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In vivo evaluation of bone tissue behavior on ion implanted surfaces

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The ion implantation process offers several unique advantages over other surfaces modifications techniques, in regard to ion release and material mechanical characteristics. The aim of this study was to evaluate the *in vivo* bone tissue response to ion implanted surfaces. Untreated and nitrogen-ion-implanted stainless steel implants were inserted in the tibia diaphysis (cortical bone) and proximal tibia epiphysis (trabecular bone) of 12 New Zealand White rabbits. The animals were divided into three groups of four animals each, which were maintained for 4, 12 and 24 weeks according to internationally accepted and standardized procedures. At sacrifice, the implants were retrieved with surrounding bone and fixed in 4% neutral buffered formaldehyde and embedded in polymethylmethacrylate (PMMA). The samples were reducted in slices and stained with hematoxylin-eosin, light-green, fuchsin acid and giemsa solution for histological evaluation; fluorescent markers were also used to assess bone apposition. Histomorphometric evaluation was used to determine the extent of bone-material contact. Results from histological and morphometrical analyses revealed active remodeling of bone around both types of implants (control and ion implanted). However, faster bone deposition was observed around the treated material (12 weeks). Both materials reached similar endpoints, as no significant differences between them were evident at 24 weeks. The results demonstrate that ion implanted stainless steel has similar, or slightly enhanced, biological compatibility in contact with bone compared to untreated material; thus it may be a useful material in biomedical applications where reduced ion release or enhanced mechanical properties (as provided by ion implantation) are required.

1. Introduction

Metallic implants have been used successfully for many years in the surgical treatment of disease and injury. However, despite their generally successful application, problems with these implants do occasionally occur [2]. For instance, fatigue fracture is a common failure mode in orthopaedic devices, particularly in traumatology, where implant breakage prior to bone healing can lead to severe complications such as non-union [1]. 316L stainless steel and cobalt-chromium alloys are used frequently for permanent and temporary implants, due to favorable friction and wear characteristics [3]. However, a primary limitation to the clinical use of these materials is their tendency to release cromium, cobalt, and nickel ions by corrosion; the ions thus released must be regarded as a likely source of long-term problems, due to their known toxic effects on human cells [4, 5].

Performance and longevity of metallic implants can be improved using various strategies, such as modifying alloy composition or employing post-fabrication bulk or surface modification techniques. One of these, ion implantation, was originally developed for semiconductor applications and later for improving wear properties of metallic machine tools and for generating electrical conductivity in polymers [8]. Now it is suggested to increase the wear and corrosion resistance of metal alloys [6, 7]. This process offers several unique advantages over other surface modification techniques [9]. Properties of the near-surface volume can be enhanced through both chemical and structural modifications performed at room temperature with no distortion or changes in surface finish. Furthermore, no chemical or structural interface is created between the enhanced surface volume and the substrate material [10]. Nitrogen ion implantation has been used as a surface treatment for titanium alloys in selected biological applications [11, 12] since it provides an effective means of reducing the wear-corrosion rates of titanium [10]. The technique

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also significantly decreases the amount of metal ions released from stainless steel, due to increased wear resistance and faster repassivation [13]. While the mechanical benefits derived from nitrogen ion implantation have been well documented, there is little published literature regarding clinical application of ion implanted materials, particularly stainless steel.

The aim of this study was the biological evaluation of stainless steel surfaces modified with nitrogen-ion implantation. We evaluated *in vivo* histological and morphometrical response of bone to these materials implanted in New Zealand white rabbits.

2. Materials and methods

2.1. Materials

Twenty-eight normalized stainless steel (approved by competent authorities for medical use in humans) cylindrical samples, 3.95 mm in diameter and 6.1 mm in length, were used.

After cleaning with acetone and isopropyl alcohol, fourteen of them were ion implanted. For treatment, the samples were mounted on a plate with the flat ends of the cylinders parallel to the plate surface. Half of each cylinder was recessed beneath the plate surface to fix it in place. To treat both the ends and cylindrical surfaces, the samples were oriented at a 45-degree angle relative to the direction of travel of the ions. The plate was constantly rotated during processing. The samples were treated in two steps, with the samples being inverted between each step so that all surfaces could be treated.

