

OSTEOGENESIS AND BONE INTEGRATION: THE EFFECT OF NEW TITANIUM SURFACE TREATMENTS

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RIASSUNTO

Il titanio rappresenta un biomateriale d'elezione in implantologia orale ed ortopedica grazie alle sue proprietà di elasticità, resistenza al carico ed inerzia in ambiente biologico.

Le modifiche di superficie del titanio sono in grado di incidere marcatamente sul processo di osteointegrazione degli impianti protesici. In particolare la microrugosità superficiale è un fattore determinante nell'adesione e colonizzazione osteoblastica durante la neodeposizione ossea nella sede perimplantare. Negli ultimi anni l'attenzione dei ricercatori si è concentrata sull'applicazione di gruppi funzionali (biomimesi) alla superficie del titanio, in grado di indurre ed accelerare i fenomeni di osteodeposizione (adesione osteoblastica, nucleazione di cristalli di idrossiapatite).

Lo scopo del nostro studio è la valutazione morfologica del potenziale di osteointegrazione di trattamenti chimici ed elettrochimici di modifica superficiale del titanio. In particolare sono stati testati un trattamento chimico ottenuto mediante doppio attacco acido ed un trattamento elettrochimico in liquidi corporei simulati (Bio-Spark™). Come modello sperimentale sono stati utilizzati conigli di razza White New Zealand e come sede anatomica di impianto si è scelta l'epifisi distale del femore.

Secondo quanto osservato, le modifiche di superficie hanno migliorato ed accelerato il processo di osteointegrazione degli impianti trattati rispetto al controllo, in modo particolarmente evidente e precoce nel titanio trattato chimicamente con doppio attacco acido. Al termine dei tempi sperimentali considerati, i due trattamenti di superficie hanno prodotto un grado di osteointegrazione comparabile.

I risultati inducono a considerare positivamente questi trattamenti al fine di sempre migliori performances degli impianti protesici nell'applicazione clinica.

Parole chiave: biomateriali - titanio – trattamenti di superficie – coniglio - tessuto osseo — osteointegrazione

SUMMARY

Titanium represents a choice biomaterial in oral and orthopaedic implantology due to its properties of elasticity, load resistance and inertness in

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biological environment.

Titanium surface modifications can considerably affect the osteointegration process of prosthetic implants. In particular, surface micro-roughness is a key factor in osteoblast adhesion and colonization during bone neodeposition around the implant. In the last years researchers have focused their attention on the application of functional groups (biomimesis) to titanium surface, able to induce and speed up bone deposition processes (osteoblast adhesion, hydroxylapatite crystal nucleation).

The aim of our study is the morphological evaluation of osteointegration potential of chemical and electrochemical treatments aiming at titanium surface modification. In particular, a chemical treatment, obtained by a double acid attack, and an electrochemical treatment in simulated body fluids (Bio-Spark™) were tested. White New Zealand breed rabbits were used as animal model and the femur distal epiphysis was chosen as implant anatomical location.

According to our findings, surface modifications have improved and speeded up the osteointegration process of treated implants compared to control, in a marked and early way in double acid attacked titanium. At the end of experimental times, both surface treatments produced a comparable osteointegration rate.

Our results allow to positively consider these treatments in order to improve prosthetic implant performances in clinical application.

Key words: biomaterials - titanium – surface treatments - rabbit – bone tissue — osteointegration

1-INTRODUCTION

Titanium, pure or alloyed with other elements (Ti6Al4V, Ti6Al7Nb), is a widely used material for biomedical applications.

Titanium is a bio-inert material with intermediate characteristics of workability and load resistance. Biotolerated metallic alloys, like surgical steel or CoCrMo alloy, present excellent workability and load resistance, but give unsatisfactory bone response and implant osteointegration, while bioactive materials (hydroxylapatite (HA) ceramic, bio-glasses) can provide good osteointegration but have bad characteristics of load resistance. Titanium is more elastic than metallic alloys and provide high mechanical performance. Moreover, it presents high corrosion resistance and excellent biocompatibility. The minimal tissue reaction induced by titanium and the favourable tissue response is attributed to the protective and stable oxide layer naturally formed by spontaneous passivation on its surface in contact with air or water, isolating implant from external environment (1, 2).

Osteointegrated dental and orthopaedic implants are useful and commercially relevant clinical tools. Dental implants are made of pure titanium, while orthopaedic implants require the use of titanium alloys for higher load involved.

