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ORIGINAL ARTICLE

Does milling one-piece titanium dental implants induce osteocyte and osteoclast changes?

Le meulage d'implants dentaires en titane entraîne-t-il des modifications du nombre d'ostéocytes et d'ostéoclastes?

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KEYWORDS
One-piece implant;
Osteocyte;
Heat;
Bone histomorphometry

Summary One-piece dental implants avoid adverse effects sometimes associated with the traditional implant-abutment interface and may provide a suitable alternative to two-piece implants; however, one-piece implants often need in situ milling, which may exacerbate cell apoptosis from excessive heat at the bone-implant interface and induce secondary crestal bone loss. Twelve implants were placed in the metaphyses of two sheep under general anesthesia. Six implants were milled with a diamond bur while the other six implants remained intact. Animals were euthanized after four days, and bone blocks were harvested. Bone samples were studied without decalcification. Osteocytes were stained with Hoechst 33342 and osteoclasts by the TRAcP reaction. Both cell types, in the cortical and trabecular bone around the implant's cervical region, were counted utilizing morphometric methods. Values were compared to areas at a distance from the cervical region. No difference was observed between milled and unmilled implants, which suggested that the amount of generated heat did not provoke osteocyte loss or induce osteoclastogenesis. Intraoral abutment preparations did not increase cellular apoptosis at the bone-implant interface after four days in the ovine model.

MOTS CLÉS
Implant monobloc ;
Ostéocyte ;
Chaleur ;
Histomorphométrie osseuse

Résumé Les implants dentaires monobloc évitent les effets indésirables rencontrés au niveau de l'interface implant-butée des implants dentaires traditionnels. Ils peuvent fournir une alternative aux implants classiques en évitant un deuxième temps opératoire; cependant, les implants monobloc ont souvent besoin d'être fraisés in situ, cela peut, en théorie, induire l'apoptose cellulaire en raison de la chaleur excessive transmise à l'interface os/implant et pouvant conduire à une perte osseuse crestale secondaire. Douze implants ont été placés dans les métaphyses de deux moutons sous anesthésie générale. Les piliers de six

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implants ont été meulés avec une fraise diamantée tandis que six autres implants restaient intacts. Les animaux ont été euthanasiés après quatre jours, et des blocs d'os ont été prélevés. Les échantillons osseux ont été étudiés sans décalcification. Les noyaux des ostéocytes ont été colorés par le Hoechst 33342 et les ostéoclastes par la détection de la TRAcP. Les deux types cellulaires ont été dénombrés dans l'os cortical et l'os trabéculaire autour de la région cervicale des implants en utilisant des méthodes morphométriques. Les valeurs ont été comparées aux zones situées à distance de la région d'implantation. On n'a observé aucune différence entre les implants meulés et non meulés, ce qui suggère que la quantité de la chaleur produite n'a pas provoqué de perte d'ostéocyte ni induit l'ostéoclastogenèse. Le meulage intrabuccal des piliers n'a pas augmenté l'apoptose ostéocytaire à l'interface os-implant, après quatre jours, dans ce modèle ovin.

Introduction

Potential complications associated with the design of traditional two-piece implant-abutment connections include screw loosening, which can lead to interfacial joint opening, abutment micromovements and component fracture, structural weakness in small-diameter implant designs, and formation of an interfacial microgap between the implant and abutment, which may theoretically become a bacterial niche that can induce periimplantitis and secondary bone loss [1,2]. With one-piece implant designs, there is no interfacial screw to loosen, no implant-abutment interface that can cause structural weakness in smaller diameter implants (3 mm), and no potential for a subgingival microgap. Since 2004, one-piece dental implants have been commercially available from several manufacturers. The concept of a one-piece implant design is not new, however. From antiquity, a metal one-piece implant dating from the 1st century was found in a Gallo-Roman necropolis in Chantambre (France) [3]. During the 1960s and 1970s, one-piece implants made of titanium or zirconium were introduced by pioneers of modern implantology, such as Ledermann [4] and Sandhaus [5].

In 2004, a new, one-piece dental implant made of commercially pure titanium (c.p. Ti) (NobelDirect, Nobel Biocare AB, Göteborg, Sweden) was first introduced, and promising short-term results were published the following year [6,7]. Beginning in late 2005, several clinicians publicly raised concerns about unusually high bone resorption observed with one-piece implants, which they later documented, in the dental literature [8—10]. This triggered an investigation by the swedish regulatory agency for medical products, which ultimately issued an alert to clinicians and recommendations to the manufacturer on protocol, instructions and required training for clinicians who use the one-piece implant.[1] Conversely, subsequent publications by other clinicians have reported positive results with outcomes similar to those generally reported for two-piece implants [11].

