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Correlation of in vitro and in vivo results of vacuum plasma sprayed titanium implants with different surface topography

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Abstract Research has proven that rough surfaces improve both biologic and biomechanical responses to titanium (Ti) implants. The purpose of this study was to evaluate the correlation between the expression of bone cell-associated proteins to Vacuum Plasma-Sprayed Titanium implants (VPS-Ti) with different surface textures in vitro and the bone integration in vivo. The biological performances of the surfaces were evaluated over a period of 8 weeks using human bone marrow cell cultures and Göttinger mini pigs. Cells were cultured on VPS-Ti with two respectively different surface-roughnesses (Ra). The level of Osteoprotegerin (OPG), Osteocalcin (OC) and alkaline phosphatase activity (ALP) were evaluated. The bone integration in vivo was evaluated by histomorphological analyses. A cancellous structured titanium (CS-Ti) construct was used as reference material in both study designs. Comparison of data was conducted using the Scheffé tests and the paired *t*-test with Bonferroni's correction. A comparative analysis was done to measure the degree of association between the in vitro and in vivo data. A total amount of OC was significantly increased for VPS-Ti for cells cultured on both VPS-Ti and CS-Ti, while OPG was only detectable after 8 weeks without any significant differences. The ALP activity on all surfaces was not statistically increased. For VPS-Ti with Ra ranging from 0.025 mm up to 0.059 mm, bone integration response was increased, but there was no statistical difference between the VPS-Ti. Expression of OPG, OC and ALP correlated with the histomorphological data over the 8-week period. The in vitro data suggest the superiority of VPS-Ti over CS-Ti, but more importantly, the biocompatibility of testing an in vitro model to predict the outcome and possible integration of implants in vivo.

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Introduction

The beginnings of orthopaedic implant research can be traced back to the sixteenth century. The first internal bone fixation was done by Hansmann et al. in 1886 [16]. In 1890, the first knee endoprosthesis, made from ivory, was implanted by Gluck [13]. During the decades and centuries that ensued, experiments were done with different materials and implants, where chromium–nickel–steel alloys, cobalt alloys and titanium alloys in particular provided new possibilities.

The steadily increasing number of patients who are in need of a partial or total joint replacement is reflected in the rapid development and research of orthopaedic implant materials for clinical application of orthopaedic implant material. Today, research focuses on the development of implant materials that have osteoconductive and osteoinductive abilities. It has been reported that titanium possesses an excellent in vitro and in vivo biocompatibility that allows osseointegration or direct bone-to-implant contact [5, 25, 29]. Several methods for modifying the implantation surface such as surface machining, acid etching, electro polishing, anodic oxidation, sand blasting or plasma spraying have been used to improve the osseointegration of titanium implants [1]. In vitro studies show that roughness of surfaces influence basic biological responses and that the cell response is improved by

roughened titanium surfaces [3, 26]. Wennerberg et al. reported that rough implants have greater bone contact compared with a turned surface and that a surface blasted with 75 μm showed more bone contact than a surface blasted with 25 μm or 250 μm [34]. In vitro studies have also indicated that rough surfaces would favour the development of cell activities, which increases cell attachment [2, 19]. Collagen synthesis, production of ECM, expression of cytokines, growth factors and bone-like formation are improved by rough surfaces [26].

However, differences in the cell origins of experimental animals and varying methods make it difficult to make direct comparisons of the results.

Human bone marrow cells were used as the culture medium [9, 10, 36]. The culture contains stem cells that have the ability to differentiate into various cell types depending on the culture condition.

Animals used for biocompatibility comparison of integration responses to orthopaedic implant materials are mice, rats, dogs, rabbits, pigs, sheep and different kinds of primates [6, 12, 14, 17, 18, 22, 23, 27, 30, 33]. In long-term investigations of the toxic effects, changes in the tissue morphology and the carcinogenic potential may be evaluated.

The "Göttinger" mini pig was chosen because of several favourable experiences in the past and because the protein structure and bone biology is comparable to humans' [11, 28, 35, 37]. The histomorphological analyses were done upon the euthanized animals.

New implant surfaces have been developed with the purpose of avoiding stress shielding as much as possible, thus prolonging the lifespan of the prosthesis. Presently, there is still a lack of information on the in vitro and in vivo behaviour of Vacuum Plasma-Sprayed Titanium implants (VPS-Ti).

The purpose of this study is to compare the in vitro effect of an ultra-high roughness and open-porous titanium surface (Ra = 0.096 mm) in comparison with medium-level (Ra = 0.025 mm) and high-level (TI60, Ra = 0.059 mm) roughness and open porous coatings such as hydroapatite (HA).