Further to biomechanical factors related to implant geometry and shape, the interaction between the implant and surrounding biological tissues plays a key role to achieve a good implant osteointegration. In this connection, the topography

and chemistry of implant surface become relevant aspects for the performance of biomaterials *in vivo* (3-6, 7).

The osteointegration of an implant is the procedure by which mature bone is deposited directly on implant material without any intervening soft or fibrous tissue (8).

An ideal implant surface should be able to conduct or induce osteointegration, irrespectively of implant site, available bone volume or bone quality. As a consequence, the main attempt of research in implantology has focused on the surface modifying techniques to be applied to biomaterials, to obtain adequate biological responses.

Many procedures have been applied to titanium to improve tissue compatibility, making it suitable for dental and orthopaedic implants (1). Indeed, bone integration of titanium implants is modulated by its surface characteristics (9). Titanium implant surface can be smooth, rough or coated. Untreated surfaces possess at any rate titanium properties of enhancing osteogenesis (inertness in biological environment).

Surface micro-architecture can be optimised by the presence of rugosities or pores (10). Rough surfaces are obtained by chemical attacks (acid attack), sandblasting (with aluminium dioxide, titanium dioxide, HA, glass microspheres) (11) or titanium plasma spray coating. The resulting micro-roughness and high pureness improves prosthesis anchorage to bone tissue and mechanical stability to obtain a favourable load distribution. The surface roughness is one factor which helps in determining the balance between bone formation and resorption at the bone-implant interface, since it has been shown to affect the synthesis of biological factors by osteoblasts (9) and their cellular response to growth factors or cytokines (12).

Coated surfaces benefit by the coating bioactivity to obtain the strongest bone-implant adhesion at an early stage after implantation. A bioactive material is able to exert a physical, chemical or pharmacological action on the host tissue metabolism. Titanium metal substrate can be coated with bioactive substances, that is calcium phosphate ceramics, like hydroxylapatite (HA) and tricalcium phosphate (TCP), and bio-glasses (7), by means of plasma spray techniques. These coatings are reported to promote direct bone-implant contact without intervening fibrous tissue (13). Nevertheless, bioactive coatings can present defective adhesion to metallic substrate for fragmentation, dissolution or delamination (14), that is, detachment between substrate and coating for their lack of homogeneity. The problem is probably related to the difficulty to control the kinetics of deposit dissolution, which hampers the long term stability of such coatings (15). As a consequence, chronic inflammatory reactions can develop and cause implant failure.

Different causes of failure have emerged, hampering implant durability. First of all, implant mobilization can occur for defective osteointegration associated with formation of fibrous connective tissue at the bone-implant interface in the first period after implantation (14). This phenomenon is more likely to occur using smooth surface devices (2). A bad osteointegration can be caused by bacterial attack, inflammatory reaction or exceeding loading/movement at the implant site during the phase of primary integration. Also, an allergic sensitization can take place after

release of metallic ions.

Focusing on physicochemical properties and morphology of implant surfaces (15), the causes of failure can be contrasted modifying titanium surface and in particular the thickness, structure and composition of titanium oxide film. Titanium surface treatments promote osteoblast adhesion and proliferation and inhibit bacterial adhesion and growth, enhancing osteointegration.

An essential requirement for a good osteointegration is the formation of “bone-like” apatite on the implant surface. Biomimesis is the presence of superficial functional groups able to promote apatite nucleation. In particular, the aim of biomimetic treatments is to create a new interface inserting proper functional groups, biological coatings with osteoconductive function (phospholipids) or biomolecules (biochemical functionalization) involved in bone regeneration process. These treatments enhance the mineralization process around the implanted material and improve the compatibility between implant and surrounding tissues.

Anodic oxidation is reported to be a preferred method to form rough, porous and thick oxide films by spark discharges at a high electrolytic voltage (1). The treatment induces the formation of micropores and microprojections on the oxide film. Increasing the oxide film thickness usually increases its crystallinity and dissolution resistance (2). In the last years, anodic electrochemical treatments have allowed to supplement the titanium oxide film with calcium and phosphorus, forming a thin and porous calcium phosphate-enriched surface layer. This technique is known as Anodic Spark Deposition (ASD) (1, 2, 10, 15). Coatings obtained by this process show good chemical and mechanical properties, as well as controlled microporosity (15). Some Authors (1) added a final hydrothermal treatment to the ASD process aiming at changing the outer amorphous layer into a crystalline hydroxylapatite. The incorporation of calcium and phosphorus into the anodic oxide film provide the compositional basis for the formation of calcium phosphates, primary inorganic phases of hard tissues, and has osteoinductive properties in physiological fluids (2). These characteristics allow a more stable bond between metallic substrate and newly formed bone.