Several pathophysiological mechanisms have been suggested to explain the excessive bone resorption observed in the earlier studies [8—10] such as the required in situ milling step used for height reduction of the one-piece implant [11].

[1] Source: Letter to Nobel Biocare AB from Läkemedelsverket Medical Products Agency, Sweden, dated 6 December 2006.

It has been demonstrated in vitro that bone tissue damage can occur after heating bone to 47-50 °C for one minute during osteotomy preparation [12—16]. In situ high-speed drilling for the placement of bone orthopaedic implants is a well-recognized cause of overheating followed by osteocyte death and subsequent bone loss [17—19]. Electrosurgery or drilling without coolant are also well know to have deleterious effects on alveolar bone [20,21]. The aim of the present study was to evaluate whether in situ milling of a one-piece implant would generate enough heat to induce cell apoptosis.

Material and methods

Implants

One-piece c.p.Ti implants from one manufacturer (Zimmer one-piece, Zimmer Dental Inc., Carlsbad, CA, USA) were selected for this study (Fig. 1). Implant surface morphology was verified by scanning electron microscopy (Fig. 2). The titanium implants were carbon-coated by evaporation (10 nm thick) with a MED 020 (Bal-Tec, Balzers, Liechtenstein) to provide the material surfaces with uniform electrical properties. Samples were examined with a JEOL 6301 F field emission scanning electron microscope equipped with an energy dispersive X-ray microanalysis machine (EDX-Link ISIS-Oxford). EDX was done by point analysis at the surface of the material to determine its composition. Images were taken with a five kilovolts acceleration voltage.

Surgical procedure

The experiment was conducted in a veterinary surgical school in accordance with ethical principles for animal studies and good clinical standards. The implantation procedure was conducted in animals in the same way that it is performed in humans. Two sheep of the Vendeean breed, four to six years old, were the study subjects. Before surgery, animals were pre-medicated with an intravenous injection of benzodiazepam and the posterior limbs were prepared as for classical surgery. Natrium amoxicillin 500 mg was given by intravenous injection as a prophylactic antibiotic. General anesthesia was induced with intravenous perfusion of ketamine and, after an endotracheal intubation, maintained

