Italian Society
of Periodontology
and Implantology

Analysis of human alveolar bone cell behavior on a hydroxyapatite nanotechnological substrate by means of Real Time – PCR, SEM and atomic force microscope

Analisi del comportamento degli osteoblasti umani alveolari su substrati di idrossiapatite nanotecnologica per mezzo di PCR-Real Time, SEM e microscopio a forza atomica

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PROCEEDINGS BOOK RESEARCH SESSION "H.M. GOLDMAN PRIZE" 2013
ATTI DELLA SESSIONE DI RICERCA "PREMIO H.M. GOLDMAN" 2013

Summary

Introduction: Nano-hydroxyapatite (nHA) is a potential ideal biomaterial for bone regeneration. However, no data are present as yet on the characterization of human osteoblasts behaviour upon to nHA exposure. Thus, aim of the present study is to evaluate the response of osteoblastic cells in the presence of nHA. *Materials and Methods:* Differentiation of primary human osteoblasts, collected from the alveolar ridge of a periodontal patient during osseous resective surgery, was monitored upon piastration and cultured on a polylysine/nanohydroxyapatite (POL/nHA) substrate as compared to cells grown on polylysine (POL) substrate. Potential modification of osteoblasts homeostasis in response to different substrates was evaluated by quantitative reverse transcriptase–PCR (qRT-PCR), scanning electron microscope (SEM) and atomic force microscope (AFM). *Results:* Real time-PCR survey showed that several genes, markers of osteoblast cell differentiation (BMP-2,-5,-7, ALP, COLL-1A2, OC, ON), were significantly stimulated in a higher exponential manner by the POL/nHA substrate as compared to the control substrate. In addition, nHA substrate demonstrated better osteoinductive properties, related to surface roughness, and stimulation of adhesion and spreading of osteoblasts. *Conclusions:* Our data demonstrate that nanosize-hydroxyapatite material enhanced human osteoblast proliferation and differentiation, as expressed by an increased production of BMP's and other specific differentiation markers, known to be osteoinductive factors in bone regeneration.

Key-words: nanohydroxyapatite, human osteoblasts, alveolar bone regeneration.

Riassunto

Introduzione: *La nanoidrossiapatite (nHA) rappresenta un biomateriale potenzialmente ideale per la rigenerazione ossea. In ambito parodontale ad oggi sono scarse le evidenze scientifiche a questo riguardo. Lo scopo di questo studio è osservare il comportamento biomolecolare di queste cellule all'esposizione con tali nanostrutture.* Materiali e metodi: *Sono stati prelevati*

osteoblasti umani primari dalla cresta alveolare di un paziente parodontale durante chirurgia resettiva. Successivamente sono stati piastrati e coltivati in due diversi terreni: polilisina/ nanoidrossiapatite (POL/nHA)(test) e polilisina (POL)(controllo). La risposta degli osteoblasti ai due terreni è stata monitorata tramite analisi quantitativa di trascrittasi inversa-PCR (qRT-PCR), microscopio elettronico a scansione (SEM) e microscopio a forza atomica (AFM). Risultati: *La PCR real time ha mostrato che diversi geni, marcatori di differenziazione degli osteoblasti (BMP-2,-5,-7, ALP, COLL-1A2, OC, ON), sono significativamente stimolati nel terreno POL/ nHA rispetto al terreno di controllo. Inoltre, il terreno con nHA ha mostrato caratteristiche osteoinduttive migliori per quanto riguarda la rugosità di superficie, l'adesione e lo spreading degli osteoblasti.* Conclusione: *Questi dati dimostrano che il biomateriale idrossiapatite quando impiegato in nanoforma stimola la proliferazione e la differenziazione di osteoblasti umani della cresta alveolare, come mostrato non solo da un' aumentata espressione delle BMP ma anche di altri marcatori della differenziazione cellulare, noti come fattori di osteoinduzione nella rigenerazione ossea.*

Introduction

The main concern of periodontology is the rehabilitation of periodontally compromised teeth, possibly aiming at the complete periodontal tissue regeneration[1]. Ideally this process would include the formation of new bone, new periodontal ligament and new cementum, or rather a new attachment over a root that has previously been deprived of all the attachment apparatus due to infection, or for a combination of the latter with traumatic or iatrogenic factors[1,2]. The therapeutic approaches attempting to achieve this objective include the use of different grafting materials (autogenous, allogeneic, xenogeneic and alloplastic grafts), physical barriers for Guided Tissue Regeneration (GTR), enamel matrix derived protein as well as exposure to growth factors[3, 4].

