Biological behavior of sol-gel coated dental implants

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The biocompatibility of dental implants coated with titania/hydroxyapatite (HA) and titania/bioactive glass (BG) composites obtained via sol-gel process was investigated using an *in vitro* and *in vivo* model.

A device for the *in vitro* testing of screw-shaped dental implants was developed, in order to well compare the two experimental models studying the behavior of human MG63 osteoblast-like cells seeded onto a particular geometry. The expression of some biochemical parameters of osteoblastic phenotype (alkaline phosphatase specific activity, collagen and osteocalcin production) and some indications on cells morphology obtained by scanning electron microscopy were evaluated.

The *in vitro* and *in vivo* models were compared after implants insertion in rabbit tibia and femur. The removal torque and histomorphometric parameters (percentage of bone in contact with implant surface and the amount of bone inside the threaded area) were examined.

A good agreement was found between the *in vitro* and *in vivo* models. These experiments showed better performances of HA and BG sol-gel coated dental implants with respect to uncoated titanium; in particular, it was found that *in vitro* the HA coating stimulates osteoblastic cells in producing higher level of ALP and collagen, whereas *in vivo* this surface modification resulted in a higher removal torque and a larger bone-implant contact area.

This behavior could be ascribed to the morphology and the chemical composition of the implants with rough and bioactive surfaces.

Introduction

The knowledge of events occurring at the bone-implant interface (reviewed by Puleo and Nanci [1]) has lead to find out strategies to shorten the healing time, producing a stable bone-implant fixation through the development of a matrix at the interface with bone-like compositional, structural and biomechanical properties.

The performance of an implanted device depends on several factors such as the material, the shape and in particular the surface (chemistry, morphology, crystallinity, topography, hydrophobicity, surface charge density, surface free energy) [2–5].

The development of bioactive materials, hydroxyapatite, Bioglass® and calcium phosphate ceramics [6–9] is essential to the achievement of a bone-implant integration. The sol-gel process is one of the surface modification techniques available for obtaining well-defined surfaces [10–13] from existing biomaterials. In particular, the sol-gel process is a simple and low cost method, that allows to obtain coatings of micrometer dimension, with a high degree of purity and homogeneity and low processing temperatures. Moreover, it has been reported as to yield bioactive surfaces [14–17]. In our laboratories we have prepared and characterized coatings of a titania matrix encapsulating hydroxyapatite (HA) and bioactive glass (BG), respectively, deposited on titanium via sol-gel as reported elsewhere [18, 19].

In vitro studies on biocompatibility [19–22] demonstrated that these materials are very promising for the development of more effective orthopaedic and dental implants. Moreover, sol-gel coatings showed better biological performances than the ones obtained via

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sputtering and plasma-spray [23]. In this work, two different surfaces of dental fixture coated via sol-gel with HA and BG, respectively, were investigated by means of *in vitro* and *in vivo* tests. Then, some uncoated titanium cp (Ti) implants were included as a control.

In vitro tests were performed using the human osteoblast-like MG63 cell line; the expression of some biochemical parameters of osteoblastic phenotype (alkaline phosphatase activity, collagen and osteocalcin production) and some indications on cells morphology were evaluated by means of scanning electron microscopy.

Generally, for *in vitro* models it is necessary to employ biomaterials with planar geometry to seed the cells onto. In order to well compare the biocompatibility of screw-shaped dental implants by means of *in vitro* and *in vivo* experimental models, a device has been developed to allow the *in vitro* seeding of the cells onto substrates with a particular geometry.

The *in vitro* results have been compared with the *in vivo* responses after implants insertion in an animal model (rabbit), evaluating the removal torque (the force required to loosen the screws from the bone bed) and the histomorphometric parameters (percentage of bone in contact with implant surface and the amount of bone inside the threaded area).

Materials and methods

Materials and sample preparation

Commercially pure grade 2 titanium (Goodfellow, Germany) was used for the production of implants by means of a numeric control microscrew machine (AM-2000, Freer Engineering, USA). All implants were screw-shaped with a nominal outer diameter of 4.1 mm. The pitch height was 1.83 mm and the length was 10 mm. The cleaning was performed in an acetone bath, using an appropriate tool for the cavity cleaning, followed by an ultrasonic rinse in acetone for 20 min, in 70% ethanol solution for 20 min and then in distilled water for 15 min.