Samples were ion implanted with nitrogen ions at an energy of 80 keV. Total dose on the parts was approximately 6×10^{17} ions/cm^2. Under these conditions, the maximum depth of penetration of the nitrogen ions was approximately 150 nm, with the implanted ions residing in a region extending from the surface. The dose rate was maintained at a low level so that temperature of the parts did not exceed 150 °C during processing. Note that ion implantation is not a coating process. All implanted ions residue sub-surface, and material dimensions are not affected by the process.

Before surgery, all implants were cleaned ultrasonically in 100% ethanol to remove any loose debris, dried at 50 °C and then sterilized in an autoclave at 121 °C for 20 min.

2.2. Surgical methods

Fourteen mature, female, New Zealand white rabbits, aged 10 months and weighing 3.9–4.4 kg were used in the study. The animals were premedicated with Ketasol and rompun; general anesthesia was then induced with a halothane-oxygen mixture. The lower tibiae were shaved, prepared using sterile techniques, and draped to expose both legs. Surgery was performed by Dr E. Rizzo at AO Research Center, Davos, CH, by courtesy of Prof. S. Perren, Director of the center at the time.

After making skin, subcutaneous, and periosteal incisions to reveal the tibia, a hole was made in the proximal tibia metaphysis and a test sample inserted using plastic forceps. Implants were inserted in medial site and allowed to penetrate the first cortical layer, never entering the opposite site. In each rabbit, a control sample was implanted in the contralateral leg using the same medical and surgical procedures. After implantation, tissues were irrigated with saline and closed in layers: periosteal and subcutaneous layers were closed with 5.0 Vicril, and the skin was closed with 5.0 Supramid sutured in interrupted fashion. Sterile dressings were applied to the wound. Anteroposterior and lateral radiographs centered at the implant area were taken immediately following implantation. The rabbits were then housed in individual cages and fed a standard chow diet. The animals were divided into 3 groups with 4 rabbits per group (2 rabbits remaining as reserves); the groups were followed for 4, 12 and 24 weeks after surgery. Implants with inflammatory or infective response were not included in the evaluation.

2.3. Histological and morphometrical analysis

During the follow-up period, fluorescent markers were injected to evaluate the remodeling activity of bone around the implants. Calceine, X-Orange, and Tetracicline (all from Fluka, Buchs, Switzerland) were used.

At the end of the follow-up period (either 4, 12, or 24 weeks depending on the group), the animals were sacrificed. The upper tibiae were harvested bilaterally for histomorphometrical evaluation. The specimens were fixed at room temperature for 2 days in 4% phosphate-buffered formaldehyde pH 6.9, and dehydrated in ascending ethanols for one night prior to three-step impregnation in a methylmethacrylate (MMA) monomer (Merk) for at least 3 days. For embedding, specimen blocks were impregnated in 80% (vol/vol) stabilized MMA, 20% (vol/lol) Plastoid N (Rohm Pharma, Germany) for 2 h in uncapped vials under vacum and embedded in capped 10 mL glass vials (water bath) at 37 °C overnight. After the polymerization, the glass vials were removed and moistened sections (50 μm) were cut on a Leica SP 1600 Saw Microtome with a rotating diamond saw blade for high-quality sample preparation of hard materials for microscopical analysis and mounted on polyethylene slides. Cut was performed on the long axis of the implant and the sections were stained using hematoxylin-eosin, light-green, fuchsin acid, giemsa and Van Gieson solution for histological evaluation.

The microradiographic analysis was performed on the slides used for fluorescence microscopy with a Philips crystallographic X-ray diffraction instrument: the sections were mounted in a vacum camera in intimate contact with the film 25 cm from the X-ray source. The prepared sections were examined histomorphologically and morphometrically by an investigator blinded to the identity of the material. The morphometry was performed both on the histologic sections and microradiographs, in order to measure the bone-implant contact. These measurements were performed using a semiautomatic image analyzer (GIPS Image processing software for the I.T.I. PCV board) connected to a Leitz Laborlux S light microscope. All measurements were performed at a magnification of 40×. Calculations were made of the total bone-metal contact around the entire

implant; new bone areas emanating from the endosteum; and average bone layer thickness for regions of bone contact. The following three indices were used to quantify the amount of the new bone formed at the implant surface:

1. Bone contact: this index evidences the percentage of the implant's extracortical length covered by new bone;
2. Average bone layer thickness: this index evidences the average layer thickness of new bone over the entire extracortical length of the implant;
3. Average bone layer thickness in bone contact regions: this index evidences the average thickness of new, extracortical bone per unit length of the implant on which bone has formed to analyze variability, morphometrical data were processed by Mann–Whitney U non-parametric test for independent data.