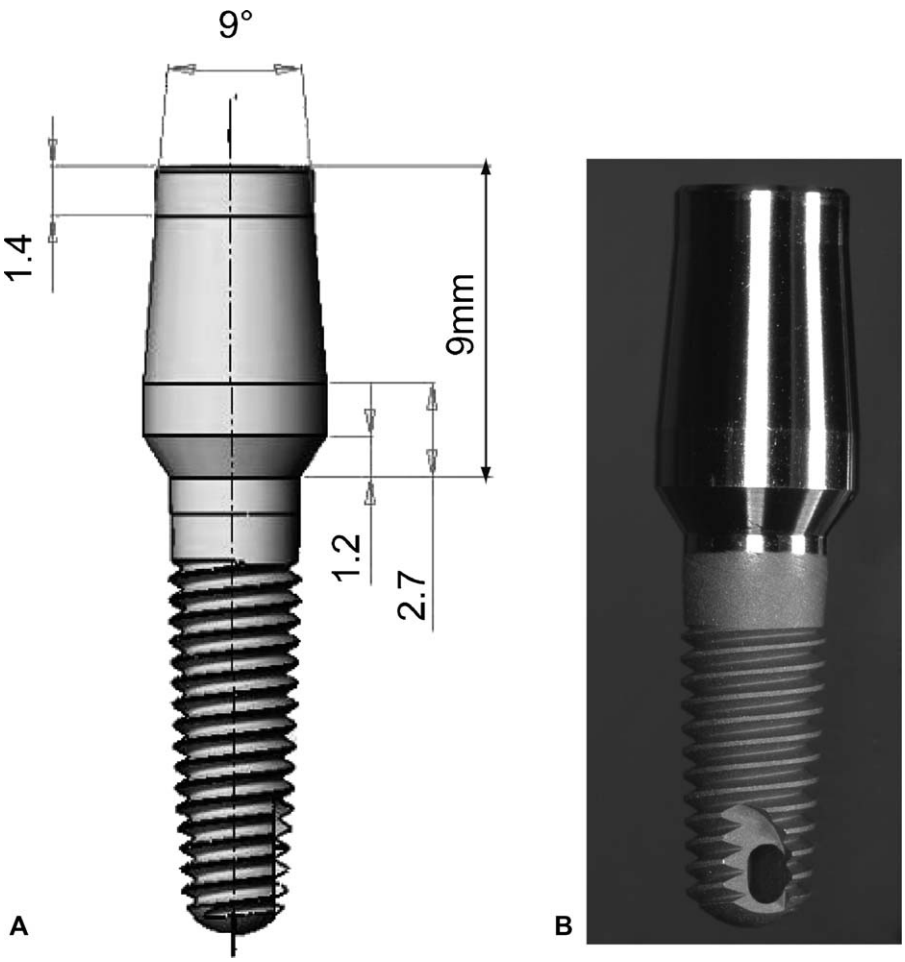

Figure 1 The one-piece titanium implant used in this study. A. Schematic drawing. B. Macroscopic aspect.
L'implant de titane utilisé dans cette étude. A. Dessin schématique. B. Aspect macroscopique.

with halothane provided by an anesthetic apparatus. The femur was exposed using a standard lateral approach at the distal epiphysis. Three dental implants were placed in the femoral distal metaphysis on each limb. The recipient sites were created with an electric rotary instrument at low speed (400 rpm), under physiologic saline as previously reported [22,23]. Ti implants were placed with a dynamometric control (Fig. 3). In each animal, one implant was milled with a diamond bur (ref. S6856 314 018, Komet/Brasseler; West One Dental Supplies Ltd; Croydon, UK) on one side and two on the opposite site were left unmilled. Milling was done at a rotation speed of 120,000 rpm under cooling with saline. The titanium was always milled in the same way (i.e. 1 mm in both vertical and horizontal direction). The incision was closed in sequential layers with resorbable sutures. An additional injection of amoxicillin trihydrate (500 mg) was administered immediately after surgery and two days after implantation at the same dose level. Sheep were sacrificed with an intravenous injection of natrium pentobarbital four days after implantation and bones were harvested. The implantations and milling times were recorded with a video camera and the milling process was about 45 seconds per implant.

Histologic technique

After surgery, bones were immersed in an alcohol/formalin fixative for 24 hours at 4 °C. Before removing implants, bones were radiographed on a numeric system (Faxitron Edimex, Angers, France) in order to determine the exact position of the implants. Implants were carefully unscrewed and blocks containing a single implant were sawn with a precision electric saw (Struers, Accutom-50, Copenhagen-Denmark). Contact radiographs of each block were taken (Fig. 4A) and blocks were processed undecalcified. Bones were dehydrated in absolute acetone for 72 hours at 4 °C, immersed in acetone/xylene for 24 hours and defatted in xylene for 72 hours at room temperature. Blocks were embedded in purified methyl methacrylate in a cold environnement [24].

Embedded blocks were dry-cut parallel to the implant axis using a heavy-duty microtome equipped with 50° tungsten carbide knives (Leica Polycut S, Rueil-Malmaison, France.). Sections were cut parallel to the long axis of the implant's cervical region and centered on the maximal diameter (Fig. 4B). For each specimen, four serial sections (7 μm in thickness) were obtained at six different levels with a 50 μm space between each level. For each stack of sec-

