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In vitro response of primary rat osteoblasts to titania/hydroxyapatite coatings compared with transformed human osteoblast-like cells

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The biocompatibility of titania/hydroxyapatite (TiO_2/HA) composite coatings, at different ratio obtained by sol–gel process, was investigated studying the behavior of primary cultures of rat osteoblastic cells, isolated by femoral trabecular bone tissue. Moreover, the results have been compared with the response of human osteoblast-like MG63 cell line. Cytotoxicity of coatings was assessed by lactate dehydrogenase activity (LDH). The cellular behavior was analyzed by the cell proliferation (MTT test), cell morphology (SEM) and the biochemical markers evaluation of osteoblastic phenotype, such as alkaline phosphatase activity (ALP) and osteocalcin production. The results showed that TiO_2/HA coatings have no toxic effects and seemed to be a good support for cell adhesion and proliferation. Moreover, these materials allowed the differentiation of osteoblasts, stimulating the expression of alkaline phosphatase activity. The responses of the primary rat osteoblasts and human osteoblast-like MG63 cell line grown onto these coatings were similar in terms of proliferation and ALP activity. Differences were found considering the osteocalcin production. The results show that these coatings, thanks to their chemical composition and the deposition technique, are very promising for the potential orthopedic and dental applications.
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1. Introduction

Cell and tissue culture models are of fundamental importance as a preliminary screening in the investigation of tissue response to implant materials. *In vitro* approach supplies an ideal system for studying cell–material interactions, without the complexity and interference of the *in vivo* model. In orthopedic and dental fields, investigations have been performed on transformed cell lines (Saos-2 and MG63, for example) [1, 2] or on primary cultures of osteoblastic cells, of animal or human origin (bone marrow cells, calvarian or adult osteoblasts) [3–6]. Generally transformed cell lines are much more resistant to stress conditions than cells isolated from biopsies; moreover, they have own limitations due to some of the cell characteristics, such as proliferation, different from those of normal cells. On the other hand, primary cell culture system does not always exhibit reproducible results, due to variation in phenotypic expression of cells from each isolation and the loss of the osteoblastic phenotype with the time in culture. Moreover, primary cells are not always readily available.

Several authors have compared *in vitro* the ability of cells derived from different species (rat, rabbit, and human) to develop a bone-like tissue [7] and have studied the behavior of primary and immortalized osteoblasts cultured on biomaterials [8], reporting that the human cells are particularly sensitive to metal ions exposure as well the immortalized rat osteoblast cell line.

The performance of a material when is in contact with the bone tissue depends on factors such as chemical composition, crystallographic structure, roughness, surface topography [9–11]. The development of coatings with hydroxyapatite, calcium phosphate ceramics and bioactive glass [12–16] for orthopedic and dental applications offers a larger number of advantages, such as surface metallic covering, bond to surrounding osseous tissue and bone formation enhancement.

In our laboratory, we have prepared a composite coatings constituted of a titania (TiO_2) matrix encapsulating hydroxyapatite (HA) by sol–gel process, in order to obtain a thin, stable and bioactive film onto a titanium substrate [17].

In the present work, the evaluation of the biocompatibility of TiO_2/HA composite coatings at different ratio (1 : 1, 1 : 2, 2 : 1), is reported by studying the behavior of primary cultures of rat osteoblastic cells, isolated by femoral trabecular bone tissue. Moreover, the results

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have been compared with the response of human osteoblast-like MG63 cell line.

The biocompatibility has been assessed by the cell proliferation (MTT tests), cell morphology (SEM) and the biochemical parameters evaluation of osteoblastic phenotype, such as alkaline phosphatase activity (ALP) and osteocalcin production. Cytotoxicity of coated titanium samples has been also evaluated by assessment of lactate dehydrogenase activity (LDH).

2. Materials and methods

2.1. Sample preparation

Commercially pure titanium (Goodfellow, Germany) was used as substrate, cut into pieces 20×10 mm size, ultrasonically rinsed in acetone for 20 min, in 70% ethanol solution for 20 min and then in distilled water for 15 min.