3. Results

In three animals the implants loosened before the end of the experiment; one animal sustained a tibial fracture during implantation, one animal was euthanized because an inflammation of the soft tissue over the implant was observed; and one case was excluded because implant displacement was observed before the end of the experiment period. Two of these rabbits were replaced with the two animals remained as reserves; the only animal excluded from the study, come from the 4 weeks group where data are expressed as media of three experiments.

3.1. Histological evaluation

3.1.1. Fluorescence microscopy

The accumulation of calcein-, X-orange-, and tetracycline-labeled bone demonstrated an active remodeling in the vicinity of the implants. Newly formed bone was deposited on both the nitrogen implanted and the untreated implant surfaces. Differences were noted only at 4 weeks, where more new bone deposition and active remodeling activity was present around the surface treated implants, compared to the untreated material. However at the end of the follow-up, no particular differences were evident between the two tested materials.

3.1.2. Light microscopy

The light microscopic evaluation of the bone/implant slices (giemsa and eosin and fuchsin acid and light-green) demonstrated bone maturation around the implant at all three observation times (Fig. 1 referred as an example to a 12 weeks surface treated sample). A collar of bone, emanating from the medial and lateral endosteal surfaces formed a buttress around the implants. At the first observation time (4 weeks), this collar was continuous with the original cortex around only two samples (both surface treated). The bone deposition around the implants was similar for the treated and control materials at 12 weeks of observation. At the end of the follow-up period (24 weeks), all of the implants were surrounded by newly formed bone, and the bone collar around the implants were continuous with the original cortex in all the treated samples and in two untreated samples, and in some cases, completely surrounded the implant. The newly formed bone was mature and lamellar, undergoing active remodeling.

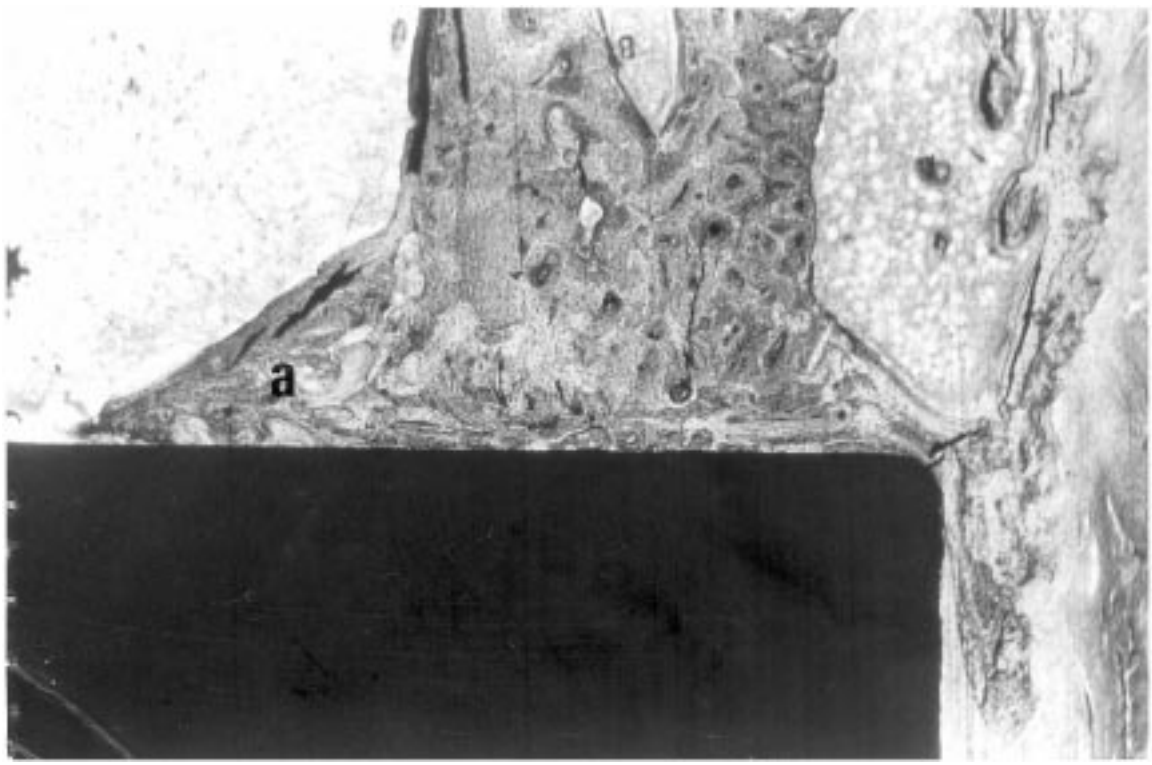
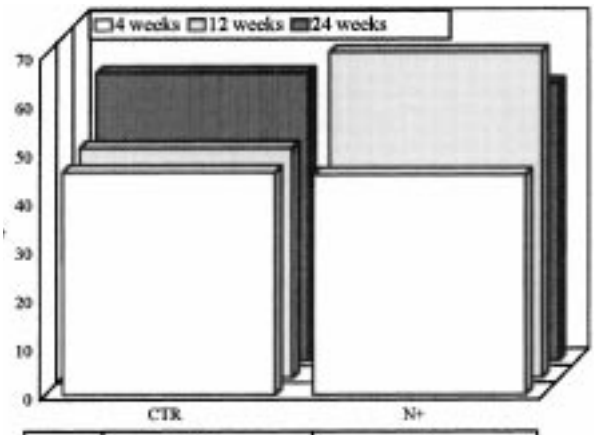