Finally, a comparative analysis was conducted in order to establish a possible correlation between in vitro and in vivo data.

Methods

Implant materials

The implant core consisted of commercially pure titanium. The implants measured 20 × 14 × 2 mm. Sulzer Metco (Germany) fabricated the surface modifications. The modified implants were supplied in individual gamma-sterilized surgical packs. The surface topography and roughness of the VPS-Ti-Ti implants was determined using a FRT MicroProf® and evaluated by the software package FRT Mark III V3.7.16.1. The FRT MicroProf® works as an optical profilometer (2D) as well as an imaging measuring instrument (3D). The measuring range of the sensor runs from 300 μm up to 3 mm with a cut-off at 2.5 mm. Three measurements were done and mean values were calculated.

Cancellous structured titanium (Trademark of Centerpulse Inc., Austin, Texas)

Cancellous structured titanium (CS-Ti) was used as a reference material. The material thickness of the 55% porosity was approximately 1,000 μm with an average pore size of 500 μm, maximal implantation thickness of 3.00 mm; maximal roughness measuring at 0.836 ± 0.1629 mm with a mean roughness of 0.096 ± 0.0147 mm).

VPS-Ti (Sulzer Metco, Wohlen, Switzerland)

The thickness of the vacuum plasma sprayed titanium surface (ISO-5832-2; degree 4) was approximately 700 μm. Two types of VPS-Ti were evaluated and used in this study, VPS-Ti-25 and VPS-Ti-50; and these differed in pore size and porosity (Figs. 1–3).

- VPS-Ti-Ti-25 had an average pore size of 500 μm with a porosity of 25%. (overall implantation thickness 2.50 mm; maximum roughness: 0.191 ± 0.01417 mm; roughness average: 0.025 ± 0.001 mm)
- VPS-Ti-Ti-50 had a pore size of 200 μm with a porosity of 50%. (overall implantation thickness 2.50 mm; maximum roughness: 0.463 ± 0.0953 mm; roughness average: 0.059 ± 0.0025 mm)

In vitro study design

Cell isolation and culture

Human bone marrow was taken under sterile conditions from the metaphyseal portion of the proximal femur during total hip arthroplasty (Performed according to the guidelines for experimentation with human cells and approved by the ethical commission AZ 44/99) from ten healthy donors (four male and six female patients; age range of 45–70 years).

Donors with acute chronic infections or malignant diseases were excluded. Donors included in the study were 45–70 year old Caucasian males or females who were designated for autologous transfusion and bone banking.

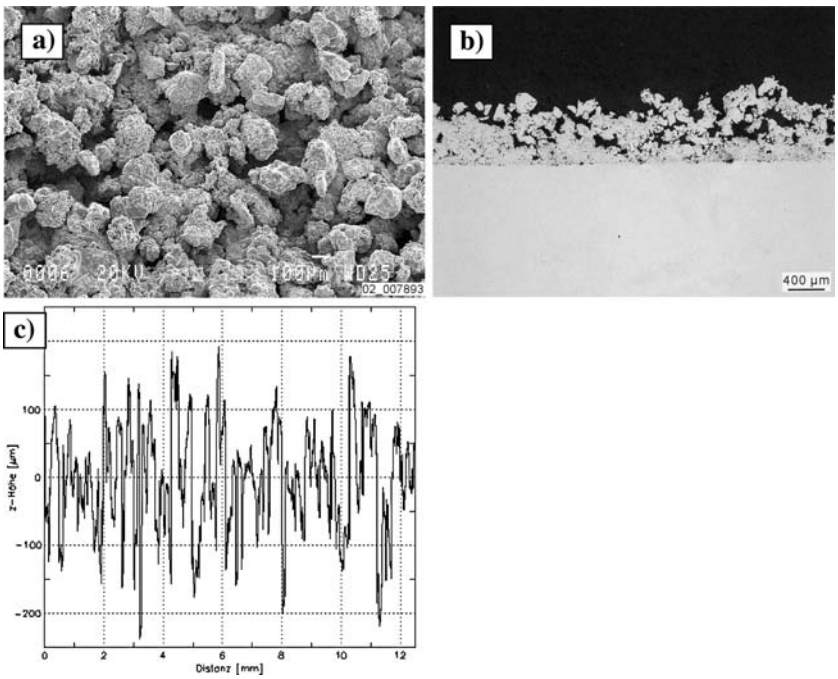


Fig. 1 (**a**) SEM of the surface of VPS-Ti-50; (**b**) lateral view of VPS-Ti-50; (**c**) example of the determination of the roughness (FRT Mark III)