It is assumed that the use of bone grafts or alloplastic materials would result both in the regrowth of alveolar bone and the formation of new cementum layer with inserted collagen fibres on previously periodontitis-involved root surface[5]. The mechanism of action may be either the stimulation of osteogenesis (new bone formation from the bone-forming cells contained in the graft), osteoconduction (when the graft serves as a scaffold for bone formation from the adjacent host bone), or osteoinduction (the matrix of the bone grafts contains bone-inducing substances that results in bone formation in the surrounding tissues even if they are other than bone tissue)[5,6]. Indeed, alloplastic materials are supposed to promote bone healing through osteoconduction and subsequently behaving as scaffold[5].

An ideal scaffold is a biocompatible material that provides an appropriate mechanical support[7]. It should present a similar structure to the native extracellular matrix, exhibit favourable surface properties leading to an increased adhesion, proliferation and differentiation of cells without changing their phenotypes[7].

Hydroxyapatite (HA) [$(Ca_{12}PO_4OH)_6$] is an alloplastic material, chemically similar to the inorganic component of bone matrix translating this property in a valuable and optimal biocompatibility[7]. A fundamental factor that allows integration of this material with bone is the dimension of the crystals. In fact it was possible to obtain kinds of HA with dimensions closer to the natural crystals found in hard tissues of vertebrates (i.e. between 1 and 10nm) known as nanophase hydroxyapatite[8]. Increased new bone formation has been observed as early as 2 weeks on nano-hydroxyapatite-coated scaffolds compared with micron-sized hydroxyapatite-coated scaffolds and uncoated scaffolds when implanted into rat calvarial bone[9].

Moreover, recent findings report that nHA can stimulate both differentiation of stem cells towards the osteogenic pathways[9] and human osteoblast like cells proliferation[10] resulting in bone formation. Furthermore, it has been reported that nHA is a strong stimulator of PDL cell attachment and proliferation[11].

Little evidence is available to date on the response of human osteoblasts exposure to nHA. Thus, the aim of our study was to evaluate the behaviour of human osteoblasts in presence of nHA *in vitro* in order to further explore the mechanisms at the basis of periodontal bone regeneration induced by nHA grafting materials.

Materials and Methods

Isolation of primary human osteoblasts

Primary human osteoblasts (hOBs) were obtained from a 50 year-old patient scheduled for resective periodontal surgery in the region 4.4, 4.5, 4.6. Prior to enrolment in the study the patient signed an informed consent. Primary human osteoblasts were prepared using a previously validated protocol [12, 13].

Bone samples from the alveolar ridge during periodontal osseous resective surgery were collected using a bone chisel and immediately placed in a Hank's salt-balanced sterile solution, to which 100 U/ml of penicillin and 100 mg/ml of streptomycin were added in order to optimize conservation.