Titania sol (TiO_2) was prepared by mixing titanium isopropoxide, acetyl acetone, nitric acid, *n*-propane alcohol and distilled water (solution A).

Hydroxyapatite (HA) powders (Fin Ceramica, Italy) were added to anhydrous ethanol in the same ratio (solution B).

Solutions A and B were mixed in different ratios (w/w): 2 : 1 (TiO_2/HA 0.5), 1 : 1 (TiO_2/HA 1) and 1 : 2 (TiO_2/HA 2).

The coatings were obtained by the dip technique at a speed of 15 cm min^{-1} and sinterized at 500 °C for 30 min. The dip-coating process was repeated four times for every sample.

2.2. Cell cultures

Primary rat osteoblasts were isolated from trabecular bone obtained from the femoral condyles of inbred rats aged 12 months. All animals were used, handled, maintained and euthanized by strictly following International and European Laws on animal experimentation.

Immediately after euthanasia, in aseptic conditions, the soft tissues were cleaned from the femurs; the cortical area of the condyles was removed with a bone cutter in order to expose the trabecular tissue. Cell cultures were obtained following a method described in literature [18]. Briefly, bone trabecular fragments were washed with DMEM : F12 serum-free, digested in the same medium added with 1 mg ml^{-1} collagenase for 90 min, at 37 °C. The enzymatic reaction was stopped by adding an equal volume of medium with 10% of fetal calf serum (FCS) and the supernatant containing the released cells was collected. Washing and collecting were repeated three times. The cells obtained were pelleted by centrifugation, resuspended, seeded in 35 mm dishes and incubated at 37 °C in a humidified atmosphere of 5% CO_2/95% air. In order to assess the ability of rat osteoblasts to mineralize their extracellular matrix, confluent cultures were grown in chamber slides, in additional presence of 1,25(OH_2)D_3 (10^{-9}) M. Supernatant was collected to measure Osteocalcin and ALP activity. Cells were then fixed, tested for type I collagen (monoclonal immunofluorescence, Sigma, UK) and stained by the von Kossa method (BioOptica, Italy).

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% FCS (Mascia Brunelli, Italy), called complete medium, and kept at 37 °C in an atmosphere of 5% CO_2 and 99% humidity. Media were changed every three days.

2.3. Biocompatibility studies

Cells were released at confluence with trypsin/EDTA, then seeded for experiment: a cell suspension of 100 μl (5×10^4 cell cm^{-2}) was applied on every sample placed in 6-multiwell plates. Cells were allowed to attach for 2 h, then 3500 μl of complete culture medium, containing ascorbic acid 50 μg ml^{-1} and β-glycerophosphate 10 mM, were added. Cultures were maintained for 7 or 10 days and the medium was replaced every two days.

At the end of the experiment, all cultures were tested in triplicate for lactate dehydrogenase (LDH Boehringer Mannheim), alkaline phosphatase activity (ALP, Sigma kit 104, UK) on cell lysates in order to evaluate cytotoxicity and cell metabolism respectively. Cell lysates were prepared releasing the osteoblasts from the culture surface by the addition of trypsin/EDTA solution for 10 min. The reaction was terminated by the addition of DMEM containing 10% serum. A second trypsinization was performed to ensure the complete cell detachment from the substrate. After centrifugation, the cell pellet was resuspended in 1 ml of sterile distilled water, sonified for 10 s and undergone three cycles of freezing at −80 °C for 20 min followed by thawing at 37 °C for 30 min to lyse the cells. The specific activity was calculated referring to protein concentration of lysates. The protein content was determined by the BioRad reactive.

Osteocalcin (OC, Novocalcin Kit, Metra Biosystem, CA, USA), a specific product of osteoblasts, was evaluated on supernatant. MTT test (Sigma, UK) was performed to evaluate cell proliferation and viability [19]. A random sample for each group was fixed with 2.5% glutaraldehyde in pH 7.4 phosphate buffer 0.01 M for 1 h, and dehydrated in a graded ethanol series. Then samples were critical point dried (Bal-Tech, Liechtenstein) and gold sputtered prior to observation with a scanning electron microscope (SEM, Philips XL 40) at 5 kV acceleration voltage.