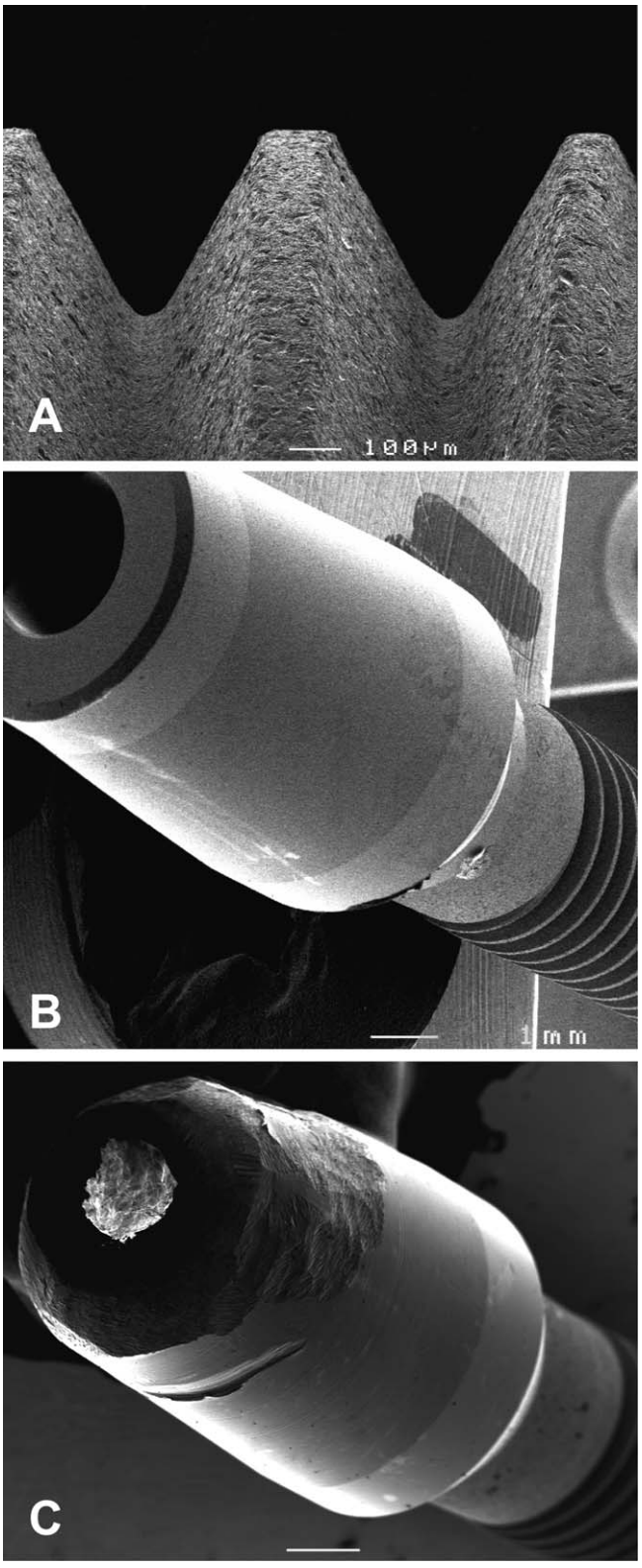

Figure 2 Scanning electron microscopy of the one-piece titanium implant. A. The threads with a rough surface (bar = 100 µm). B. The abutment with a smooth surface (bar = 1 mm). C. The abutment of a retrieved implant which had been milled in vivo (bar = 1 mm).

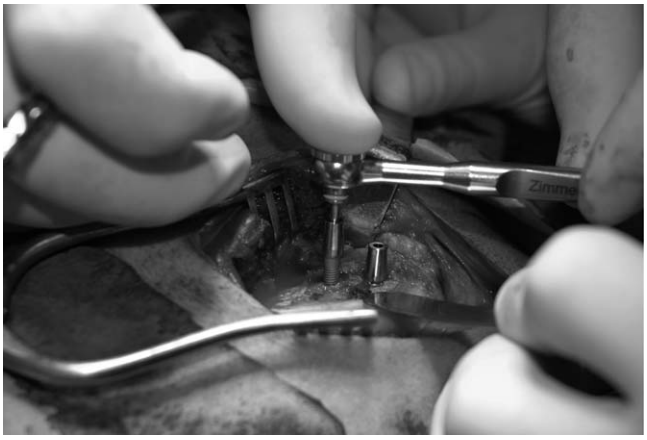

Figure 3 Surgical view of two implants placed in the femoral metaphysis of a sheep.
Vue chirurgicale de deux implants placés dans la métaphyse fémorale d'un mouton.

tions, one section was used for histochemical identification of osteoclasts by tartrate resistant acid phosphatase (TRAcP) [25] and one for a precise identification of living osteocytes. Osteocytes were detected by an intense nuclear staining with Hoechst 33342 (2 mg dissolved in 1000 ml of distilled water, staining time: 30 minutes) and counterstained in red using nuclear yellow fast R (1 % in formalin containing phosphotungstic acid) during 30 minutes (Fig. 5) [26]. Hoechst 33342 has been used widely as a fluorescent dye for staining the nuclei of living cells [27]. It preferentially binds to AT regions of DNA and shows no cytoplasmic staining. Observations and measurements were made under UV fluorescence microscopy with a WU near-ultraviolet fluorescence cube on an Olympus BX51 microscope equipped with a DP71 digital camera.