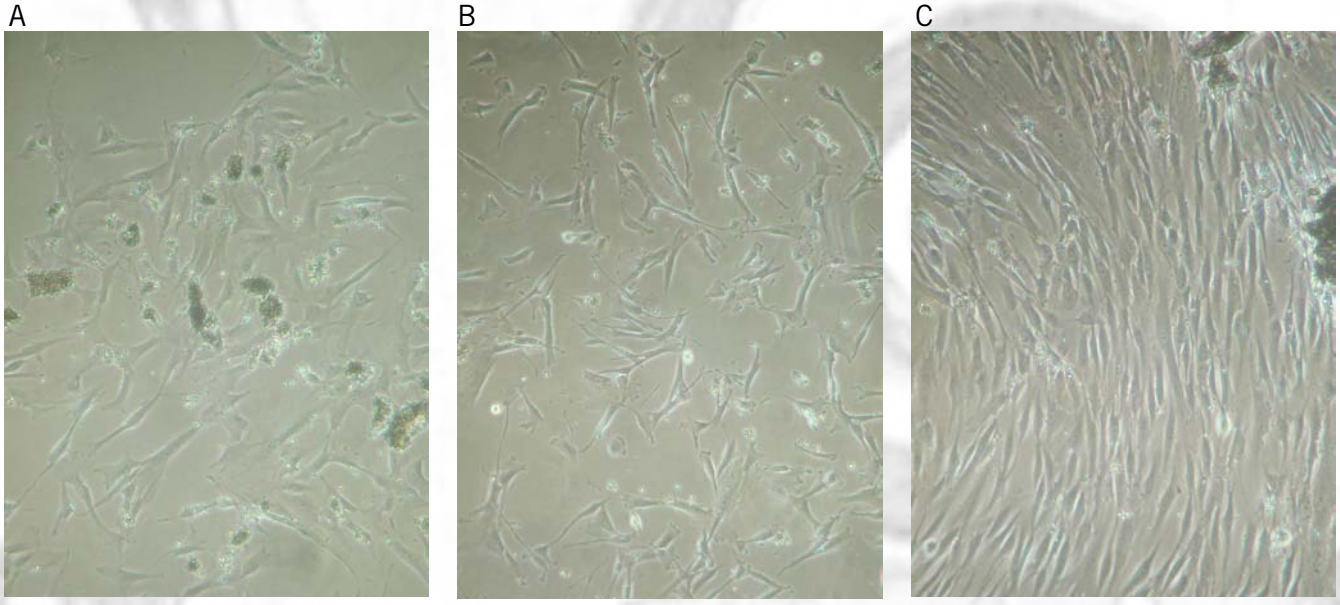
Within 24 hours of tissue collection, samples were processed by a modification of the sequential collagenase/trypsin digestion method as described elsewhere and later[14].

Bone samples were rinsed in sterile saline solution (phosphate buffered saline, PBS), fragmented with a scalpel and digested with agitation in 1 mg/ml of type IV collagenase and 0.25% tripsin at 37°C for 30 minutes, subsequently 40 minutes for second digestion and finally 90 minutes for final digestion.

Cells obtained from the second and third digestions were collected, centrifuged and resuspended in 25cm^2 diameter agar plates DMEM (Dulbecco's Modified Eagles Medium, Gibco-BRL, Rockville, MD) in addition to 50 U/ml of penicillin, 50 µg/ml of streptomycin, 4 mmol/l L-glutammin and fetal bovine serum (FBS, Hyclone, Logan, Utah) at 10%.

After 4-5 days of culture, capsules were observed by optical microscope to evaluate the proliferation of hOBs from the bone fragments (Fig. 1 - A,B). At 14 days of culture, osteoblasts were then observed at the optical microscope (Fig. 1 - C).

Fig. 1 Human primary osteoblasts taken from the alveolar ridge of periodontal patient: after 4-5 days (A,B) and 14 days (C).



2. Preparation of layers

A 1ml/25cm^{2}POL solution (Poly-L-lysine solution 0.01%, Bioreagent, mol wt 150,000-300,000, sterile-filtered, Sigma-Aldricht) was aseptically transferred to 6 cm^2 culture dishes to coat the culture surfaces. Each capsule was gently shaken to homogenize stratification of the substance

in each point. The excess solution was removed after 5 minutes, plated surfaces were rinsed with sterile water and allowed to dry.
Subsequently 10 mg of nHA powder (Ghimas Spa, Casalecchio di Reno, BO, Italy) was re-suspended in 1 ml of sterile water and the solution transferred to the plates previously covered in POL. After 5 minutes the excess powder was removed and the capsule surface rinsed with sterile water and allowed to dry for several hours.