2.4. Statistics

A statistical evaluation of data was performed using the software package SPSS/PC$^+$ Statistics™ 7.1 (SPSS Inc., Chicago, IL, USA). Data, results of three replications of experiment, are reported as mean ± standard deviation (SD) and the significance level was set at $p < 0.05$. After the assessment of significant differences by one-way variance analysis (ANOVA), differences among groups were established with Scheffé's *post hoc* multiple comparison test.

3. Results

The TiO_2/HA coatings toxicity was evaluated measuring the LDH activity on the supernatant of primary rat osteoblastic cells cultured onto coatings. The results, reported in Table I, showed no significant differences between the LDH level of TiO_2/HA coatings and the controls, represented by untreated titanium and polystyrene standard culture plate, suggesting that these materials are not toxic. The viability and proliferation of primary rat osteoblasts was assessed after seven days of incubation by MTT test (Fig. 1). All coatings seemed to support viable cells and promote their proliferation. Alkaline phosphatase specific activity was affected by substrate composition (Fig. 2): ALP of rat osteoblasts seeded onto polystyrene was higher than control titanium. TiO_2/HA 0.5 and TiO_2/HA 1 samples showed a trend to increase the ALP production with respect to untreated titanium, resulting similar to polystyrene.

Moreover, the cells grown on TiO_2/HA coatings did not show significant differences in osteocalcin production compared to controls (Fig. 3).

Scanning electron microscopy (Fig. 4) showed the rat osteoblasts well spread on the samples surface and flatted. Individual cellular elements were difficult to recognize because the cells developed a very close layer. Moreover, the cells grown onto the coatings seem to form a more compact layer.

In Table II are summarized the results concerning the behavior of MG63 cell line cultured onto the same substrates. MTT test did not reveal significant differences between the proliferation of osteoblastic cells on the various materials.

ALP specific activity of osteoblasts seeded on TiO_2/HA coatings was significantly higher than controls. Moreover, an increase of osteocalcin production for the cells grown on titanium and titanium coatings was observed compared to polystyrene. In particular, cells grown on TiO_2/HA 1 sample showed an increased osteocalcin level with respect to the other coatings and untreated titanium.

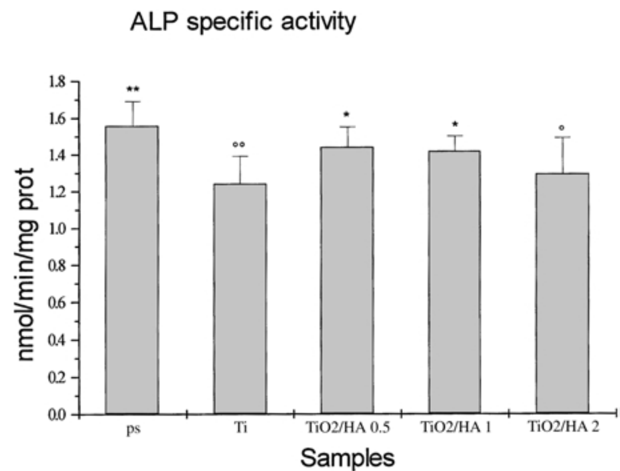

Figure 2 Alkaline phosphatase specific activity of primary rat osteoblasts grown on different substrates tested after seven days of incubation, compared with controls, untreated titanium (Ti) and polystyrene (ps). Difference from Ti: $*p < 0.05$, $**p < 0.01$. Difference from ps: $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$.

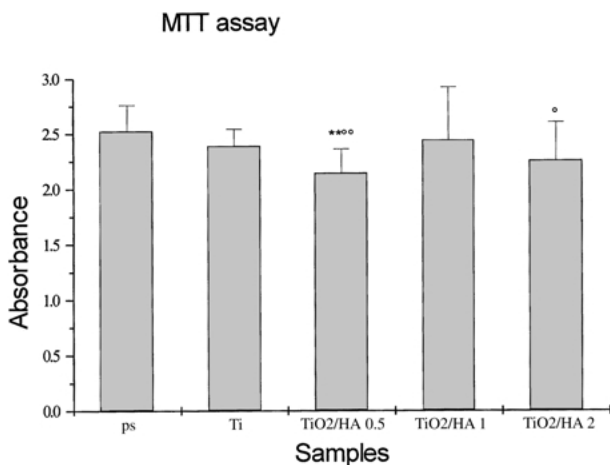

Figure 1 Proliferation of primary rat osteoblasts cultured on various substrates estimated by the MTT assay after seven days of incubation, compared with controls, untreated titanium (Ti) and polystyrene (ps). Difference from Ti: $**p < 0.01$. Difference from ps: $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$.