Histomorphometry

Bone volume and osteocyte count were determined on Hoechst 33342 stained sections with an image analyzer (Leica Quantimet 550, Leica, Rueil Malmaison, France) with a specially written software in Quips™. The program measures the following parameters (the ASBMR—American Society for Bone and Mineral Research—nomenclature is used hereafter):

- the trabecular bone volume (BV/TV, in %) represents the amount of trabecular bone inside the cancellous space. It was measured after thresholding the trabeculae;
- the number of osteocytes (N.Ocy/B.Ar, in ¢/mm^2) was determined by automatically counting the number of osteocytes identified as highly brilliant spots inside the red trabecular bone matrix.

Microscopie électronique à balayage d'un implant en titane. A. Le filetage avec une surface rugueuse (bar = 100 µm). B. La zone avec une surface lisse (bar = 1 mm). C. La zone supérieure d'un implant prélevé qui avait été fraisé in vivo (barre = 1 mm).

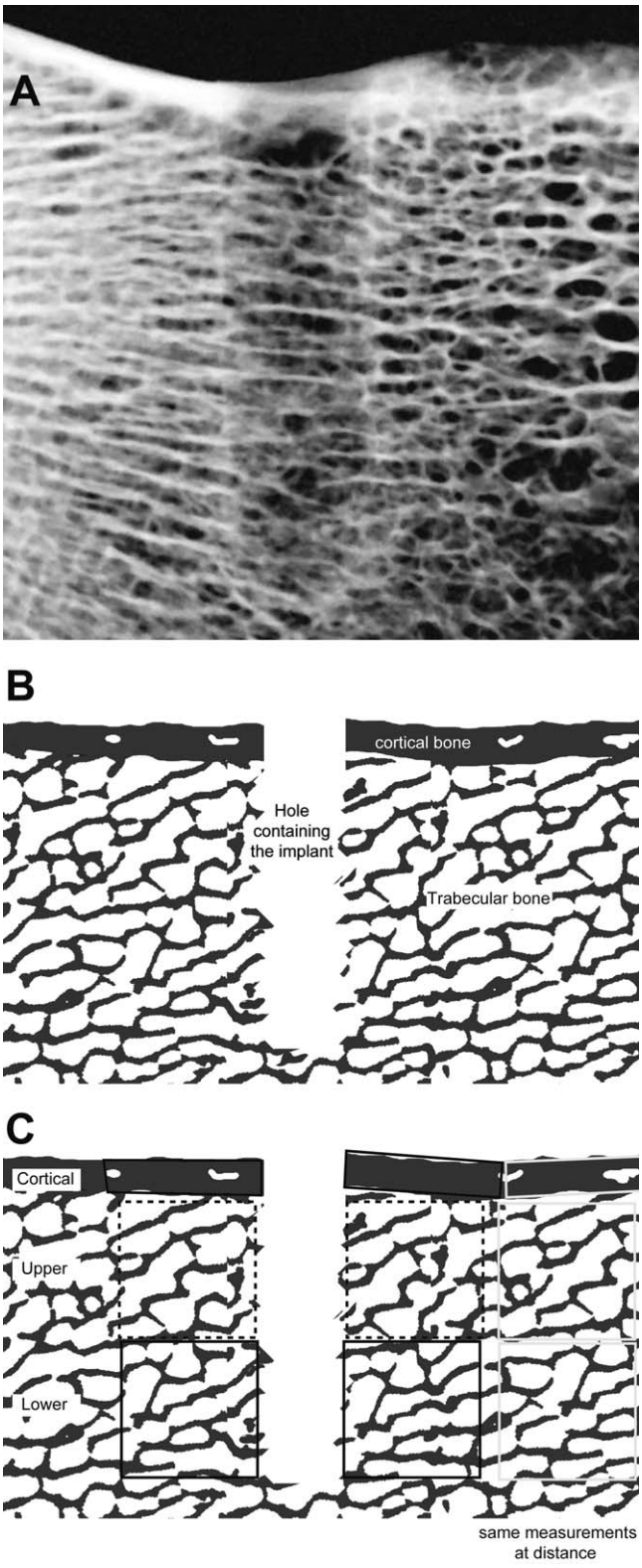

Figure 4 Principles of histomorphometric measurements: A. X-ray image of the implantation bed after removal of the implant. B. Digitized histological section showing the cortical and trabecular bone, and the hole which contained the implant. C. The various regions of interest used for morphometric measurements, cortical, upper trabecular on both sides of the hole margins in dotted lines, lower trabecular areas with plain lines.

Osteocyte number was determined in three different locations:

- the cortical bone region in direct contact with the implant abutment (an area a priori exposed with the maximum heat generated during milling);
- in the upper trabecular region close to the milling site, and;
- in the lower trabecular region at distance from the milling zone (Fig. 4C).

