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Osseointegration is improved by coating titanium implants with a nanostructured thin film with titanium carbide and titanium oxides clustered around graphitic carbon

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ABSTRACT

Titanium implants coated with a 500 nm nanostructured layer, deposited by the Ion Plating Plasma Assisted (IPPA) technology, composed of 60% graphitic carbon, 25% titanium oxides and 15% titanium carbide were implanted into rabbit femurs whilst into the controlateral femurs uncoated titanium implants were inserted as control. At four time points the animals were injected with calcein green, xylenol orange, oxytetracycline and alizarin. After 2, 4 and 8 weeks femurs were removed and processed for histology and static and dynamic histomorphometry for undecalcified bone processing into methylmethacrylate, sectioned, thinned, polished and stained with Toluidine blue and Fast green. The overall bone-implant contacts rate (percentage of bone-implant contacts/weeks) of the TiC coated implant was 1.6 fold than that of the uncoated titanium implant. The histomorphometric analyses confirmed the histological evaluations. More precisely, higher Mineral Apposition Rate (MAR, μm/day) ($p < 0.005$) and Bone Formation Rate (BFR, $\mu m^2/\mu m$/day) ($p < 0.0005$) as well as Bone Implant Contact (Bic) and Bone Ingrowth values ($p < 0.0005$) were observed for the TiC coated implants compared to uncoated implants.
In conclusion the hard nanostructured TiC layer protects the bulk titanium implant against the harsh conditions of biological tissues and in the same time, stimulating adhesion, proliferation and activity of osteoblasts, induces a better bone-implant contacts of the implant compared to the uncoated titanium implant.

1. Introduction

Titanium (Ti) is the most used metal for the fabrication of orthopedic and dental implants due to its high biocompatibility with human tissues, its low price and large availability on the market. However, these features do not give to this metal the title of ideal material due to its high affinity for oxygen which allows the spontaneous formation, on the Ti surface, of a layer of Ti oxides, mainly TiO_2. Those oxides represent a non metallic layer on the Ti surface that, in the harsh conditions of biological fluids, have a tendency to grow up, constituting a brittle interface between the implant and the bone [1–3].

This interface may be fractured, promoting the formation of particles [4–5], which may induce toxicity [6] or the induction of fibrotic reactions [1–3,7]. Furthermore, the chemistry of these non-metallic Ti layers could stimulate the cells to produce a large number of small adhesion points which induce fibrinogenesis and lead to soft tissue encapsulation of implants, micromotion, implant loosening and finally to implant failure [1,3,7,8].

In order to avoid these drawbacks, which produce human distress and social costs, many strategies have been developed, such as the physical modification of the Ti surface or the coating of the implant with different chemical structures as Ti dioxide, Ti nitride, hydroxyapatite and various ceramic materials. New deposition techniques have also been adopted, such as Physical Vapor Deposition (PVD), Chemical Vapor Deposition (CVD) and Plasma Spray Electrolysis (PSE) [2,9–13]. Another

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strategy is to cover Ti surface with biomimetic molecules to increase the bioactivity of the implant. Very good results have been obtained using short peptide chains present in adhesion protein sequences, such as fibronectin [14–15]. This strategy is very efficient because it is based on a natural pathway, however it presents some drawbacks as the high costs of the peptide synthesis and its deposition on the metallic surface of the implant.

An alternative strategy is to cover the implant surface with a layer to protect from further oxidation and in addition to promote osteoblasts stimulation to produce new bone. During last years, we have developed a simple method to cover the Ti surface of implants with a layer that should be protective for the Ti implant and at the same time able to stimulate the production of new bone, and may induce a more rapid and efficient osseointegration of the implant into bone [16]. The first device used, the Pulsed Laser Deposition (PLD) [16] evolved into a simpler and more easily managed device, the Ion Plating Plasma Assisted (IPPA) [17–18]. This device is composed of a vacuum chamber equipped with a magnetron sputtering on which is located a target of titanium carbide (TiC), powered by a direct current (DC), and of a sample holder containing the implants to be coated, powered by a radiofrequency (RF). A plasma cloud is formed between the sample holder and the magnetron that allows the deposition of a hard and compact film on the surface of Ti implants whose thickness is a function of DC and RF applied, and of the deposition time.

In order to select the layer that induces the best biological effects (*i.e.* the increase in gene expression of proteins involved in bone turnover), previously many deposition conditions were tested. The layer, experimentally defined optimal, had a thickness of 500 nm, a hardness five times higher than that of the Ti and was composed of 60% graphitic carbon clustered with 25% Ti oxides and 15% Ti carbide to form a well defined nanostructure [19]. The layer had a protective effect on the bulk Ti of the implant, preventing further Ti oxidations, and was effective in the stimulation of either human primary osteoblasts or osteoblast-like osteosarcoma cell line Saos 2. Indeed, the genes encoding for proteins involved in bone turnover were overexpressed and the osteoblast adhesion was stimulated through the increase of integrins on the surface of osteoblasts [20]. The positive results obtained in *in vitro* studies were predictive of a better *in vivo* osseointegration of the implants coated with the nanostructured TiC layer compared to the uncoated Ti implants.