4. Discussion

TiO_2/HA composite coatings prepared by sol–gel process were described and characterized in a previous paper [17]; the coatings resulted to be uniform, clean, adherent on titanium substrate and with a homogenous thickness. In the present work the behavior of primary rat osteoblasts cultured on these materials has been

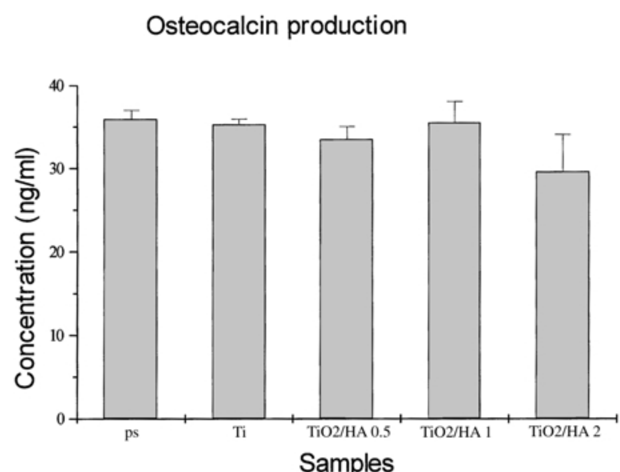

Figure 3 Osteocalcin production after seven days of incubation by primary rat osteoblasts during culture on different substrates and controls, untreated titanium (Ti) and polystyrene (ps).

TABLE I Evaluation of enzyme activity of LDH (U/ml) obtained from primary rat osteoblasts cultured onto TiO_2/HA coatings (means ± SD)

Polystyrene (ps)	Titanium (Ti)	TiO_2/HA 0.5 coating	TiO_2/HA 1 coating	TiO_2/HA 2 coating
114.33 ± 8.62	122.67 ± 5.03	111.33 ± 10.02	115.67 ± 9.29	112.67 ± 8.50

Statistical analysis: the means are not significantly different.