Before the coating process, the implants were subjected to chemical etching, dipping the implants thread in a HF 20% solution for 1 min, followed by a HNO_3 30% solution for 1 h. After this treatment, the implants were coated with hydroxyapatite/titania (HA) and bioactive glass/titania (BG) composite coatings by sol-gel process.

All coatings resulted to have a thickness $< 10\,\mu m$ [18] that does not influence the final implant diameter.

Hydroxyapatite/titania coating (HA)

The coating preparation has been reported elsewhere [18]. In short, hydroxyapatite powder (Fin Ceramica, Italy) was added to anhydrous ethanol in the same ratio. A titania solution was prepared by mixing titanium isopropoxide, acetyl acetone, nitric acid, n-propane alcohol and distilled water. Hydroxyapatite and titania solutions were mixed in the ratio 1 : 1 (w/w) and used for spray deposition by means of an aerograph. The implant was fixed to the mandrel of a rotating system and the aerograph was oriented during the rotation phase perpendicularly to the lateral surface of implant and then according to the inclination angle of the screw thread (43° and 17°). After the coating deposition in the indicated directions for 2 s each, the sample was dried at 80 °C for 30 min and sintered at 500 °C for 30 min. This process was repeated three times for every implant. Operative parameters, such as the nozzle hole, the substrate-aerograph distance, the exposure time of the substrate to the sprayed solution and the number of treatments (i.e. the number of deposition cycles, drying and sintering process) were optimized in order to obtain a complete and uniform coating of the substrate.

Bioglass/titania coating (BG)

Bioactive glass powder (with composition SiO_2 65%, CaO 31% and P_2O_5 4%) was prepared via sol-gel in the laboratories of PASTIS-CNRSM, using as precursors tetraethyl orthosilicate (TEOS), calcium nitrate and phosphoric acid, respectively. Three solutions were prepared: TEOS in ethyl alcohol in the ratio 3 : 1 w/w (solution A); phosphoric acid in distilled water and ethanol alcohol in the ratio 1 : 5 : 2.5 w/w (solution B); calcium nitrate in distilled water in the ratio 1 : 1 w/w (solution C). Solutions A and B were mixed and stirred for 30 min; then, the solution C was added and stirred for 1 h. The final solution was dried for 20 h at 60 °C and then subjected to a thermal cycle: 5 h at 90 °C, 5 h at 180 °C and 3 h at 600 °C. After the cooling, the sample was milled and sieved to provide a particles size distribution between 45 and 24 μm.

Bioactive glass powder was added to distilled water (ratio 1 : 1). The sol-gel solution was prepared mixing the bioactive glass with titania solution (reported above) in the ratio 1 : 1 in an ultrasonic bath for 30 min and deposited on the implants as described for the HA coating.

Surface topographical analyses

TopScan 3D® (Heidelberg Instruments, Germany) was used for the topographical description of the three different surface modifications used in the present study (as-machined, HA and BG coated implants). This measuring equipment is based on the confocal principle. A laser beam is situated below the surface to be measured, so allowing measurements of arbitrary shaped objects. During scanning the objective is moved along the X, Y and Z axis. The movement along the X and Y axis is controlled by a precision scanning mechanical unit and the movement in Z axis by a piezoelectric motor. The maximum measuring area is 2×2 mm. The maximum sampling points in the X and Y axes are 512×512. The minimum sampling interval is 1 μm. Possible vertical resolution is 6 nm. Three screws were topographically characterized for each surface modification. Each screw was measured on nine sites of the threaded area, three tops, three valleys and three flanks.

In vitro evaluations

Cell cultures

MG63 osteoblast-like cells (ATCC, USA), originally isolated from a human osteosarcoma, were cultured in

Dulbecco Modified Eagle's Medium (DMEM, Biowhittaker, Belgium), containing penicillin/streptomycin (100/100 U), amphotericin B (2.5 μg/ml) and gentamycin (100 μg/ml), supplemented with 10% foetal calf serum (Euroclone, UK), and kept at 37 °C in an atmosphere of 5% CO_2 and 99% humidity. Media were changed every three days.