3. Cell culture

After completion of the above procedures the hOBs were used for the in vitro experiments. Cells were plated during the first transit on POL capsules (test 1) and POL/nHA (test 2) at a density of $2\times10^4/cm^2$ and cultured in DMEM with addition of 10% FBS and incubated at standard conditions (95% humidity, 5% CO_2, 37°C) for different periods of time.

4. Preparation of samples for microscopic observation

In order to assess the real cellular adhesion to the specific POL/nHA substrate, chemical fixing was carried out on each sample.
Tests 1 and 2 containing the hOBs were cultured on POL and POL/nHA for 24 hours in standard conditions. At the end of incubation cells were rinsed twice with PBS, subsequently fixed in 2,5% glutaraldehyde for 1 hour, rinsed with distilled water and dehydrated with graded concentrations of ethanol (i.e. 30%, 50%, 70%, 90%) and air dried.
Samples were subsequently coated with gold using sputter coating prior to examination by SEM, using a Leo 1450VP (Carl Zeiss, Jena, Germany) running at 15 kV.
Atomic Force Microscope (AFM) analysis was carried out using a Nanonics Imaging AFM system (Jerusalem, Israel) in no contact mode and a reference voltage of 0.8-1.0 V between the tip and the sample surface during all experiments.

5. RNA extraction and Real-Time PCR

The hOBs were cultured on the two different substrates (POL e POL/nHA) for 14 days. At the end of incubation, cells were rinsed twice with cold PBS and the whole RNA extracted using Trizol reactant (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed by adding 2 µg total RNA and 0,1 µg of oligo (dT) to 20 µl reaction buffer. RNA was denaturated by heating at 70°C for 10 minutes. First strand buffer, 2 µl 0,1 M DTT, 1 µl 10 µM dNTP Mix and 1 µl of ribonuclease inhibitors (Invitrogen) was added to the reaction tube followed by incubation at 42°C for 2 minutes. At the end of this reaction 200 units of Super Script II (Invitrogen) were added and incubated at 40°C for 50 minutes.
The qRT-PCR analysis was performed using an ABI Prism 7300 (Applied Biosystems, Foster City, CA, USA) and amplification was carried out with 50ng of cDNA , in a 96-well plates, using SYBR green PCR Master Mix (Applied Biosystems) in 25µl volume. Each sample was analyzed in triplicate. PCR conditions were: 94°C for 10 minutes followed by 40 cycles at 94°C for 15 seconds, 60° C for 1 minute. Primer sequences were designed using Primer Express software (Applied Biosystems) and synthesized by Primm (Milano, Italy). Results were analyzed using Sequence Detection System software (SDS, Applied Biosystems).
Generation of double-stranded DNA was measured in real time by the increase in fluorescence due to the spontaneous binding of SYBR green. Target genes Ct values were normalized against GAPDH. Data were analyzed using the $2^{-\Delta\Delta C}$ method (Livak, Pfall) and expressed as fold change compared to POL layer.

6. Statistical analysis

Statistical analysis was performed using Student's *t* test, and differences were considered to be statistically significant when a $p < 0.05$ was obtained. Each experiment shown in this study was repeated three different times to be considered statistically significant.