The aim of this study is to compare the biological response of bone tissue to differently treated titanium implants in rabbit femur. T1 is chemically-treated rough titanium achieved by a double step acid etching, and T2 is bioactive titanium obtained by Bio-Spark™ (BSP) treatment (15, 16). Tc is untreated machined titanium. Double step acid etching aims at providing a proper rough surface, after a previous decontamination by strong alkali etching, while BSP treatment involves a double ASD treatment and a final alkali etching to modify the superficial titanium oxide film. BSP treatment results in a thickened titanium oxide layer doped with calcium and phosphorus. This layer exhibits a high bioactivity resulting in an enhanced calcium-phosphate nucleation from simulated body fluids. Also, the presence of hydroxile groups on the oxide film surface seem to affect the mineralisation potential (15).

2-MATERIALS AND METHODS

2.1-Implants and surface treatments

Cylindrical implants of commercially pure grade 2 titanium with a diameter of 3 mm and a length of 13 mm were used for testing 2 different titanium surface modifications *in vivo*.

Implant surfaces were obtained as follows: chemically-treated titanium, T1, resulted from a decontamination by strong alkali etching followed by a double step acid etching, the first one carried out in 1M NaOH containing 2% H₂O₂ at 80°C for 10 minutes, the second in an acid water solution at 28°C for 1 hour. Electrochemically-treated titanium, named Bio-Spark™ (BSP), T2, was prepared in an electrochemical cell by 2 ASD treatments, the first one performed in a solution containing calcium and phosphate ions, the second in a solution containing calcium ions only. A final treatment in concentrated potassium hydroxide water solution at 60°C was performed. Untreated machined titanium, Tc, was used as control material. Materials were kindly supplied by the Department of Chemistry, Materials and Chemical Engineering "G.Natta", Politecnico di Milano, Milano, Italy.

Materials were sterilized using gamma ray irradiation (25kGy for 14 hours).

2.2-Animals and surgery procedure

Approval was obtained from Health Ministry prior to performing the study. European and Italian regulations on animal experimentation were strictly followed according to the EC rules (EU 86/609). White New Zealand breeding age male rabbits of average weight $3,3 \pm 0.2$ Kg were used to evaluate osteointegration, biocompatibility and osteoinduction properties of testing materials. The study design was composed of two different experimental times: 45 and 90 days. For each experimental time, 4 animals were used and divided in two experimental groups of 2 animals each, group 1 for T1 and group 2 for T2. In each animal, T1 or T2 implants were inserted in the right femur distal epiphysis, while contralateral femur served as a control for the insertion of Tc implant.

Prior to surgery rabbits were given antibiotic therapy I.M. (Baytril, Bayer, 5 mg/kg) and analgesic therapy S.C. (Rimadyl, Pfizer, 4 mg/kg). The specimens were inserted during surgical procedure in general anaesthesia (Domitor, Pfizer, 0.1 ml/kg; Ketavet 100, Gellini, 0.3 ml/kg; Isoflurane-Vet, Merial) with sterile techniques. After arthrotomy and luxation of the rotula, a hole was performed in the throclear groove. The procedure was performed with attention to the specimen collocation in order to avoid motility. In the control segment of the left femur a hole was performed and the Tc implant was positioned in a stable way. Postoperative X-rays were taken to verify implant placement.

In order to assess the osteogenic activity, vital fluorochromic bone markers were used, allowing to mark the areas of bone growth within the administration period (17, 18, 19). Labelling of bone deposition was performed by subcutaneous injections of two fluorescent markers: Calcein Green (CG) (5 mg/Kg body weight, Sigma) and

Xylenol Orange (XO) (90 mg/Kg body weight, Fluka). The animals belonging to the experimental time of 45 days were marked with CG on the 13th and 14th day after surgery, and with XO on the 31st, 32nd and 33rd after surgery. The animals belonging to the experimental time of 90 days were marked with CG on the 13th and 14th day after surgery and with XO on the 60th, 61st and 62nd day after surgery. CG shows a bright green fluorescent glow (λ of absorption: 495 nm and λ of emission: 550 nm), while XO shows an orange fluorescent glow (λ of absorption: 540 nm; λ of emission: 620 nm).