Experimental design

Osteoblastic cells were suspended at a concentration of 4.0×10^5 cells/ml in the complete culture medium containing sodium ascorbate (50 μg/ml) and β-glycerolphosphate (10 mM) and put in vials containing 5 ml each. The implants, fixed on the middle of the vials cap by means of suitable adapters, were inserted in the vials and sealed with O-rings. A rotation device, consisting of one horizontal rotation axis with four housings for the vials, was developed in our laboratory. In order to ensure an even distribution of osteoblastic cells on the implants surface, the vials were rotated at a velocity of one rotation per hour for 24 h at 37 °C. Then, the implants were incubated for another eight days. After the incubation period, the biochemical parameters were evaluated on cell lysates, obtained detaching the cells from implants surface with the addition of trypsin/EDTA solution for 10 min and repeating the treatment a second time. As the reaction was interrupted by the addition of DMEM containing 10% serum and the centrifugation, the pellet was suspended again in 1 ml of sterile distilled water, sonified for 10 s and subjected to three freezing cycles at − 80 °C for 20 min followed by thawing at room temperature for 30 min to lyse the cells.

Alkaline phosphatase activity (ALP) was evaluated from the transformation of p-nitrophenylphosphate into p-nitrophenol at 37 °C and pH 10.2 using appropriate reactives (Sigma, USA), while the specific activity was calculated with regard to the protein concentration of lysates determined by means of a commercially available colorimetric assay (BioRad, Germany). The collagen assay was based on the binding of the dye Sirius Red F3B (BDH, UK) to the triple helical collagen fibril [24].

The production of osteocalcin from cultures in the cell medium was measured by a commercially available enzyme immunoassay (BioResearch Ireland Diagnostics, Ireland) employing highly specific monoclonal antibodies and peroxidase-labeled osteocalcin.

For SEM observations, samples were fixed with 2.5% glutaraldehyde in phosphate buffer and dehydrated through a graded series of ethanol. Then the specimens were critical point dried (Bal-Tech, Liechtenstein), sputter-coated with gold and examined with a scanning electron microscope (Philips XL 20) at 25 kV acceleration voltage.

Three replicates were performed for each experiment and at least three samples of the same typology were included in each experimental run.

Results were expressed as mean values ± standard deviation. Comparison were made by ANOVA and *t*-test analysis by a two population comparison. Statistical significance was considered at a probability $p < 0.05$.

In vivo evaluations

Animal and anaesthesia

A total of 10 New Zealand White rabbits have been used for the experiment. These animals, who were all adult females, were kept in one specially designed room with a free access to tap water and fed with standard pellets. Immediately after operation, the rabbits were kept in separated cages for a better monitoring of the healing process, and put together in the specially designed room as soon as they recovered.

During surgery, general anaesthesia was obtained with intramuscular injections of Hypnorm® (Janssen Pharmaceutical LTD, Oxford, England) and intraperitoneal injections of Apozepam® (Apothekernes Laboratorium, Norway). The hind legs were shaved and antibiotics were administered prophylactically. Immediately before surgery 1 ml Xylocain® (Astra, Sweden) was injected to each insertion site.

Surgical technique and implant insertion

A total of 60 implants of which 25 HA coated, 25 BG coated plus 10 titanium ones (used as a control), were used for *in vivo* experiment. All implants were sterilized in an autoclave before surgery. The operation was performed under aseptic condition, the holes were drilled with low rotatory speed and under copious irrigation of saline. The screw holes were tapped to a final diameter corresponding to the implant diameter. A total of six implants were inserted in each rabbit. One implant was inserted in the distal femoral metaphysis and two were inserted in the proximal tibial metaphysis. Each animal served as its own control. A comparison of torque measurements and histomorphometrical evaluations was performed between implants inserted in the left and right legs of the same animal. In Table I is reported the schedule used for implant insertion. Two animals had to be killed after 4.5 weeks due to an infection occurred on the operated area. After 12 weeks of healing period, the remaining eight rabbits were killed with an overdose of barbiturate.

Removal torque

The implants inserted in the left and right femur and in the left proximal tibiae (24 in total) were evaluated with peak removal torque, in order to get information on the force needed to loosen the screws from the bone bed.