Figure 1 Example of light-green/fuchsin acid stained section (40× magnification). New bone areas emanating from the endosteum is well rapresented (a) and an Intimate bone-implant contact is evident in the upper side of the figure while in the right side a layer of fibrotic tissue separate bone from implant.

There was no evidence of foreign body reaction or inflammatory responses around the implants. Material surfaces were frequently characterized by the presence of an intimate bone-implant contact; remodeling lacunae full of bone marrow were also visible. A non-continuous, thin layer of fibrotic tissue was found in the proximity of some implants, but only in one case was its presence predominant on the bone tissue.

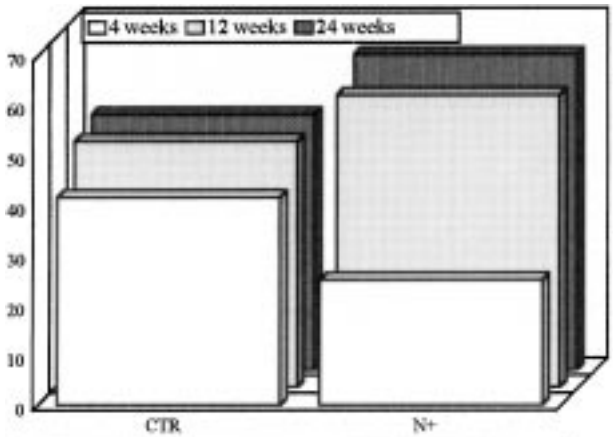
3.2. Histomorphometric and microradiographic analysis

As indicated above, the three outcome variables analysed in the histomorphometric analysis were bone contact, average bone layer thickness, and average bone layer thickness in bone contact regions. The evaluation of microradiography, conducted on the same fluorescent slide, demonstrated at all times observations active remodeling activity of bone around the implants with no differences between the two materials. The mean values of these variables for treated and untreated materials at 4, 12 and 24 weeks are shown in Fig. 2. As shown in Fig. 2(a), the amount of bone contact was similar for N^+ treated and untreated material. The percentage of contact increased from 4 to 24 weeks, with a more rapid bone deposition for the nitrogen ion implanted materials (evidenced by the higher contact values at 12 weeks). Furthermore, bone layer thickness (Fig. 2(b)) increased with time of implants, with no statistical differences between the two materials studied. Data of bone layer thickness in bone contact regions (Fig. 2(c)) showed no statistical differences between the