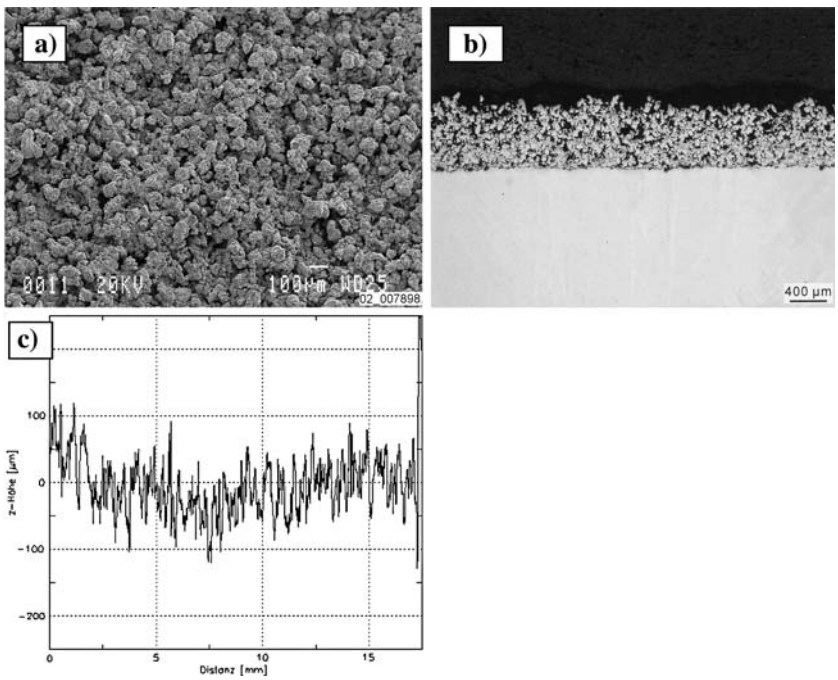


Fig. 2 (**a**) SEM of the surface of VPS-Ti-25 (**b**) lateral view of VPS-Ti-25; (**c**) example of the determination of the roughness (FRT Mark III)

The human bone marrow cells were isolated by differential centrifugation as described previously [9, 10, 36]. Afterwards, the cells were suspended in culture dishes with 1.5 mL medium. One and a half million cells suspended in 1.5 mL of medium were planted on each implant (6 well bottom 9.81 cm^2) and incubated at 37 °C, 12.5% O_2, 5% CO_2 and 0% N_2 over a period of 8 weeks.

The supernatants were sampled and centrifuged to remove any debris and non-adherent cells every 48 h. All donors were individually tested and the results were expressed as the mean of each of these values. Supernatants were stored at –80 °C for less than 3 months before being tested for the mediators, using immunoassays. Alkaline phosphatase activity was tested immediately.

Medium

IMDM (Iscove's modified Dulbecco's Medium) was supplemented with 12.5% foetal calf serum, 12.5% horse

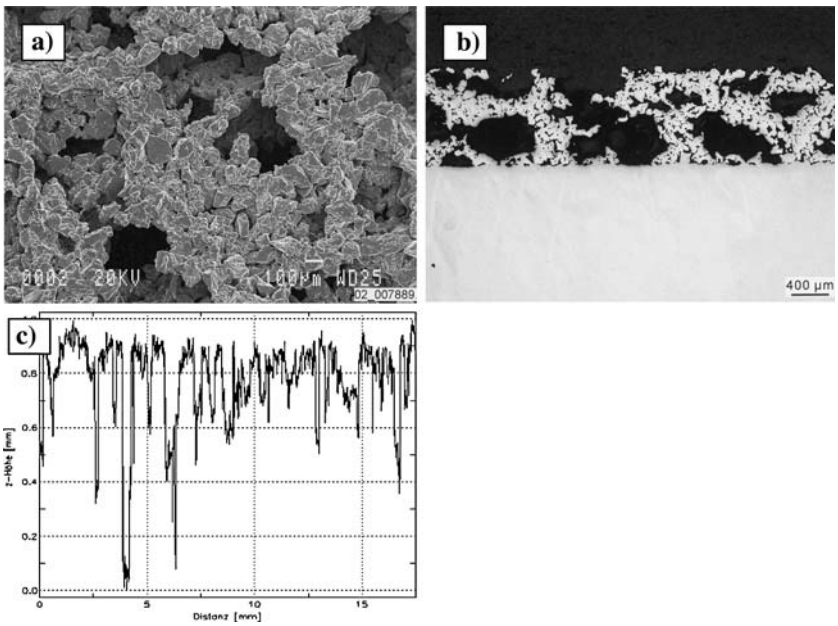


Fig. 3 (**a**) SEM of the surface of CS-Ti; (**b**) lateral view of CS-Ti; (**c**) example of the determination of the roughness (FRT Mark III)