Histomorphometry

The remaining 36 tibial implants (left distal and right proximal and distal implants) were removed en bloc, fixed in 4% neutral buffered formaldehyde, dehydrated in alcohol solutions and embedded in light curing resin (Technovit 7200 VLC, Kultzer & Co, Germany). An Exakt™ sawing and grinding machine was used to prepare sections for histomorphometrical analyses. These sections (one central section per implant) were examined with a light microscope with regard to the bone-metal contact and the amount of bone inside the threaded area.

Results were expressed as mean values ± standard

TABLE I Schedule of implants insertion in the femur and tibia of each animal

Animal	Femur		Tibia			
	Left	Right	Left prox	Left dist	Right prox	Right dist
1	BG	HA	BG	CTR	BG	HA
2	BG	HA	BG	HA	BG	CTR
3	BG	HA	BG	HA	CTR	BG
4	BG	HA	BG	BG	HA	CTR
5	BG	HA	BG	CTR	HA	BG
6	HA	BG	HA	BG	CTR	HA
7	HA	BG	HA	BG	HA	CTR
8	HA	BG	HA	CTR	BG	HA
9	HA	BG	HA	HA	CTR	BG
10	HA	BG	HA	CTR	BG	HA

BG = Bioactive glass, HA = Hydroxyapatite, CTR = control (Titanium).

deviation. Statistical analyzes were performed using Wilcoxon signed rank test (femur) and Mann W u-test (tibia). Statistical significance was considered at a probability $p < 0.05$.

Results

Surface topographical characterization

The surface of titanium implant had the smallest height deviation surface (Sa) among the investigated surface modifications, but had also the most dense structure as evidenced by the parameter Scx. The increased surface area was 17.74% for the titanium surface and 14.73% for the bioactive glass, while the corresponding value for the hydroxyapatite coated surface was 16.33%. However, the standard deviation of the titanium surface was greater with respect to the other two surface modifications investigated (Table II).

In vitro evaluations

The evaluation of some biochemical parameters regarding osteoblasts differentiation is reported in Fig. 1. Alkaline phosphatase specific activity of the osteoblasts grown onto HA and BG coatings was significantly higher with respect to the uncoated titanium implant. The collagen synthesis was higher in HA coating whereas it was similar in titanium and BG coating. On the contrary, the osteocalcin production appeared higher in BG coating and lower in HA coating when compared with titanium implant.

SEM analysis showed a uniform distribution of osteoblasts grown on the threaded area of implants with seeded cells, both on the tops and on the valleys and flanks. After nine days of incubation, the osteoblasts grown on the implants developed a very close layer (Fig. 2). The cells grown onto titanium implant were oriented parallel to the grooves of the substrate. The cells seeded onto HA and BG coatings were not oriented and seemed to follow the substrate topography, characterized by numerous particle aggregations.

In vivo evaluations

The experimental design was based on a 12 week follow-up time. However, two animals had to be killed 4.5 weeks after implant insertion due to a severe infection occurred in the soft tissue covering the left tibial implants. The implants inserted in these two rabbits (for a total of 12 implants) were removed together with the surrounding tissue, and sections for histomorphometric evaluation were prepared as described earlier. However, the data were not included in the statistics. Yet another animal developed an infection over the tibial implant during the healing phase. However, this infection was treated with antibiotics and the animal recovered.

As far as the torque analysis is concerned, the fracture occurred at the bone-implant interface. No bone was visible at the implant surface. The removal torque used to loosen the hydroxyapatite coated implants from the bone bed in femur and in tibia was significantly higher with respect to the BG coated implants. In the femur a force of 81 N cm was needed for the HA coated implants and of 53 N cm for the BG coated implants, while in the tibia the corresponding values were 73 N cm and 61 N cm, respectively (Table III).