In the present *in vivo* study we report the histological and histomorphometric results of bone-implant contacts and new bone formation of uncoated and TiC coated Ti implants driven in rabbit femurs.

2. Materials and methods

2.1. Implants

In order to evaluate whether the architecture of the titanium implant may influence the deposited nanostructured layer, two types of implants were used, one (4 × 8 mm, 2 mm coil screw) obtained from Orvit (Bologna, Italy) was used for the pilot *in vivo* study. The AnyRidge implants (4 × 8.5 mm, 0.5 mm coil screw) obtained from Megagen (MegaGen Implant Co. Korea) were used for the *in vivo* study with fluorochromes.

All the implants were sandblasted with 120 μm zirconia particles. The treated implants were coated with an uniform coating by continuous rotation of the implants for 1 h on a rotating sample holder powered by 100 W RF of the IPPA chamber described elsewhere [19]. Briefly, the IPPA apparatus is composed of a high-vacuum chamber hosting a TiC target on a magnetron sputtering source, powered by a 900 W DC. The implants were located in the IPPA chamber at 10 cm from the TiC target. XPS (X-ray Photoelectron Spectroscopy) quantitative analysis of the layer deposited on the two types of implants, as previously reported [19], revealed the same chemical composition. Coated and uncoated Ti implants were thoroughly cleaned by immersion for 30 min in a 0.1% solution of Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA), followed by sonication and rinse with ultrapure water and with hexane, and the latter was repeated three times followed by air drying and autoclaved.

2.2. In vivo study

In vivo study was performed at the Department of Animal Experimentation of Istituto Superiore di Sanità of Rome. It was carried out in accordance with Italian Law DL.116/92, following the ethical procedures defined by the ethical committee of Sapienza University of Rome and the approval of Italian Ministery of Health (decree number 254/2012-B of 22/10/2012): the number of animals was reduced to a minimum following the above mentioned law.

A total of 7 New Zealand rabbits (Harlan Italy, Correzzana, Italy, b.w. 4.3 ± 0.3 kg) were premedicated with dexmedetomidine (0.15 mg/kg; Dexdomitor, Orion Pharma, Finland) and ketamine (10 mg/kg; Ketavet, Intervet, Italia) *via* intramuscular injection in the lumbar muscles. An intravenous catheter was placed in the right cephalic vein and 0.9% NaCl solution was administered at 10 ml/kg/h with an injection pump throughout the anaesthesia. Under endoscopic guidance, rabbits were intubated with 2.5 or 3 mm uncuffed endotracheal tubes. The anaesthetic plan was maintained with isoflurane in the range of 3–4% in pure oxygen at 2 l/min.

The rabbits were placed in lateral recumbence and the area over the femur was shaved and aseptically prepared for sterile surgery. A longitudinal incision of approximately 4 cm was performed on the skin, directly over the femur and the quadriceps was retracted in order to achieve visualization of the femoral cortex. By use of a manual surgical drill a 4 mm hole on the cortex was made in each femur. Implants distal to the greater trochanter of both femurs were slowly and manually screwed into the holes. Each animal received one TiC coated implant, into their left femurs, and one uncoated (Ti) implant, acting as a controlateral control, in the right one.

At the end of the surgical procedure, premedication was antagonized with atipamezole (0.5 mg/kg; Antisedan, Orion Pharma). Meloxicam (1 mg/kg; Metacam, Boerhinger Ingelheim am Rhein, Germany) and enrofloxacin (5 mg/kg; Baytril, Bayer, Leverkusen, Germany) were administered *via* intramuscular injection for post-operative analgesia and antimicrobial coverage, respectively.

In the first pilot study, 3 rabbits were employed and one animal was euthanized after 2 weeks, another after 4 weeks and the third after 8 weeks from implantation. Then the trend and the formation rate of bone-implant contacts were analyzed.

In the second part of the study, 4 rabbits were used to associate bone formation with each implant design following a previously described schedule [16,21]. In detail, four different intravital fluorochromes were administered postoperatively over a period of 8 weeks at four time points after their implantation: calcein green (Sigma C0875) (10 mg/Kg), 2 weeks after implantation; xylenol orange (Fluka 33,825) (90 mg/Kg) 4 weeks after implantation; oxytetracycline (Terramicin long acting, Zoetis, Italy) (25 mg/kg), 6 weeks after implantation; alizarin red (Sigma A388) (30 mg/kg), 8 weeks after implantation. All fluorochromes, but oxytetracycline, were subcutaneously injected two daily consecutive and two days after the final injection, the rabbits were euthanized.

2.3. Histological process

At the end of experimental times, all the rabbits were anesthetized with a mixture of Ketamine and Dexdomitor, and euthanized with Tanax (Intervet Italia, Segrate, Italy). The femurs were removed, stripped of soft tissues and bone density around the implants was evaluated by an X-ray mammograph (GE Senograph 200D) using a focal spot of 0.1 mm.