serum, 2.4 ng/mL hydrocortisone, 50 ng/mL certomycin, 292.2 ng/mL L-glutamine, 0.3% $NaHCO_3$. As growth factors for cell stimulation, interleukin 3 [IL-3] (Behring AG, Marburg, FRG) and granulocyte-macrophage-colony-stimulating-factor [GM-CSF] (Behring AG, Marburg, FRG) in concentrations of 10.0 ng/mL medium were added when the supernatants was re-evaluated every 2 days.

Osteocalcin, Osteoprotegerin measurement and determination of alkaline phosphatase activity

The levels of osteocalcin (OC) were measured using commercially-available immunoassay kits purchased from DPC Biermann, Germany (Immulite). Osteoprotegerin (OPG) was measured using a commercially-available ELISA (enzyme-linked immunosorbant assay) from Immundiagnostik (Bensheim, Germany). The activity of alkaline phosphatase was measured using a standardized photometric method in the Department of Clinical Chemistry and Molecular Diagnostics—Central Laboratory—of the University of Marburg, Germany.

CLSM

After 8 weeks, the implants were fixed with acetone for 10 min at –20 °C and washed three times with TBS. The implants then were incubated with anti-h-collagen I antibody (Biodesign, Germany—rabbit) for 20 h at a temperature of <8 °C. A second anti-h-collagen III antibody (Biodesign, Germany—mouse) was applied and re-incubated for 20 h at <8 °C.

Anti-mouse-FITC and anti-rabbit-RedX conjugated antibodies were admitted and each incubated for 1 h at room temperature. Finally, the implants were fixed with glycerine-gelatine.

Some implants were used as negative controls and only processed with anti-mouse-FITC and anti-rabbit-RedX conjugated antibodies without being incubated with the anti-h-collagen antibodies as previously described.

The analysis was done by using CLSM (Leica DMRXE, Leica TCS SP) and evaluated by the software package Leica TCS NT, version 1.6.587.

In vivo study design

Experimental animals

A total of 14 male Göttinger mini pigs with a mean age of 25 months (range 12–40 months) and a mean weight of 50.2 kg (range 42–66 kg) [11, 28, 35, 37] were purchased from Ellegard (Göttingen, Germany). The pigs were acclimatized for 1 week under standard stable conditions (25 m^2). Drinking water and commercial pig feed were available ad libitum throughout the 12-week study. No morbidity was induced by the research protocol. The experimental protocol was approved by the ethics review committee for animal experiments (Ref. 17a-19cj20-15(1)-MR20-1/93).

Surgical technique

The implantation was performed under general anaesthesia induced by intraperitoneal injection of Hypnomidate® and intravenous injection of Narcoren®. The lateral surface of

proximal femurs metaphysis were exposed on both sides. Proximal longitudinal grooves were prepared using a specially-designed drill. The dimension of the final groove was slightly smaller than the dimension of the implants, and the implants were inserted with an initial press-fit. The implantation site and procedure provided initial contact between bone and the plasma-sprayed surface of the implants. The grooves were prepared in a reproducible fashion by using a dental drill constantly irrigated with sterile 0.9% NaCl. At the completion of the preparation, the grooves were then flushed and cooled with sterile 0.9% NaCl to remove bony debris. After surgery, full weight bearing was allowed. Each mini pig received bilateral proximal femur metaphyseal implants. Close observation of the mini pigs followed surgery.

A total of 14 Göttinger mini pigs received bilateral implants. Seven mini pigs were respectively allocated to one of the following groups to allow for 4 and 8 weeks of healing. After the allowed healing time, the mini pigs were pharmacologically euthanized and the femurs were taken out and the soft tissue removed. X-rays were taken at time of death and assessed by an expert radiologist to evaluate for loosening of the implants or fracture of the bones.