As far as the histomorphometrical analysis is concerned, the HA surface was found to have the highest percentage of bone in contact with the implant surface after a healing period of 12 weeks, in the measure of 29% against 24% of the BG surface and 17% of the titanium surface. The difference was significant between HA

TABLE II Surface roughness described by one height descriptive parameter Sa, one spatial descriptive Scx and one parameter the increased surface compared with a total flat reference area. The values are a mean of 18 measurements each. A gaussian filter sized 50 × 50 μm was used to separate roughness from errors of form and waviness before evaluation

Surface modification	Sa	Scx	Sdr
Titanium	0.64 ± 0.41	10.33 ± 1.37	17.74 ± 15.92
Hydroxyapatite	0.76 ± 0.17	12.55 ± 1.27	16.33 ± 5.18
Bioactive glass	0.70 ± 0.16	13.88 ± 2.22	14.73 ± 4.91

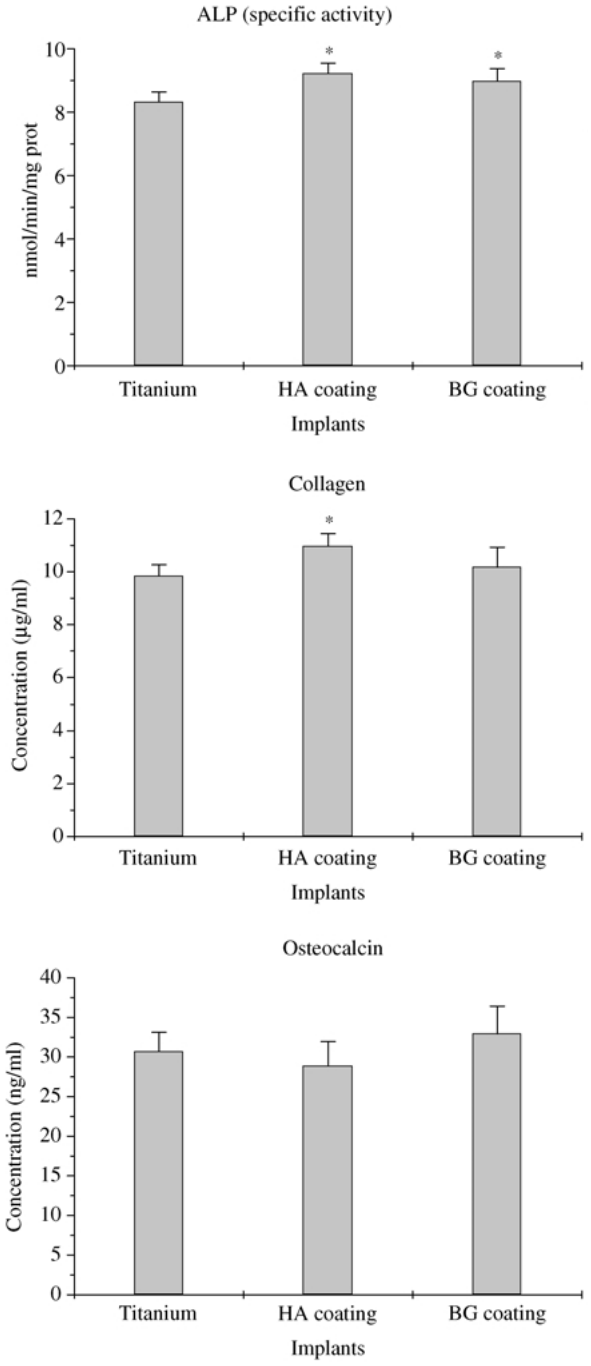

Figure 1 Evaluation of biochemical parameters (ALP, Collagen and Osteocalcin) obtained by MG63 osteoblastic cells cultured onto Titanium, HA and BG coatings. The data were expressed as mean ± standard deviation. Difference from titanium: $*p < 0.05$.

TABLE III Peak removal torque (N cm) value as measured in eight animals after 12 weeks of healing

Femur		Tibia	
HA	BG	HA	BG
81 ± 17	53 ± 7	73 ± 22	61 ± 29

Femur $p = 0.0178$ (Wilcoxon).
Femur + tibia $p = 0.001$ (Mann W. u-test).

coated and the titanium control fixtures. The amount of bone inside the threaded area was the highest for the titanium surface and the lowest for the HA surface (Table IV); however, the difference was significant only between the control and the HA coated implants. After