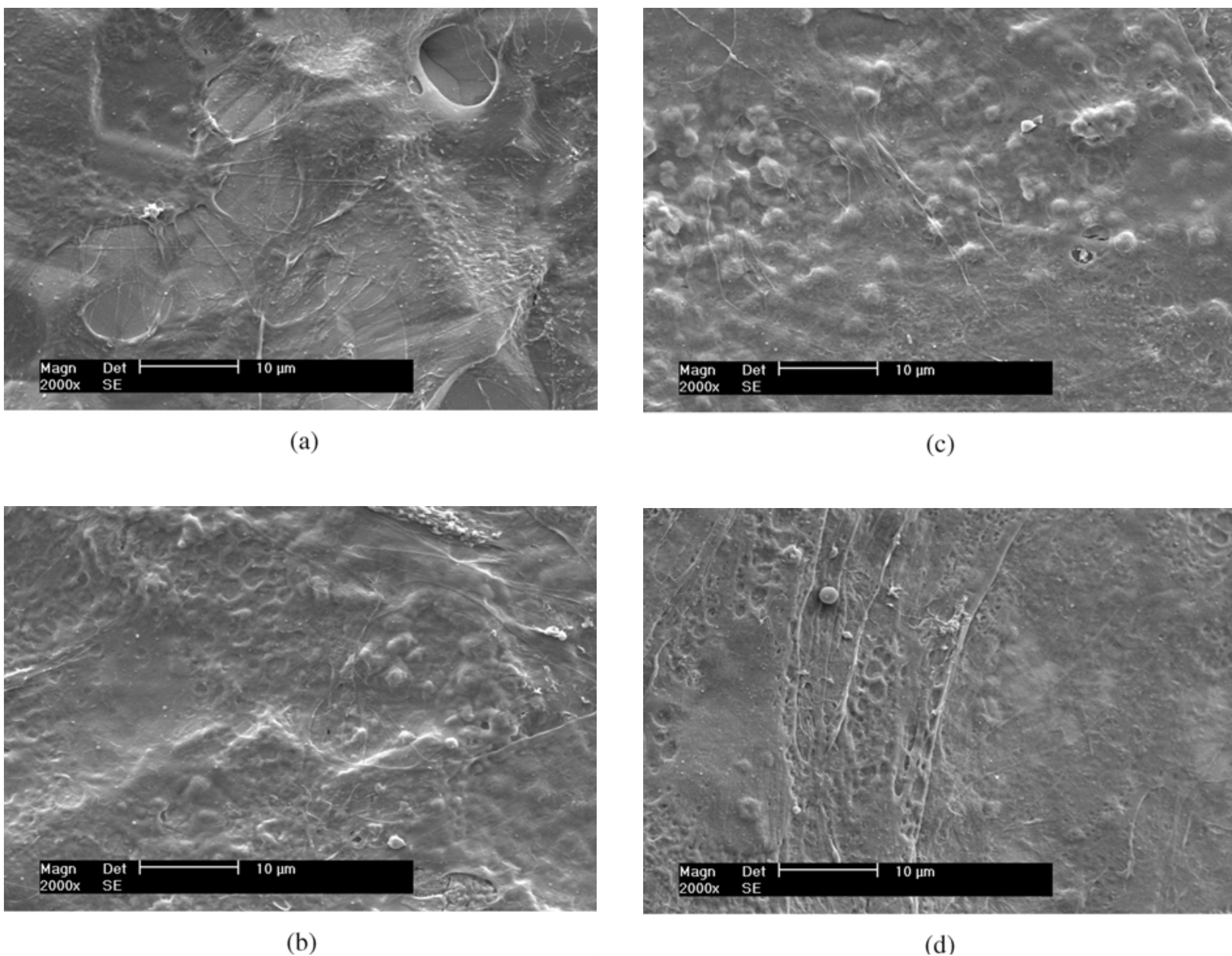

Figure 4 SEM images of primary rat osteoblasts grown on materials tested after seven days of incubation: (a) Titanium, (b) TiO_2/HA 0.5, (c) TiO_2/HA 1, (d) TiO_2/HA 2. Magnification 2000 ×.

investigated, compared with the response of MG63 cell line. The results showed to have no toxic effects on viability of primary rat osteoblasts. The cytotoxicity tests performed with MG63 also suggested the absence of cellular damage, as reported elsewhere [20]. These two cell lines cultured onto TiO_2/HA coatings exhibit a similar behavior after seven days of incubation, at least regarding the cell proliferation and ALP dosage. In previous experiences, further times of incubation have not revealed significant differences. The samples supplied a good substrates for cell adhesion and proliferation. Moreover, the TiO_2/HA coatings stimulated the cells to produce ALP, an early marker of osteoblasts differentiation, more than untreated titanium. Only TiO_2/HA 2 sample seeded with primary rat cells was similar to titanium control.

Considerable differences between the primary rat and established osteoblasts behavior were detected in osteocalcin level. The MG63 cells were affected from the different composition of substrates: osteoblasts grown onto TiO_2/HA 0.5 and TiO_2/HA 1 coatings and untreated titanium increased the osteocalcin production with respect to polystyrene; in contrast, the primary osteoblasts were not influenced from substrates.

The different origin of cells could explain their different behavior. The MG63 osteoblast-like cells show features typical of the relatively immature osteoblasts, such as the stimulation of alkaline phosphatase activity and osteocalcin synthesis, but they do not mineralize their matrix. Then, they are a good model for studying the early stages of osteoblasts differentiation [10].