Histological examination

The implants and surrounding bone were explanted from the femoral metaphysis with the use of a water-cooled diamond saw. The retrieved implants, along with the surrounding bone, were fixed in 10% buffered formalin and send for histological examination. Subsequently, they were dehydrated with acetone and embedded into Technovit® 7200. The specimens were sectioned by the Donath cutting-grinding technique [8]. Finally, four slides of 30 µm-thick specimens were stained with toluidine blue to distinguish between mature (violet colours) and new bone (blue colours). Each specimen was examined under a light microscope (Leitz) and a digital photo was taken (Leica). The images of the bone areas were magnified 200× to perform the bone integration histomorphometry.

The percentage of bone integration concerning the different surface modifications were measured by using the image analyzing tool Image Pro Plus 4.51 from Media Cybernetics on a personal computer.

The mature bone area was identified by the colour staining characteristics and morphology. The implant and integration area of each section were also identified to allow for the calculation of possible percentage of new bone integration. The bone integration was determined according to the following equation:

$$Bl = Anew/Aall - Aimplant - Aold * 100$$

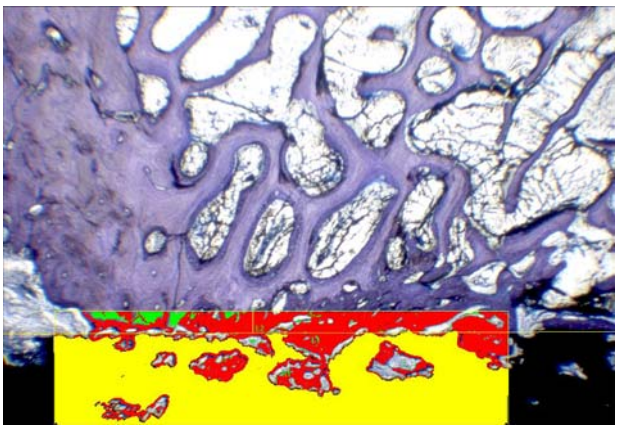


Fig. 4 Healing site after 4 weeks for VPS-Ti-25. Stained with toluidine blue. Original magnification 200×. *Yellow* = biomaterial; *red* = new bone; *green* = mature bone; *white spots* = remaining space for integration of bone into the porous implant surface

BI represents the bone integration, A new represents the new extra cellular matrix, [A all—A implant—A old] represents the available space for bone integration within the porous implant surface (Fig. 4).

Statistical analysis

The SPSS statistical package for personal computers was used to calculate the means. The data were statistically analyzed considering the variables (independent variables were implant surfaces, time after implantation). Post hoc assessment of significant differences ($p < 0.05$) was performed with the Scheffé tests and the paired *t*-test with Bonferroni's correction.

A comparative analysis was used to measure the degree of correlation between the in vitro and in vivo data. A value of *r* near or equal to 0 implies little or no linear relationship between the two sets of data. Thus, if *r* is closer to 1 or to –1, the stronger the linear relationship between the in vivo and in vitro data.

Results

In vitro results

OC

Over the period of 8 weeks, a significant difference between the response to CS-Ti and VPS-Ti-implants was seen ($p < 0.001$ = ***). Comparing VPS-Ti-25%/50 µm and VPS-Ti-Ti 50%/200 µm between the eighth day and fourth week the expression of OC was significantly higher for VPS-Ti 50%/200 µm. However, no difference was

detectable after 8 weeks of being suspended in a culture medium. The maximum release of osteocalcin was 18.39 ng/mL for VPS-Ti 50%/200 µm after 4 weeks (Fig. 5).

OPG

The release of OPG was only detectable after the eighth day. The amounts of OPG increased steadily over the period of 8 weeks without demonstrating any significant differences between the implant materials during the first 4 weeks.

After 8 weeks, there was a significant difference in the levels of OPG between the CS-Ti and the VPS-Ti-Ti implants ($p < 0.05$), but no statistical differences were found between the VPS-Ti-Ti implant materials. The maximum release of OPG was 1844.84 pg/mL for CS-Ti after 4 weeks (Fig. 6).

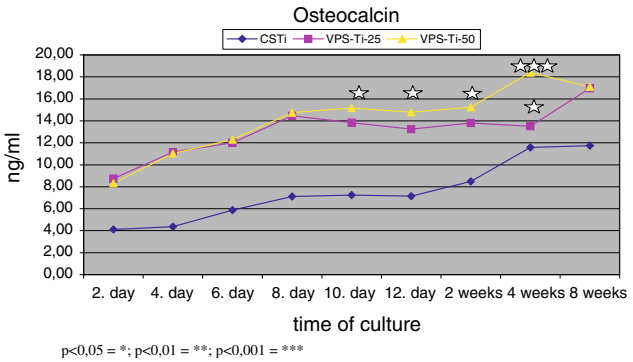


Fig. 5 The release of osteocalcin (means) by human bone marrow cells during a period of 8 weeks in response to CS-Ti, VPS-Ti-Ti 50%/200 µm and VPS-Ti-Ti 25%/50 µm with level of significance