Animals were pharmacologically euthanised (Tanax, Roussel Hoechst, 0.3 ml/kg/bw) under general anaesthesia (Domitor, Pfizer, 0.1 ml/kg; Ketavet 100, Gellini, 0.3 ml/kg).

2.3- Histological evaluation

The femoral distal epiphysis were harvested and processed for histological evaluation. Undecalcified bone specimens were fixed in 70% ethanol, dehydrated in graded ethanol (70%-100%) and embedded in MMA (Osteo-Bed, Polysciences).

Undecalcified bone sections (thickness 100 μ m) of the embedded specimens were obtained with a diamond-edged blade in a rotating saw (Leiz 1600) following a section plane perpendicular to the implant long axis.

The histological evaluation was carried out taking into consideration at least six sections of each explanted bone segment at different heights; thus, corresponding sections from the left femur and from the right one at the same heights were compared (20). Controlateral implant allowed a paired evaluation, so that testing and control materials were compared within each animal. Thereby, the biological difference between individuals, which can be significant, was essentially eliminated.

The analysis was performed by means of a stereomicroscope (Nikon SMZ 1000) with combined visualizing systems of polarised and fluorescent light. The stereomicroscope was equipped with a digital camera for microscopic use (Nikon mod. DS-5M) connected to a PC with LUCIA G software, version 4.81 (Laboratories Universal Computer Image Analysis).

The Von Kossa stain technique was performed on 40 μ m-thick deplasticized sections to differentiate mineralized bone, fibrous tissue and osteoid matrix.

3- Results and Discussion

In the first phase of analysis, polarised light was used due to its value in the morphological-structural study of bone tissue. Such analysis provided the following information: the correct position of the implant, which was centrally located within the femur distal epiphysis and surrounded by spongy bone tissue; the contact between bone tissue and the majority of the implant's surface area in both control and testing materials, which showed a good interpenetration degree between the implant and bone tissue.

Bone tissue surrounding the implanted cylinders showed an alternate organization of wavy and parallel fibers. The presence of wavy fibers organization attests new bone deposition, since it represents the first bone tissue forming in areas

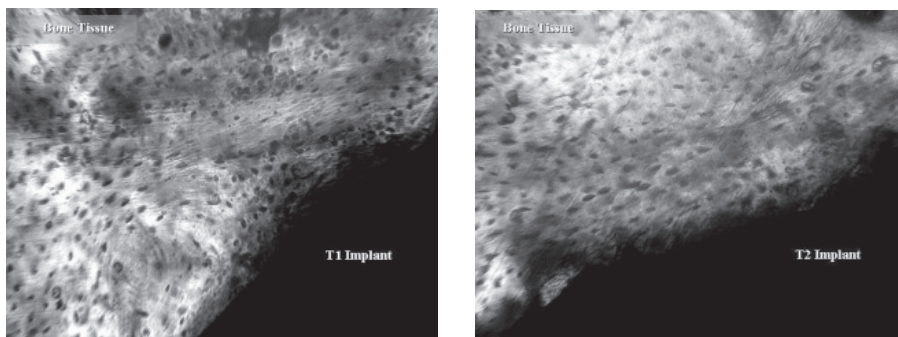
of quick reparation. No postoperative complications were seen.

After performing Von Kossa stain on deplastized sections, no fibrous tissue ongrowth was observed around both testing and control implants.

In the samples T1 and Tc from experimental group of 45 days, there was contact between bone tissue and most of the implant surface; in the samples T2, the contact between bone tissue and implant was discontinuous.

The bone tissue surrounding the implanted cylinders showed an alternate organization of waved and parallel fibers, but a waved fiber organization prevailed at the interface with T1 and T2.

Analysis of samples from experimental group of 90 days showed a more extensive bone deposition around T1 samples than around T2 and Tc. After 90 days, the bone ring around T1 implant was thicker than the rings present around T2 and Tc, and than the ring around T1 after 45 days, too. After 90 days, the ring bone around T2 implant was continuous but thinner than the one around T1. At this experimental time the bone tissue surrounding implanted cylinders showed a prevalent organization of parallel fibers characterized by regular birefringence. We hypothesize that the bone ongrowth could be remodelled after the first phase of osteodeposition.