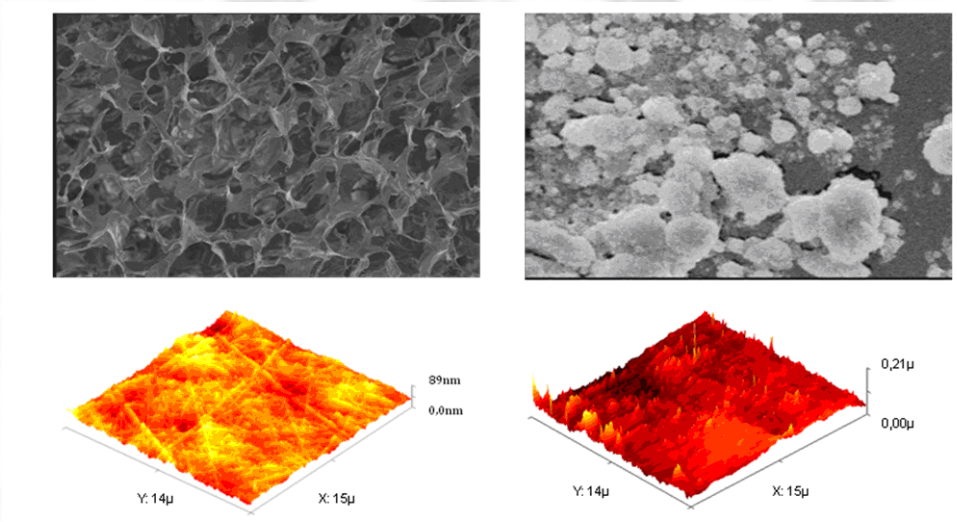
Results

Surface properties

The roughness of both substrate surfaces was evaluated by SEM and AFM. The nanometric surface roughness examined with AFM was 49.7 rms;nm for POL/nHA and 6.3 rms;nm POL (Table 1). Thus the plate covered in POL/nHA showed a 7.89 fold higher nanometric superficial roughness than POL alone [Figure 2 (A-D)].

Additionally, the morphology of the nHA particles, in rod-like shape 70-90 nm in diameter could be seen by SEM.

Fig. 2 Scanning electron microscopy (A, B) and atomic force microscopy (C, D) images of the POL substrates (A, C) and POL/nHA (B, D). The major nanometric superficial roughness is observed at POL/nHA level.



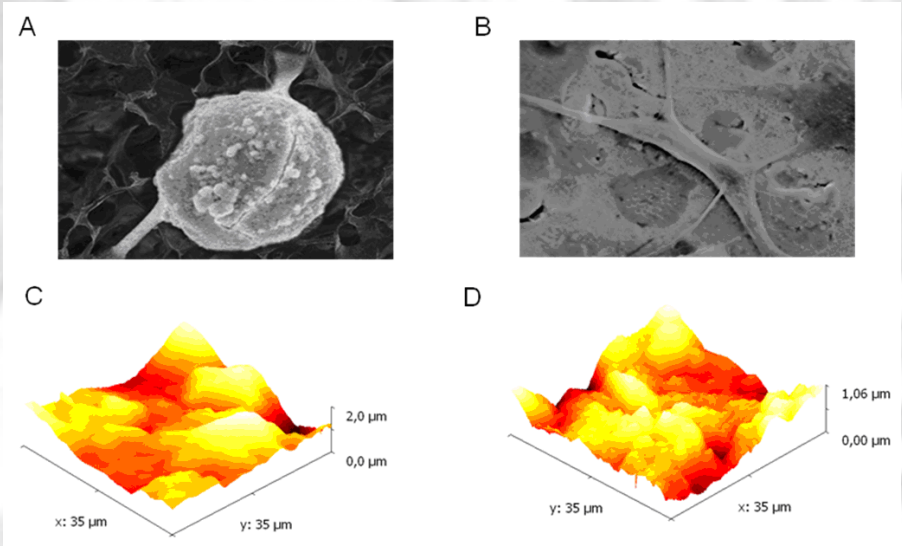
Effect of nanohydroxyapatite on osteoblasts adhesion and proliferation

After plating on layers covered whit POL and POL/nHA, the potential morphological modifications of hOBs were evaluated.

After 24 hours of incubation, cells plated on POL/nHA had firmly adhered to the substrate as demonstrated by the presence of numerous filopodia, whereas those plated on POL had maintained a spherical form (Fig. 3, A-B), suggesting that the particles of nHA considerably improved osteoblastic cells adhesion and spreading.

Additionally, images obtained by AFM showed that hOBs cultured on POL/nHA adhered in a substantially stronger manner than on POL alone, as shown by the increase in nanometric superficial roughness (Fig. 3, C-D).