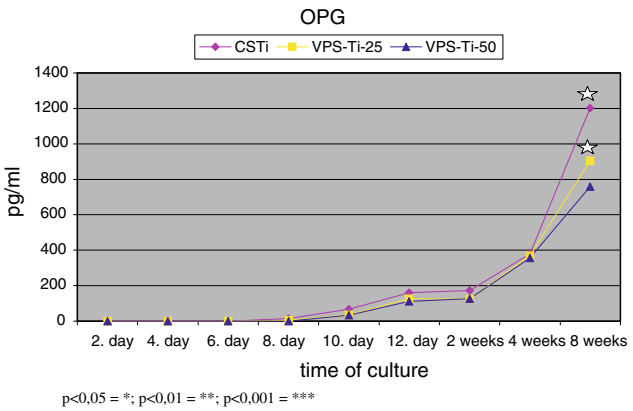


Fig. 6 The release of OPG (means) by human bone marrow cells during a period of 8 weeks in response to CS-Ti, VPS-Ti-Ti 50%/200 µm and VPS-Ti-Ti 25%/50 µm with level of significance

Alkaline phosphatase

The activity of alkaline phosphatase demonstrated no significance between the implants but the activity steadily increased within the period. The maximum of the average activity of alkaline phosphatase was 46.57 U/L for CS-Ti after 4 weeks (Fig. 7).

CLSM

In the event of CS-Ti, only scarce matrix tissue was observed, which was removed by the preparation steps as previously described. Figure 8 shows two cellular structures. The image on the right-hand side demonstrates the co-localization of collagen I and III.

VPS-Ti-Ti 50%/200 µm showed the highest amount of matrix tissue compared to CS-Ti and VPS-Ti-Ti 25%/50 µm. In the case of the VPS-Ti-Ti implant material, the collagen III detection was superior to collagen I. Both display high amounts of detectable vital cells and fibrous tissue and the co-localization was evident. The overlay of collagen I and III shows some intracellular marking (arrow) (Fig. 8).

In vivo results

Surgery was tolerated well by all animals and no infections or complications were recorded in the post-surgical period. With retrieval of the femurs, the implantation sites were macroscopically evaluated for mal-positioning or infection and no complications were noted. The histology demonstrated that all implants were placed correctly within the femoral metaphyseal bone, and neither inflammatory cell infiltrate nor signs of infection were observed.

The radiological films also showed the correct position of the implants in the proximal femur metaphysis and

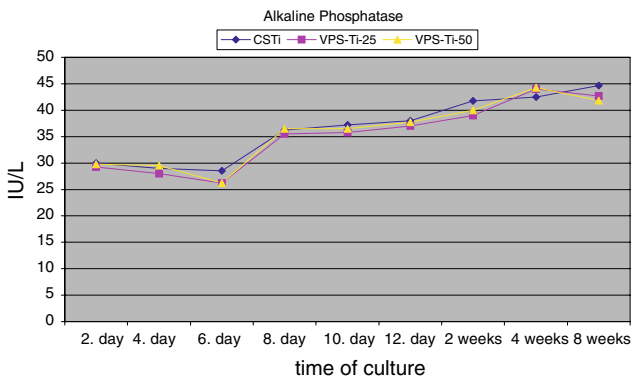


Fig. 7 The activity of alkaline phosphatase (means) by human bone marrow cells during a period of 8 weeks in response to CS-Ti, VPS-Ti-Ti 50%/200 µm and VPS-Ti-Ti 25%/50 µm with level of significance