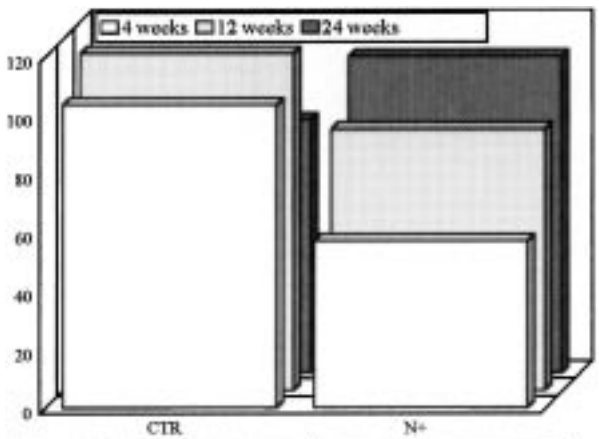
	CTR	N+
4 weeks	45,8± 15,1	45,3± 10,6
12 weeks	47,3± 10,4	67,2± 22,4
24 weeks	59,3 ± 17,5	57,1 ± 5,6

(a)



	CTR	N+
4 weeks	41,6 ± 11,6	25,0 ±4,1
12 weeks	49,3 ±13,3	58,6 ± 18,2
24 weeks	51,8 ± 17,1	63,2 ± 36,7

(b)



	CTR	N+
4 weeks	103,6 ± 51,5	57,1 ± 12,1
12 weeks	115,3 ± 51,4	89,2 ± 22,1
24 weeks	86,5 ± 14,3	108,2 ± 52,9

(c)

Figure 2 Histomorphometric data of bone-implant contact measurements. Fig. 2(a) evidences the percentage of the implant's extracortical length covered by new bone (bone contact); Fig. 2(b) evidences the average thickness of new bone over the entire extracortical length of the implant (average bone layer thickness); Fig. 2(c) evidences the average thickness of new extracortical bone per unit length of the implant (average bone layer thickness in bone contact regions).

two materials tested, but only N^+ implanted samples showed increased values from 4 to 24 weeks observation.

4. Discussion and conclusions

The most common metal alloys used for cementless hip and knee replacements are cobalt-chrome-molybdenum alloy and titanium-6 alluminium-4 vanadium alloy. However, these alloys are very stiff, and have been associated with adverse remodeling of the surrounding bone. For these reasons newer materials with lower modulus of elasticity and more reliable degree of bone ingrowth/apposition are needed. Nitrogen ion implantation treatment has been found to increase significantly the fatigue life of AISI 3 1 6L stainless steel screws used in spine surgery for pedicle fixation with an improvement of the fatigue life up to 98% [2] whereas other studies evidenced that stainless steel nitrogen ion implantation did not reveal significative advantages in wear behavior [8]. Furthermore, Davidson [14] remarks that the benefit from nitrogen implantation may be temporary due to the oxidative wear process, and thus produce increased wear by third body mechanism.

Bordji *et al*. [15] recently studied *in vitro* the effect of several surface treatments on human fibroblast and osteoblast cultures. They found significant cellular reaction in contact with 316L stainless steel treated with low temperature plasma nitriding, while nitrogen ion implantation did not modify the cytocompatibility. Our study supports this finding; results show many biological similarities between N^+ treated and untreated samples. In particular, stainless steel samples treated with nitrogen ion implantation and implanted in the rabbit tibia did not adversely influence the comportment of the bone tissue around the implant. In fact, fluorescent histological evaluation evidenced bone active remodeling around both the materials with more new bone deposition around the surface treated material at 4 weeks observation, data confirmed by the light microscopic finding of a collar of bone continuous with the original cortex and histomorphometric analysis that at 12 weeks showed that bone deposition was more rapid around treated samples than around control ones. At the end of the follow-up, a bone tissue layer was detected around both materials with no significant differences in the bone comportment around them. The faster deposition around the treated material is potentially explained by the nitrogen implantation reducing the metal ion release; ion release can cause a variety of deleterious effects, interfering with the biological mechanisms of bone deposition and remodeling, and ultimately compromising survival of the implant [16].

These studies suggest that ion implanted stainless steel, shown in previous studies to provide enhanced wear and corrosion resistance, exhibits good biological reaction and could be used in clinical situations where the mechanical properties of the ion implanted material are desirable.

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