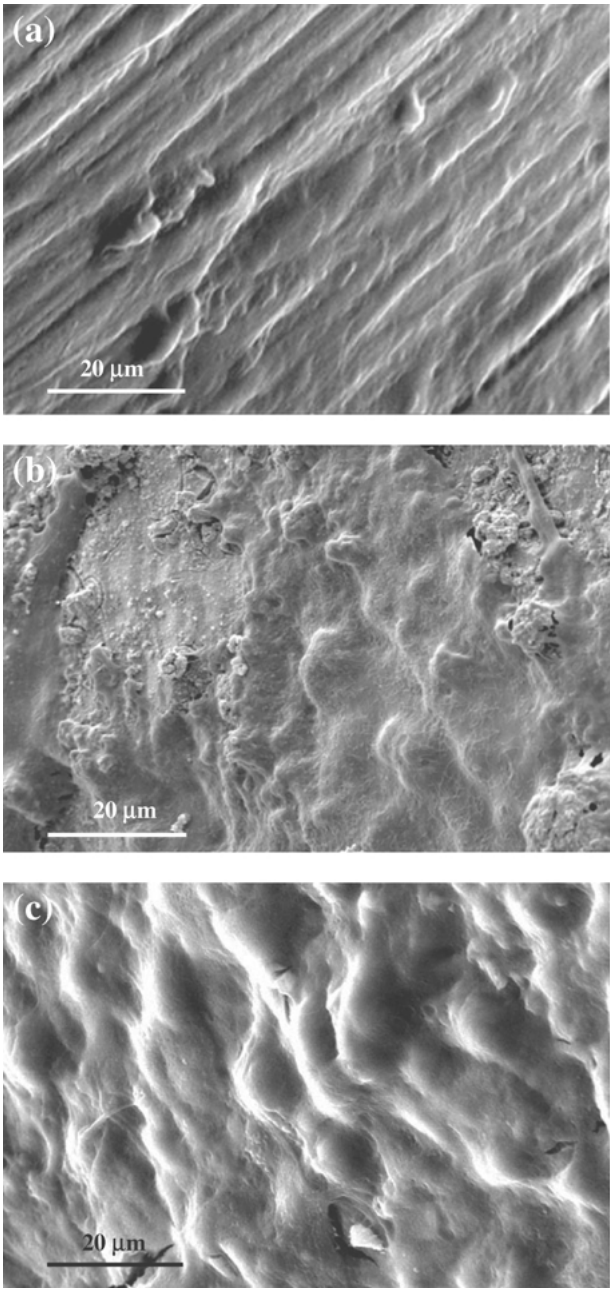

Figure 2 SEM images of MG63 osteoblastic cells grown on Titanium (a), HA coating (b) and BG coating (c). Magnification 1000 × .

4.5 weeks the BG surface showed a slightly larger bone-implant contact area and a higher percentage of bone inside the threaded area with respect to the HA surface, while for the titanium surface was recorded the lowest percentage either of bone contact and bone presence inside the threaded area at this early stage of healing (Table V).

Discussion

The *in vitro* evaluations showed that HA and BG coatings prepared via sol-gel allowed the differentiation of MG63 osteoblast-like cells. In fact, the coatings stimulated the cells in producing ALP, an early marker of osteoblasts differentiation, more than uncoated titanium. Moreover, it was noted on one hand an increase of the collagen synthesis from the cells grown onto HA coating, and on the other hand an increase of the osteocalcin

TABLE IV Bone to implant contact (BIC) and amount of bone inside the threaded area (Area), in percent, measured on both sides of each implant, after 12 weeks in rabbit bone. Eight rabbits are included and three implants in each rabbit positioned in left (one implant) and right tibia (two implants)

Control surface (Titanium)		HA surface		BG surface	
Area%	BIC%	Area%	BIC%	Area%	BIC%
60 ± 12	17 ± 7	40 ± 11	29 ± 7	49 ± 6	24 ± 13

p values BIC: Control vs. BG 0.4285 (NS); Control vs. HA 0.0044; BG vs. HA 0.0822 (NS).
p values area: Control vs. BG 0.1261 (NS); Control vs. HA 0.0074; BG vs. HA 0.0655 (NS).