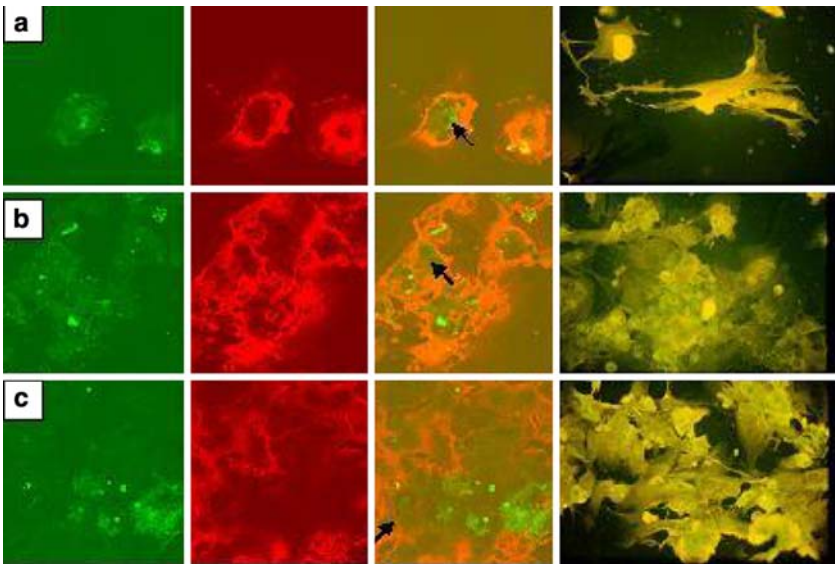


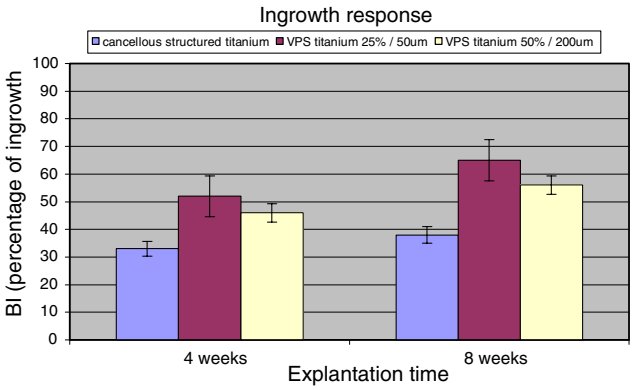
Fig. 8 From left to right—CLSM: *Green*: Collagen III marked; *Red*: Collagen I marked; Overlay. Magnification: 630× and light microscopic picture after markation against collagen III. Magnification 400×. (**a**) CS-Ti (**b**) VPS-Ti-Ti 25 and (**c**) VPS-Ti-Ti 50

demonstrated that no fracture, loosening or indirect signs of infection were visible at the time of the experiment.

Four weeks after implantation, a well-defined interface zone developed adjacent to both surface structures. At this point, there was a slight difference between the VPS-Ti-25 and VPS-Ti-50 implant. The matrix around the VPS-Ti-25 implants was more dense and extensive than that around the VPS-Ti-50 implants. The percentage of bone integration was superior for VPS-Ti-25 to the VPS-Ti-50 (52% vs. 48%) without being clinically significant.

The integration response after 8 weeks was superior in comparison to the 4-week data but significant differences between the various VPS-Ti-Ti implants were not evident.

After a healing period of 8 weeks, both VPS-Ti implant surfaces were well-covered and this covering was in the form of mineralized tissue and a dense matrix. The bone integration increased steadily with time over 4–8 weeks with minor differences between the VPS-Ti implant surfaces. However, the histomorphometry evaluation showed a lower bone integration for CS-Ti at 4 and 8 weeks, respectively, as compared with the other surface modifications ($p < 0.05$ vs. VPS-Ti-25 and VPS-Ti-50) (Fig. 9).



Level of significance after 4 weeks

versus	cs-Ti	VPS-Ti-Ti-25	VPS-Ti-Ti-50
cs-Ti	-	P<0.05	p<0.05
VPS-Ti-Ti-25	p<0.05	-	p>0.05
VPS-Ti-Ti-50	p<0.05	p>0.05	-

Level of significance after 8 weeks

versus	cs-Ti	VPS-Ti-Ti-25	VPS-Ti-Ti-50
cs-Ti	-	p<0.05	p<0.05
VPS-Ti-Ti-25	p<0.05	-	p>0.05
VPS-Ti-Ti-50	p<0.05	p>0.05	-

Fig. 9 Integration response of the different surface modifications over the period of 8 weeks with level of significance

Comparison analysis of in vivo and in vitro data

The analysis revealed the positive correlation between the bone integration index (BI) achieved by evaluating the in vivo and the in vitro evaluation of parameters like OPG and OC. These results should be reviewed with caution, as many variables exist. The low BI of the in vivo evaluation were reflected in low in vitro data suggesting that an inferior bone integration demonstrates low concentrations of OPG, OC and ALP in vitro (Table 1). A positive correlation between the in vivo and in vitro results was found even though related information is low, as mentioned previously.