The number of osteoclasts (N.Oc/B.Ar; in ¢/mm^2) was determined similarly on TRAcP-stained sections. Only TRAcP positive cells in direct contact with the bone matrix were selected. Osteoclasts were only determined in trabecular bone.

Statistical analysis

Statistical analysis was performed using Systat® statistical software release 11.0 (Systat Software, San Jose, CA). Differences among groups were searched with the Kruskal-Wallis one-way analysis of variance and differences between groups by Mann-Whitney's *U* test when the Anova revealed a significant difference. Differences were considered as significant when $p < 0.05$. Results were expressed as mean ± standard deviation.

Results

TRAcP identification revealed osteoclasts as deeply stained cells with brown cytoplasm in direct contact with the blue calcified bone matrix. On Hoechst 33342 stained sections, osteocytes appeared as intensely stained greenish spots (and only intact nuclei were stained) on a red-stained background corresponding to the calcified bone matrix. Nuclei of bone marrow cells, endothelium and lining cells were also stained but did not interfere with measurements since the image of the trabeculae was used as a mask for counting osteocytes.

Results of histomorphometric analysis are summarized in Table 1. The trabecular bone volume was unchanged in both the upper and lower trabecular regions. No difference was also observed in the cortical bone. There was no increase in osteoclast number in the upper and lower trabecular regions, regardless of whether the implant had been

At distance from the implantation area, the region of interest used to obtain control values.

Les principes de mesures histomorphométriques: A. Image radiographique du lit d'implantation après dévissage de l'implant. B. Coupe histologique numérisée montrant l'os cortical et l'os trabéculaire, et le trou qui contenait l'implant. C. Les différents régions d'intérêt utilisées pour les mesures morphométriques, corticales, trabéculaires supérieures des deux côtés des marges de trou sont en lignes pointillées, plus en dessous, les secteurs trabéculaires en lignes pleines. À distance de la zone d'implantation, la région d'intérêt permet d'obtenir des valeurs de contrôle.