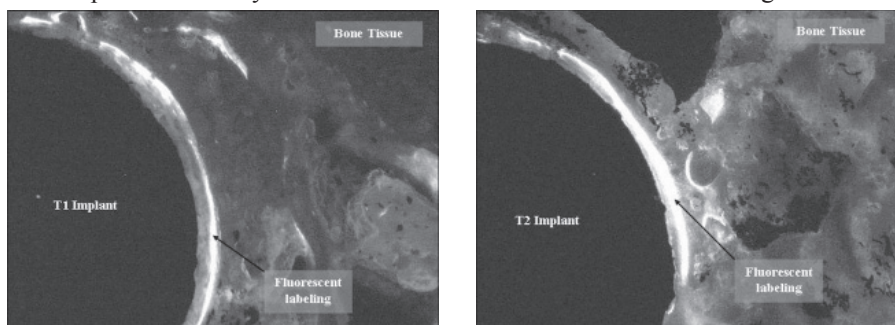
Figs. 1 and 2: *Polarised light histological sections (magnification 100X). Osteointegration of T1 implant (fig. 1) and of T2 implant (fig. 2) after 90 days.*

Also, fluorescent microscopy analysis was carried out on bone sections. Visualising marker fluorescence, it was possible to assess the osteogenic activity of treated materials and control.

CG fluorescence was present and well detectable in the samples of both experimental groups. The analysis of the samples from experimental group of 45 days showed a more extensive osteogenic activity around T1 implant than around T2 and Tc.

Nevertheless, the presence of bone tissue interposed between metal implant and fluorescent lines was observed in T1 samples, while no interposition of bone tissue was detectable at the interface with T2. One might hypothesize that T1 stimulated

bone deposition directly on surface material before fluorescent labelling.



Figs. 3 and 4: Fluorescence microscopy histological sections (magnification 40X). Calcein green labelling in the implant-ongrown bone tissue after 45 days. In fig. 3, fluorescent labelling around T1: an early deposition of unlabelled bone tissue is present between fluorescent line and implant. In fig. 4, fluorescent labelling around T2 showing labelled bone deposition directly on material surface.

The analysis performed on experimental group of 90 days showed a moderate osteogenic activity around T1, T2 and Tc samples .

At the experimental time of 45 days, bone tissue ongrowth between implant and fluorescent line in T2 was thicker than in T1, whereas at the experimental time of 90 days the osteogenic activity presented no significant difference between T1 and T2 .

XO fluorescence was poorly evident in both T1 and T2 and in control. XO low visibility could be due to a reduced osteogenic activity during the labelling period, in both T1 and T2 and in control.

This data implies that osteogenic activity is exerted within a limited time-frame after implant insertion.

4- Conclusion

The purpose of this study was to investigate whether chemically treated rough titanium and bioactive titanium, obtained by BSP treatment, had a positive effect on osteogenesis and bone integration. A positive outcome could justify their further evaluation for clinical applications.

Modifications of titanium surface play a key role in the process of bone to implant integration. In particular, titanium chemical etching by organic acids is able to induce a marked microrugosity stimulating osteoblast colonization (21). An increasing roughness of titanium implant surfaces is positively correlated with the percentage of direct bone-implant contact (12). Titanium biocompatibility can be further improved by biomimetic treatments, in particular the ASD technique, which seems to be a very promising system to obtain a proper morphology and chemical functionalization of the implant surface (1).

According to our findings, both titanium surface treatments could improve the

osteointegration in the healing of bone adjacent to the implant *in vivo*. The chemically treated rough titanium T1 demonstrated to induce an early bone formation in the first phase after implantation and the best bone apposition at the end of the experimental time. On the other hand, electrochemically-treated BSP titanium, T2, showed a good osteointegration, too, despite of the fact that bone deposition was slightly more delayed.

However, after 90 days both T1 and T2 implants achieved a good grade of osteointegration.

Both titanium surface treatments demonstrated that the achievement of a surface with similar morphological characteristics in terms of microroughness, avoiding the problems related to the coatings, could alone represent an interesting element speeding up bone contact to the implant surface.

These results prove that the tested titanium surface treatments may be considered a key type of surface modification for an increased osteogenesis and a strong and durable bone to implant connection in dental and orthopaedic implantology.

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