Discussion

The influence of porous structures on bone repair and integration has been a topic of scientific research since the early 1970s [20, 21]. An open macro porous structure similar to cancellous bone promotes the infiltration of bone

Table 1 Correlation coefficient of the in vivo and in vitro data

	Bone integration index/OPG	Bone integration index/ osteocalcin	Bone integration index/AP
Correlation coefficient after 4 weeks			
CS Ti	0.88	0.83	0.79
VPS-Ti Ti 25	0.9	0.93	0.62
VPS-Ti Ti 50	0.92	0.93	0.88
Correlation coefficient after 8 weeks			
CS Ti	0.99	0.93	0.89
VPS-Ti Ti 25	0.85	0.9	0.95
VPS-Ti Ti 50	0.84	0.97	0.96

tissue, bone marrow and blood vessels, comparable with autografts or allografts [24].

In the recent years, a large number of investigations have dealt with the effects of pore structure on implants and the rate and volume of the bone repair process [17, 18, 27, 32, 33]. There is still no absolute consensus regarding which surface structure of implants is the best, since methodological complexity, varying methods and degrees of quantification in the pore structure and volume or rate of bone apposition makes it difficult to determine.

A key question in most applications of titanium is how the material influences and is influenced by the biological response that results from the contact between the biomaterial and biological systems. Surface properties of an implant play a crucial role in the process of biocompatibility, since bone cells can recognize and respond to these surfaces [7]. Surface modifications that could improve osseointegration include HA coatings, mechanical blasting, anodic oxidations, acid-etching processes and plasma spraying.

Some authors have recently reported on significantly improvement bony tissue reactions by modifications of surface properties of titanium implants. The in vitro and in vivo results reported in the literature are somewhat controversial. While some studies describe an enhancement of in vitro osteoblastic differentiation and inhibition of cell proliferation as well as in vivo osseointegration in the instance of increased surface roughness, others indicate that the in vitro proliferation and in vivo osseointegration can be improved on specimens submitted to higher surface roughness. These results suggest that there are other aspects that influence the results of the study.

The objective of this study was to evaluate the influence of different surface topographies by comparing the in vitro results of marrow cell culture correlated with the integration response of vacuum plasma sprayed titanium and CS-Ti implants in vivo. This may help to gain more information about the capability of implants to promote bone cell activity and therefore achieve greater peri-implant bone formation. The surface must promote rapid and complete osteogenesis while delaying bone resorption, ultimately producing a larger bone volume prior to remodelling.

This study in uniqueness used open-porous vacuum plasma sprayed implant surfaces. As expected—it has shown that bone cells are able to grow on porous surfaces, as porous surfaces exposed to body fluids or culture media become rapidly oxidized and will be coated with proteins [15]. Cells attaching to the metal surfaces may not have direct contact with the implant surface but rather with the biofilm deposited on the surface.

Bone marrow cells were found to decrease proliferation and to increase synthesis of alkaline phosphatase, the formation of nodules and mineral deposition in vitro when cultured on metal surfaces with increasing roughness [3, 4]. Furthermore, the macrophage cell lineage J774A.1 was found to be stimulated to release BMP-2 upon cultures on sandblasted surfaces [31].

The VPS-Ti implants demonstrated superiority regarding the expression of OPG, osteocalcin and CLSM compared to CS-Ti. The effects seen with the in vitro investigation were statistically significant for OC when the VPS titanium implants exposed were compared to CS-Ti. Although these results were considerably significant at 4 and 8 weeks, they represent less than a twofold increase. OPG demonstrates very low differences at 4 weeks, but at the eighth week, it was statistically higher for CS-Ti compared with the VPS-Ti-Ti implants. Differences between the VPS-Ti implants were only evident in case of the OC production between the fourth and eighth week time point. VPS-Ti-50 seems superior in the expression of osteocalcin and matrix tissue as seen by the CLSM.

The in vivo data regarding the integration response was assessed in the Göttinger mini pig because of several positive experiences [11, 28, 35, 37]. The implants in this study had similar bone integration over the healing period of 8 weeks. The values of BI were not significant in the comparison of VPS-Ti-Ti-25 and VPS-Ti-Ti-50 but a significantly increased integration response was found ($p < 0.05$) when compared to CS-Ti.

In conclusion, a positive correlation between the expression of bone cell associated proteins like OC and OPG and the integration response was found, but only if low or high bone integration indices achieved by the in vivo investigation were set to corresponding in vitro data.

The in vitro data show minor differences in the biological response to bony integration.

It is thus difficult to determine the effects of the various surface topographies on the cellular response by this in vitro model and stability and bony integration of the implants can most likely only be demonstrated in vivo due to the limitations of the study.

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