Fig. 3 Scanning electron microscopy (SEM) and atomic force microscopy (AFM): cells grown on POL appear spherical with retracted fibres (A, C); cells grown on POL/nHA appear elongated and with many filopodia (B, D).



Effects of nanohydroxyapatite on gene expression of differentiation of human osteoblasts

Cells grew on both substrates (POL e POL/nHA) for 14 day. At the end of this incubation, RNA was extracted and a qRT-PCR carried out for the assessment of specific osteoblastic differentiation genes (Table 3) such as bone morphogenetic protein -2,-5,-7 (BMP-2,-5,-7), alkaline phosphatase (ALP), A2 pro-collagen type 1 chain (COLL-1A2), Osteocalcin (OC) and Osteonectin (ON).

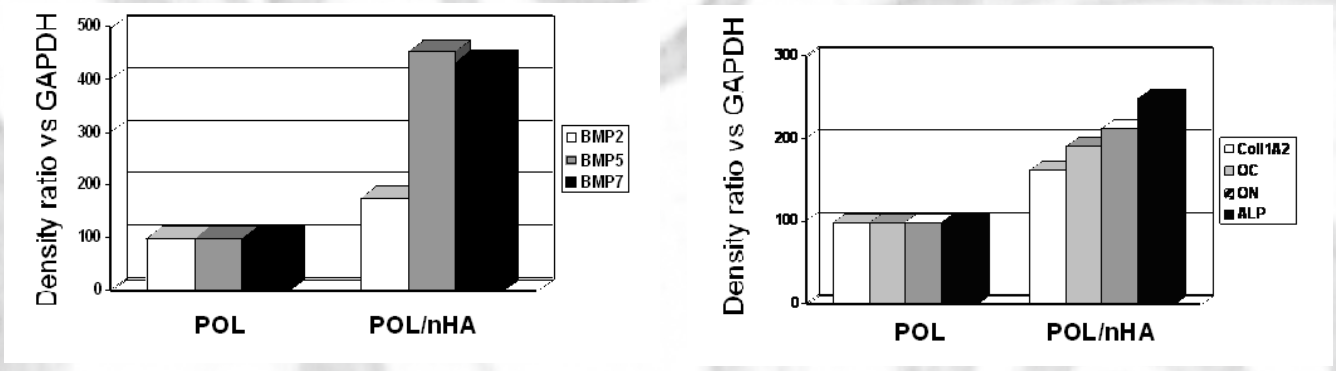
In particular, osteoblasts differentiation was evaluated based on the expression of bone morphogenetic proteins (BMPs), alkaline phosphatase (ALP), A2 pro-collagen type 1 chain (COLL-1A2), Osteocalcin (OC) and Osteonectin (ON). This choice was based on the evidence that the differentiated osteoblasts are known to synthesize and secrete these specific bone matrix proteins that have been proven to be particularly useful as osteogenic markers[10].

As shown in Figure 4, BMP-2 increased by approximately 1.7 times in hOBs cultured on POL/nHA substrates, while BMP-5 expression was increased by 4.5 times and BMP-7 by 4.3 times.

We then examined the expression of skeletal extracellular matrix proteins. Interestingly COLL-1A2, OC and ON expression in hOBs grown on POL/nHA was respectively 1.6, 1.9 and 2.1 times higher than cells gown on POL. Interestingly, the expression of the enzyme ALP was also increased 2.5 times in hOBs grown on POL7nHA, strongly suggesting that the differentiation of these cells is highly stimulated in these culture conditions.

All these results strongly indicate that, in a 14 days time interval, the POL/nHA substrate is able to significantly modulate the expression of all the analysed osteoblastic genes, therefore strongly suggesting a positive effect on cell differentiation and thus, potentially, leading to bone formation.

Fig. 4 (a), (b) Gene expression of hOBs placed in culture in POL and POL/nHA. Cells were incubated for 14 days on each substrate, before RNA extraction and generation of cDNA, as described in "Materials and Methods" section. cDNA was then subjected to Real Time – PCR analysis, to determine the expression of each mRNA species, relatively to GAPDH, using cT method. These data are representative of one of the three experiment performed.