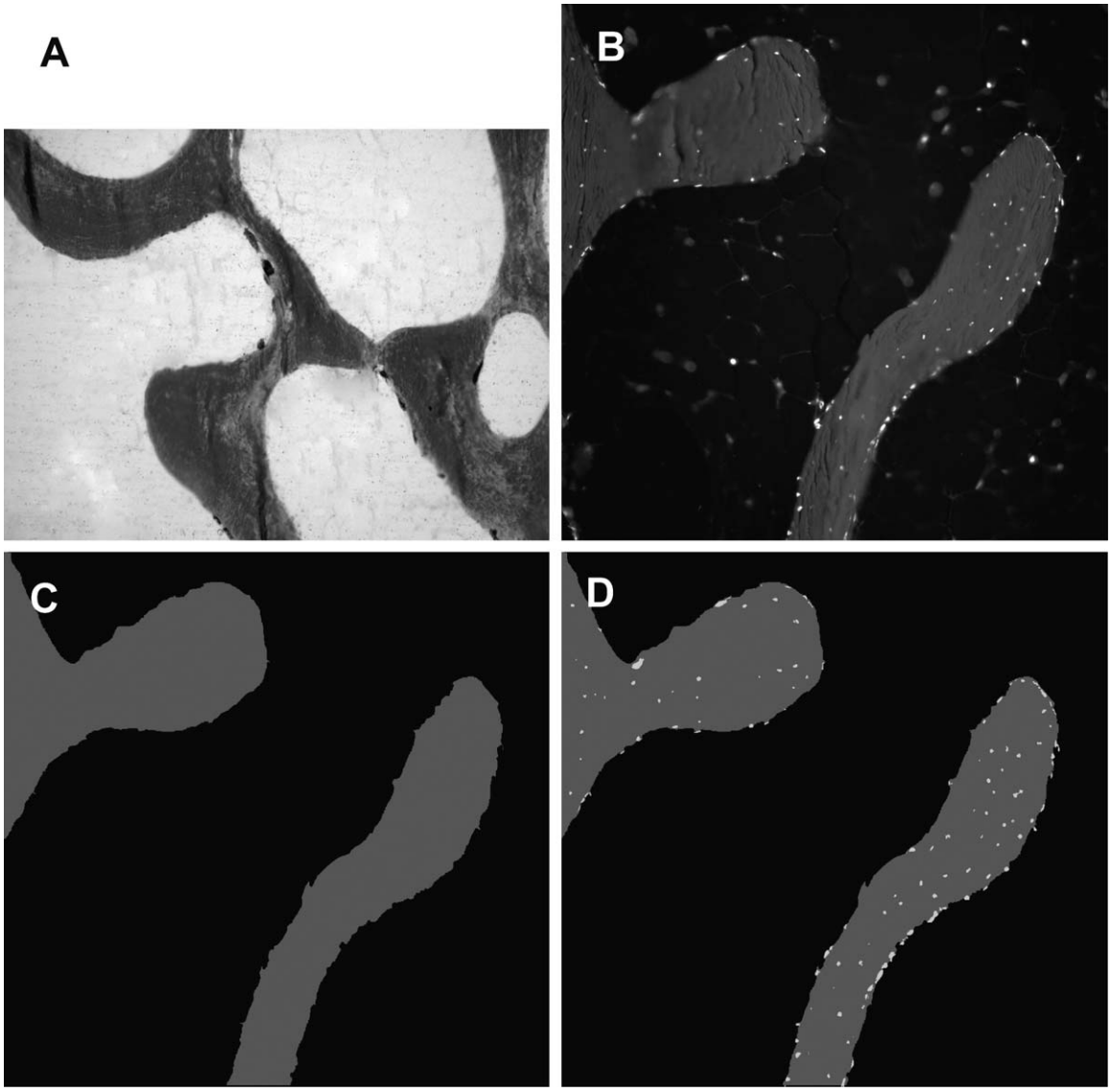

Figure 5 Histological aspects of bone cells revealed A. By the TRAcP staining which identify osteoclasts in purple, the bone matrix is lighly counterstained; original magnification × 100. B. Osteocyte nuclei identified by Hoechst 33342 with a red counterstain in fluorescence microscopy. Original magnification × 250. C. Same image treated by image analysis, the trabeculae are threshold and used as a mask to measure the bone surface, the bone marrow is eliminated. D. Same image after overimposition of osteocytes in pseudocolor.

Aspects histologiques de cellules osseuses après coloration. A. Par la détection de la TRAcP qui identifie les ostéoclastes en pourpre, la matrice osseuse légèrement contrecolorée ; agrandissement original × 100. B. Noyaux des ostéocytes identifiés par le Hoechst 33342 avec une contrecoloration rouge en microscopie de fluorescence. Agrandissement original × 250. C. La même image traitée par analyse d'image, les travées sont utilisées comme un masque pour mesurer la surface osseuse, la moelle osseuse est éliminée. D. Même image après surimposition des ostéocytes en pseudocouleur.

milled or not. In addition, the number of osteoclasts did not increase around the implants and did not differ from the mean value measured in the sheep femoral bone at distance from the implantation site. The number of osteoclasts was significantly higher in the deeper zones of the bone samples, at a distance from the cortice. Similarly, the number of osteocytes was not modified by milling in either the cortical area or the two trabecular regions. Living osteocytes were observed in bone trabeculae in the vicinity of the hole. On bone matrix debris, osteocytes were not identified in small particles but were present in larger particles intercalated with the trabeculae.

Discussion

One-piece dental implants could represent an interesting progression with the increased popularity of immediate loading. Several disadvantages of conventional two-stage implants are avoided: screw loosening, screw fracture,

Table 1 Results of histomorphometric analysis.
Résultats des analyses histomorphométriques.

	Unmilled	Milled	At distance	*p*
Cortical parameters				
BV/TV (in %)	71.0 ± 5.3	62.6 ± 7.6	72 ± 8.3	NS
N.Ocy/B.Ar (in ¢/mm^2)	232 ± 46	268 ± 75	253 ± 50	NS
Trabecular parameters, upper area				
BV/TV (in %)	26.4 ± 2.5	28.0 ± 2.0	27.2 ± 5.3	NS
N.Ocy/B.Ar (in ¢/mm^2)	424 ± 117	438 ± 38	432 ± 50	NS
N.Oc/B.Ar (in ¢/mm^2)	9.4 ± 7.0	8.3 ± 4.4	14.8 ± 6.5	NS
Trabecular parameters, lower area				
BV/TV (in %)	21.3 ± 2.9	21.8 ± 4.9	22.1 ± 4.6	NS
N.Ocy/B.Ar (in ¢/mm^2)	477 ± 96	457 ± 40	460 ± 68	NS
N.Oc/B.Ar (in ¢/mm^2)	11.6 ± 10.5	8.5 ± 8.4	14.9 ± 9.5	NS

bacterial leakage, and metal ion leakage at the implant-abutment joint [28]. One-piece implants have been found to generate the lowest maximum equivalent stress in the peri-implant bone [29].