The primary cultures develop a fully differentiated phenotype with a temporal sequence of events: attachment, proliferation, synthesis of extracellular matrix proteins, expression of high levels of ALP, modification of extracellular matrix, deposition of hydroxyapatite crystals and mineralization [21]. The mineralization induces the synthesis of osteocalcin that is therefore the marker of final osteoblast differentiation.

The differences noticed in the osteocalcin production between the two cell lines could be due to the different stage of differentiation.

However, the TiO_2/HA coatings affect on the expression of the osteoblast phenotype stimulating the ALP production, even though there are no differences in the behavior of osteoblasts cultured on coatings of different composition. Some evidence is present in osteocalcin production of MG63 cells seeded onto TiO_2/HA samples.

Generally, the influence of these materials can be explained with the characteristics of the chemical composition and the deposition technique. In fact, in order to establish an excellent tissue response to bone, the materials undergo a change of their surface properties which cause the formation of a layer of calcium

TABLE II Evaluation of biochemical parameters obtained by MG63 cell line cultured on TiO_2/HA coatings (means ± SD, $n = 4$ triplicates)

Culture cells characterization	Polystyrene (ps)	Titanium (Ti)	TiO_2/HA 0.5	TiO_2/HA 1	TiO_2/HA 2	ANOVA F; p
MTT assay (absorbance unit)	1.75 ± 0.09	2.02 ± 0.11	2.17 ± 0.08	2.13 ± 0.14	1.62 ± 0.38	$3.72; = 0.027$
ALP (nmol/min/mg prot)	1.23 ± 0.05	1.08 ± 0.04	1.39 ± 0.02	1.43 ± 0.02	1.44 ± 0.03	$53.57; < 0.0005$
OC ($ng\,ml^{-1}$)	3.75 ± 0.36	11.31 ± 1.80	9.56 ± 2.34	17.63 ± 1.14	4.60 ± 0.48	$30.39; = 0.001$

Statistical analysis: ALP: ps vs Ti ($p = 0.039$), TiO_2/HA 0.5 ($p = 0.027$), TiO_2/HA 1 ($p = 0.01$), TiO_2/HA 2 ($p = 0.008$); Ti vs TiO_2/HA 0.5 ($p = 0.002$), TiO_2/HA 1 ($p = 0.001$), TiO_2/HA 2 ($p = 0.001$). OC: Ti vs ps ($p = 0.029$), TiO_2/HA 2 ($p = 0.046$); TiO_2/HA 1 vs ps ($p = 0.002$), TiO_2/HA 0.5 ($p = 0.022$), TiO_2/HA 2 ($p = 0.003$).

phosphate at the interface with the tissues. Authors have demonstrated [22, 23] that TiO_2/HA coatings deposited by sol-gel process are bioactive, improving the calcium and phosphate precipitation onto the surface. Using *in vitro* modeling results, it was suggested that this phenomenon is related to the presence of hydroxyl groups (Si–OH and Ti–OH) or carbonyl groups (C–OH) at the materials surfaces [23–25].

Ti–OH groups were detected on TiO_2/HA coating surfaces prepared in our laboratory [17] and could be responsible of calcium and phosphate nucleation.

In any case, early response of osteoblasts to implant materials, such as the number of cell attached and ALP activity, do not necessarily predict whether calcification will be greater on a particular substrate. Therefore, further investigations are necessary in order to evaluate the behavior of osteoblasts seeded onto TiO_2/HA coatings for a long time.

5. Conclusion

The study of biocompatibility of titania/hydroxyapatite coatings of titanium, prepared by sol-gel process, suggested that these materials are not cytotoxic and allowed the differentiation of osteoblasts, stimulating the expression of a peculiar biochemical marker as alkaline phosphatase activity. The behavior of primary rat osteoblasts and MG63 osteoblast-like cells grown onto these coatings is similar at least considering the response relative to the early stages of osteoblasts differentiation.

Therefore, the obtained coatings, thanks to their chemical composition and the deposition technique, are very promising and could be good candidates for use in orthopedic and dental field.