Discussion

IIn the last three decades, hydroxyapatite has been widely investigated among all the bone grafting substitutes in periodontal regeneration [15,4]. Nanotechnology introduced by Eric Drexler in 1976 as *"...a technology at molecular level which could allow us to place each atom where we want it to be"* made it possible to obtain nanosized HA particles performing better than micro-sized HA in bone regenerative surgery [9]. These materials are designed to mimic the extracellular matrix of bone in size and structure [16]. HA alloplastic grafts are known mainly for their osteoconductive properties as they had not resulted in clinical and in vitro remarkable bone regenerative properties despite the similar chemical composition with the bone[5]. A recently published study reported that nHA particles act in a manner similar to bone morphogenetic protein-7, speculating potential osteoinductive properties[9].

Osteoblasts are the main cells responsible for the formation of alveolar bone, although they cannot

migrate and proliferate in a site where bone regeneration is required. In fact, their contribution involves production of growth factors and stimulation of the migration and differentiation of mesenchymal progenitor cells towards the osteoblast lineage[17]. The present in vitro study demonstrated that nHA stimulates the on-site osteoblasts to produce growth factors (i.e. bone morphogenetic proteins -2, -5, -7) which are known to initiate the entire process of bone formation starting from the progenitor cells.

Indeed, several types of BMPs, which belong to the transforming growth factor (TGF)-β-family have been investigated and characterized for their modulatory role in bone tissue homeostasis. BMP-2 and BMP-7 are of particular interest, since they are naturally released in response to trauma or in bone remodelling and are, to date, the only known inductive agents[18]. Stimulation of BMP-2, 5 and 7 secretion is an important feature as these proteins are the most potent growth factors enhancing bone formation. Albrektsson & Johansson suggested that pre-existing osteoblastic cells only contribute to a minor portion of the new bone needed in a fracture-healing situation and that the recruitment of immature cells and their differentiation to osteoblasts are, likely, the basic and crucial mechanism regulating bone healing [18]. Moreover, osteoblasts are located on the bone surface, meaning that the grafting material will be in contact with them when applied to induce bone regeneration. This rationale was followed when selecting the type of cells to evaluate the response to nHA. The results presented herein showed that nHA possesses osteoinductive properties, as a material able to induce secretion of specific factors, such as BMPs, which when released by the stimulated osteoblast in the site of bone defect, might be able to improve bone regeneration.

Moreover, surface roughness of graft material is considered an important characteristic related to the induction of bone formation[9]. According to physical and mechanical principles a substrate with superficial roughness shows a higher capacity for adhesion when compared to a smooth surface. ESM, AFM images showed that nHA hold a rougher surface than the control substrate surface and favoured the adhesion and proliferation of human osteoblasts better than polylysine alone. This results are in agreement with those reported by Thian et al showing that nHA enhanced adhesion and spreading in a human osteoblast–like cell model[19]. More recently, Liu et al., re-evaluated the adhesion and proliferation induction of nHA on human osteoblast–line MG-63 cell lines[10]. Using a model previously used (dispersion of particles of nHA in poli-ethylene-glicol-dimethacrylate or nHA associated with a polycarbonate, polyamide, polysaccharide) [20, 21, 22, 23,24], the authors demonstrated that nHA can significantly increase human osteoblasts adhesion, proliferation and differentiation stimulating bone formation by influencing the recruitment of progenitor cells by the on-site bone osteoblasts in contact with this material. These in vitro findings could explain, at least in part, the new bone formation obtained in bone defects filled with nHA grafting material.

Apart from these encouraging data, up to date there is only one clinical trial showing the potential benefit, up to six months, of the use of nHA in infrabony defects in humans[11]. This poses the basis for future development in the understanding and potential usage effect of nHA in periodontal bone regeneration and its role in the overall periodontal reattachment processes, including the formation of new cementum and new periodontal ligament. However further studies are needed to fully characterize the role of this promising nanomaterial in the clinical situation.

Source of support: This study was carried without any financial support.

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