TABLE V Bone to implant contact (BIC) and amount of bone inside the threaded area (Area), in percent, measured on both sides of each implant, after 4.5 weeks in rabbit bone. Two rabbits are included. A total of eight implants are positioned in left (four implants) and right tibia (four implants), whereas a total of four implants are positioned in left (two implants) and right femur (two implants)

Bone localization	Control surface (Titanium)		HA surface		BG surface	
	Area%	BIC%	Area%	BIC%	Area%	BIC%
Tibia	29 ± 10.6	7 ± 3	30 ± 11	15 ± 4	32 ± 7	16 ± 6.4
Femur	—	—	50 ± 6	17 ± 1	45 ± 2	14 ± 1

production from the osteoblasts seeded onto BG coating, even if not significant.

The morphology of osteoblasts grown on these substrates for nine days and visualized by SEM images can be related to the behavior of cells grown on plane samples for two–four days and previously analyzed by confocal laser scanning microscopy [22]. The cells grown onto titanium were flatted and oriented parallel to the grooves of the substrate, whereas the cells seeded onto sol-gel coatings were not oriented and seemed less spread, depending on different topography of the substrate. The 3-D reconstruction of images [22] showed that the bioactive coatings develop osteoblast clusters. Cell aggregation is an early and critical event leading to cell differentiation and mineralization process and could be a first signal of the tendency of bioactive coatings to stimulate cell differentiation [25].

In vivo evaluation showed that the HA coated implants needed a significantly higher torque to loosen implants from the bone bed with respect to the BG glass coated implants. This was the case for femur and for tibia although the number of tested implants in tibia was rather small. From a histomorphometrical point of view, the bone-implant contact area was larger for the coated surfaces than for titanium control implants, again only significant when the comparison was made between HA and control implants. Thus, the results achieved from the removal torque test and histromorphometry are in good agreement with the *in vitro* evaluations. However, with regard to the bone area calculation (i.e. bone percentage inside the threads) the presence of bone was higher in control implants than in HA coated implants. No other statistically significant data were observed for the evaluation of the bone area. The contradictory results obtained for the bone area and the bone-implant contact have been already reported elsewhere [26–28]. An hypothesis not yet proven could be that the presence of enough bone-implant contact can make the filling of the thread area unnecessary.

The *in vitro* and *in vivo* experiments showed better performances of HA and BG sol-gel coated dental implants with respect to uncoated titanium; in particular, the HA coating was found to stimulate osteoblastic cells in producing a higher level of ALP and collagen. This behavior could be ascribed to the morphology and the chemical composition of the implant surface. Previous studies [18] have reported that the HA coated samples have a uniform, clean and adherent coating with a well defined thickness and phase composition, a rough surface with numerous particle aggregations, and are not cytotoxic [20, 21]. Moreover, both HA and BG coatings have a higher height deviation surface (Sa) and a less dense structure (Scx parameter) in comparison with uncoated titanium implants.

It has been demonstrated that surface and roughness influence cell and tissue responses to implants. In fact, the osteoblastic cells attach more rapidly to surfaces with rougher microtopography increasing the bone apposition [4, 29, 30]. In addition, topography strongly affects the response of the body to the implant surface [31].

As far as the chemical composition is concerned, authors have demonstrated [32, 33] that titania coatings deposited with the sol-gel process are bioactive, improving the calcium and phosphate precipitation onto the surface, due to the presence of hydroxyl groups [33, 34]. Ti-OH groups were detected on HA coating prepared in our laboratory [18] and could be responsible for calcium and phosphate nucleation; an evidence is given by the decrease of calcium concentration in the medium containing HA coating and the appearance of a fine structure with needle-like morphology on the coating surface containing calcium and phosphorous [20].

Therefore, the surface roughness and the chemical composition of coatings, and in particular the one containing hydroxyapatite, could promote the interactions with cells and encourage a stable bone-implant fixation.

Conclusions

A device for the *in vitro* biocompatibility testing of screw-shaped dental implants was developed in order to obtain an early model able to provide some previsions on

the *in vivo* later mineralization process and to determine the long-term stability of the implanted prosthesis.

A good agreement was found between the *in vitro* and *in vivo* evaluations. These experiments showed better performances of HA and BG sol-gel coated dental implants with respect to uncoated titanium; in particular, it was found that *in vitro* the HA coating stimulates the osteoblastic cells in producing higher level of ALP and collagen, whereas *in vivo* it has a higher removal torque and a larger bone-implant contact area.

This behavior could be ascribed to the morphology and the chemical composition of the implants with rough and bioactive surfaces.

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