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References

1. M. AHMAD, M. MCCARTHY and G. GRONOWICZ, *Biomaterials* **20** (1999) 211.
2. R. KUE, A. SOHRABI, D. NAGLE, C. FRONDOZA and D. HUNGERFORD, *ibid.* **20** (1999) 1195.
3. C. FAUCHEUX, R. BAREILLE, F. ROUAIS, J. AMÉDÉE, A. LIEBENDÖRFER and M. DARD, *J. Mater. Sci.: Mater. Med.* **5** (1994) 635.
4. J. E. G. HULSHOFF, K. VAN DIJK, J. P. C. M. VAN DER WAERDEN, J. G. C. WOLKE, L. A. GINSEL and J. A. JANSEN, *J. Biomed. Mater. Res.* **29** (1995) 967.
5. T. MATSUDA and J. E. DAVIES, *Biomaterials* **8** (1987) 275.
6. P. J. MARIE, A. LOMRI, A. SABBAGH and M. BASLE, *In Vitro Cellular & Development Biology* **25**(4) (1989) 373.
7. H. TOMÁS, G. S. CARVALHO, M. H. FERNANDES, A. P. FREIRE and L. M. ABRANTES, *J. Mater. Sci. Mater. Med.* **8** (1997) 233.
8. R. MACNAIR, E. H. RODGERS, C. MACDONALD, A. WYKMAN, I. GOLDIE and M. H. GRANT, *ibid.* **8** (1997) 105.
9. J. LINCKS, B. D. BOYAN, C. R. BLANCHARD, C. H. LOHMANN, Y. LIU, D. L. COCHRAN, D. D. DEAN and Z. SCHWARTZ, *Biomaterials* **19** (1998) 2219.
10. J. Y. MARTIN, Z. SCHWARTZ, T. W. HUMMERT, D. M. SCHRAUB, J. SIMPSON, J. LANKFORD, D. D. DEAN, D. L. COCHRAN and B. D. BOYAN, *J. Biomed. Mater. Res.* **29** (1995) 389.
11. D. M. BRUNETTE, *Int. J. Oral Maxillofac. Implants* **3** (1988) 231.
12. D. L. WHEELER, K. E. STOKES, R. G. HOELLRICH, D. L. CHAMBERLAND and S. W. MCLOUHLIN, *J. Biomed. Mater. Res.* **41** (1998) 527.
13. E. BARTH, C. JOHANSSON and T. ALBREKTSSON, *Int. Oral Max. Imp.* **5** (1990) 227.
14. P. DUCHEYNE and Q. QIU, *Biomaterials* **20** (1999) 2287.
15. W. C. A. VROUWENVELDER, C. G. GROOT and K. DE GROOT, *J. Biomed. Mater. Res.* **27** (1993) 465.
16. K. DE GROOT, R. G. T. GEESINK, C. P. A. T. KLEIN and P. SEREKION, *ibid.* **21** (1987) 1375.
17. E. MILELLA, F. COSENTINO, A. LICCIULLI and C. MASSARO, *Biomaterials* **22**(11) (2001) 1425.
18. L. R. HALSTEAD, M. J. SCOTT, L. RIFAS and L. V. AVIOLI, *Calcif. Tissue Int.* **50** (1992) 93.
19. D. SGOURAS and R. DUNCAN, *J. Mater. Sci. Mater. Med.* **1** (1990) 61.
20. P. A. RAMIRES, A. ROMITO, F. COSENTINO and E. MILELLA, *Biomaterials* **22**(12) (2001) 1467.
21. G. S. STEIN and J. B. LIAN, *Endocrine Rev.* **14** (1993) 424.
22. T. PELTOLA, M. PATSI, H. RAHIALA, I. KANGASNIEMI and A. YLI-URPO, *J. Biomed. Mater. Res.* **48**(3) (1998) 504.
23. M. JOKINEN, M. PÄTSI, H. RAHIALA, T. PELTOLA, M. RITALA and J. B. ROSENHOLM, *ibid.* **42** (1998) 295.
24. P. LI, C. OHTSUKI, T. KOKUBO, K. NAKANISH, N. SOGA and K. DE GROOT, *ibid.* **28** (1994) 7.
25. P. M. LI, K. DE GROOT and T. KOKUBO, *J. Am. Ceram. Soc.* **77** (1994) 1307.

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