The effect of in situ modification of one-piece implants by milling could be expected at various levels:

- mechanical level: use of a diamond bur during milling can generate vibrations that can decrease the primary stability;
- biological level: pollution of the implantation site by milling debris: particles of titanium, diamond or tungsten carbide according to the bur's composition;
- thermal Level: a sudden rise in temperature in the cervical portion of the implant could be transmitted to the surrounding bone via heat conduction through the metal, a phenomenon perhaps favored by the lack of interface between the two components of the one-piece implant.

Finne et al. [30] prepared 35 implants out of 82 in mouth after the installation with carbide bur or diamond rotary cutting instruments. The stable marginal bone level and soft tissue health observed indicate that the one-piece implant tested has the ability to preserve both hard and soft tissues. Intraoral preparation of titanium at placement had no adverse effect on bone [30]. In the present study, some rapidly bone cell changes were evaluated after a short-term implantation in the sheep. There was no osteoclast increase in the margins of the implant hole only four days after drilling and implant placement. Osteoclastogenesis is a rapid phenomenon after modification of local stresses [31]. Usually three-five days are necessary to observe an osteoclast crisis. Here, whatever the procedure used at the time of implant preparation, the number of osteoclast was not modified. Heat, local acidosis and changes in the RANKL-OPG ratio or local surgical trauma are known to be potent stimulators of osteoclastogenesis [32,33]. In this study, it is likely that the local raise in temperature during milling did not increase osteoclastogenesis around the implant.

Osteocytes are fragile cells with numerous processes (about 50 per cell) running in canaliculi forming a lacelike communicating network. Osteocytes act as mechanoreceptors and can detect strain and initiate remodeling in damaged bone [6,34,35]. Osteocytes are known to be very sensitive to heat although the effects of heat on bone have received little consideration. In dental implantology, cutting or drilling under a coolant fluid is a well-known precaution [17—19]. Irrigation under saline was found to limit the temperature peak during drilling the cavity for bone implants; the temperature peak being more pronounced at the top than at the bottom of the hole [36]. On the other hand, few studies have found that a mild heat may not be detrimental to these cells [37,38]. Osteocytes are also very sensitive to anoxia or cessation the diffusion of nutrients that travel through canaliculi from the marrow cavity. In orthopedic surgery, heat generated during poly methylmethacrylate cement curing has been recognized as the main factor for inducing osteocyte necrosis [39—41]. Heat generated during drilling can reach 174 °C with a diamond bur [42] and thermographic analysis revealed that the highest frictional heat generated during drilling occurred in the cortice [43]. The traumatic section of the osteocyte processes by microcracks or microfractures occurring in the bone matrix is also known to induce osteocyte death by apoptosis [44—46]. In the present study, the four-day interval between implant placement and euthanasia was chosen to avoid spontaneous bone healing but sufficient to induce bone cell changes. We have previously reported that a 20 minutes storage of bone samples in saline resulted in a significant increase in osteocyte necrosis (evaluated by morphometry and transmission electron microscopy) [47]. Similarly, Franssen et al. reported a 50 % loss of osteocytes in the vicinity of Kirschner's wire after drilling into the femur and tibia of rabbits [48]. In vitro, more than 80 % of isolated osteocytes die within one hour after the introduction of a toxic compound (500 μM of hydrogen peroxide) [49].

In this study, the use of the Hoechst 33342 stain was found to allow a precise count of living osteocytes since only the nuclei are stained [26]. During apoptosis (or necrosis), cell nuclei do not take stain similarly and nuclei cannot be counted. The use of a red fluorescent counterstained allowed a clear-cut definition of these living cells and an automatic count of osteocytes per mm^2 of bone. Because osteocytes are the most numerous cells of bone (about

10,000/mm^3) [50]. The present method was found well adapted for bone cell morphometry. There was no difference in osteocyte number in cortical or trabecular bone in the vicinity of milled or unmilled implants. In the same way, the number of osteocytes was not modified when compared to areas at distance from the implantation hole. The four-day interval between implant placement and euthanasia was fully sufficient to induce cellular changes (if any) and sufficiently short to avoid spontaneous bone healing.

Drilling under coolant and milling the head of one-piece implants in situ with a bur did not induce osteocyte apotosis or increase osteoclastogenesis in the vicinity of the implant during a short time period.

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