correlations were found between mechanical and histomorphometric parameters: F_{max}—AI: $r = 0.626$; F_{max}—BI: $r = 0.724$; σ_u—AI: $r = 0.550$; σ_u—BI: $r = 0.723$.

The histomorphometric analysis revealed that none of the surface treatments affected the metabolism and healing capability of the surrounding bone. Therefore, the only significant improvement in de novo bone formation observed around the screws was time-related. Bone ingrowth, in fact, proved to be almost the same for all surface treatments with significant improvements for MACH, HAVPS and ANODIC implants between 8 and 12 weeks. On the contrary, different behavior in terms of apposition of re-grown bone to the implant was seen depending on the surface treatment applied: MACH, HAVPS and ANODIC strongly accelerated the formation of the bone contact area as compared to HF, even if a similar amount of bone had re-grown inside the screw threads as demonstrated by the BI values. At the longest experimental time the HF surface still showed a lower

AI when compared to the other materials, as observed at 8 weeks.

In the current authors' opinion, a partial explanation for the present results may be found in the data obtained with the superficial chemical analysis performed by means of the Auger electron spectroscopy (AES) and energy-dispersive micro-analysis (EDS) at a previous stage of the research [32]. This analysis demonstrated the presence of superficial contaminating agents (C, O, Ca, Na, P, S, Cl, Si, Ni and Fe) on MACH implants, associated with a considerable quantity of Fe (6–14%) and F traces on HF implants even after the sputtering process.

Regarding the surface morphology characterization, Wennerberg A. et al. have pointed out that the use of a Gaussian filter highly affected roughness calculations in the three-dimensional measurements of surface topography by means of a confocal laser scanner, and surface deviations of form and waviness were excluded [31]. The laser profilometer used for the present study did not make it possible to avoid surface deviations of form and waviness, and surface roughness measurements were therefore taken on the lateral surface of 3 mm-diameter cylindrical rods specifically prepared with the same surface treatment as the implant in question. Although this method appears to be unable to transfer results from one design (rod) to another (screw) and to provide absolute measurements on roughness, the current authors tried to perform these measurements on the same design for all the superficial treatments selected and were able to compare roughness of the various surfaces, also in consideration of the fact that the introduced variables referred only to superficial treatments.

In the present authors' opinion, these insignificant correlations between surface profile and histomorphometric and mechanical results, which contrast with a previous report by the current authors [25], could be due to the method used for taking surface roughness measurements. However, Vercaigne et al. have observed similar insignificant correlations, but they have stated that they may depend on different factors affecting bone integration, such as excessive roughness and minimal damage of bone tissue during surgery which slow down bone healing [30].

The present experimental times and implant sites were selected on the basis of previous studies and following ISO 10993 standard specifications, also taking into account that the biological bone response to implants depends on the material properties and the trauma of surgery [33]. Since implantation in bone tissues may need longer observation periods, the choice of 8 and 12 weeks was considered suitable. However, some authors have also evaluated shorter experimental times, above all within the first 15 postoperative days, and have shown improvement in mechanical strength and bone osseointegration during the first 4 weeks of implantation [2,34,35]. If shorter periods, such as 2 and 4 weeks, had been considered, the behavior of ANODIC and MACH implants might have been better clarified, especially during the initial phases of osseointegration. Regarding the implant site, the forces acting on diaphyseal cortical sites have been found to be different from those acting on condylar trabecular sites [36]. The former are mainly shear forces which appear to have a negative effect on bone response, while the latter are a combination of shear and compression forces; compression forces have moreover been demonstrated to have a beneficial effect on the remodeling process of the bone–implant interface [36].

The authors decided to perform mechanical testing by using the push-out test on account of the anatomical site selected and the geometry of implants whose screw heads were not suitable for the torsional clamp device. The interfacial shear strength between bone and implant is usually measured with various mechanical tests, such as the push-out, pull-out and removal torque tests [6,18,37–44]. The choice of these tests depends on the clinically most significant failure mode of the tested device and its shape; nevertheless, the best definition of clinical failure remains a matter of debate since correlation results have given different outcome [41]. The push-out test is generally used for cylindrical implants inserted in the cortex of femur, tibia or mandible, the removal torque for screw implants and the pull-out test for both cases when inserted in the cancellous bone of the vertebral body, proximal and distal portion of long bones [29,41,45,46]. In the past the push-out test has also been used for the evaluation of screw holding power, since this force is equivalent to that of a pull-out force applied to the screw head [47]; the capability of the bone tissue to be a mechanical constraint for threaded implants has also been assessed [48]. Other variables, however, contribute to screw holding power such as the extent of cortical purchase, the depth of screw penetration, thread angulation, pitch diameter, the screw placement within the bone, the physical changes in the screw or bone between insertion and withdrawal, bone failure, the speed at which the screw is withdrawn, the presence of predrilled holes, and bone quality [45,49]. SEM is currently considered the actual mode to analyze the failure in order to interpret the measured forces correctly and obtain additional information on the type of mechanical property measured during the push-out test [29].

In conclusion, the present histological, histomorphometric and mechanical findings confirm that appropriate surface roughness and bioactive ceramic coating may improve bone osseointegration. However, the current findings do not highlight specific differences in the behavior of MACH, ANODIC and HAVPS implants in terms of osseointegration process and time. In addition, it is interesting to observe that although MACH implants show superficial contaminating agents which may have affected the process of mechanical bone-bonding to the material at the shortest experimental time, they can nevertheless successfully osseointegrate both mechanically and histomorphometrically within 12 weeks, as do ANODIC and HAVPS implants. This may be related to the fact that the effects of the superficial treatments on implants are no longer visible at both experimental times, as well as to the selected implant site. The site has in fact been reported to greatly affect the rate of both osseointegration and biodegradation of the ceramic materials with higher osteogenesis in cortical versus trabecular bone and higher material degradation in trabecular versus cortical bone [50]. Finally, it should be remembered that bone remodeling is already apparent after 12 weeks. This process may be enhanced by some types of implants which can also partially modify perimplant bone in terms of quantity and quality, with subsequent worsening of mechanical and histomorphometric features. To obtain a deeper insight into bone healing around the physico-chemically modified surfaces, further studies on bone micro-hardness, mineralization and bone-(coating)–implant interface strength around implants are now required.

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