A Xenogenic Bone Derivative as a Potential Adjuvant for Bone Regeneration and Implant Osseointegration: An *In Vitro* Study

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Abstract Several clinical conditions may limit the success of bone regeneration and/or implant osseointegration. For this reason, many compounds have been tested for their ability to stimulate this biological process. Synthetic hydroxyapatite (HA), mimicking natural bone hydroxyapatite, and extra-cellular matrix proteins, such as type I collagen, are potential candidates. However, the synthetic origin of HA and the denaturing conditions required for extracting collagen from skin and derma are sources of potential drawbacks. This study examines the *in vitro* effects of a natural bone derivative (NBD) extracted from equine bone and containing both natural, non-synthetic bone hydroxyapatite and native, non-denatured, type I bone collagen as a possible active compound for stimulating bone regeneration and implant osseointegration. The activity of NBD was tested on bone marrow stromal cells (BMSCs), evaluating their growth/viability by the methylthiazol tetrazolium (MTT) assay and their migration potential by a scratch assay. Moreover, expression of the hyaluronic acid receptor (CD44) and the C-X-C chemokine receptor type 4 (CXCR4, CD184) on the surface of BMSCs was assessed by flow cytometry, and the release of Transforming Growth Factor (TGF)- β , Interleukin (IL)-1 α and IL-6 was quantified using an enzyme-linked immunosorbent assay (ELISA). The effect of NBD-coated implants on human osteoblasts was tested by measuring alkaline phosphatase (ALP) activity with the p-nitrophenyl phosphate (pNPP) degradation test. NBD stimulated BMSC growth/viability, migration, CD184 surface expression and the release of TGF- β 1. NBD-coated implants increased ALP activity of human osteoblasts. These results indicate that NBD may be an adjuvant to accelerate both bone regeneration and osseointegration.

Keywords Osseointegration · Type I collagen · Dental implants · Implant coating · Hydroxyapatite

1 Introduction

Prosthetic-guided implant treatment in recent years has become the standard procedure to rehabilitate edentulous patients [1]. This also often involves the application of bone augmentation procedures. Implant placement is safe and effective, with success rates dependent on the position of the surgical site, anatomical conditions and bone density at the implant site [2]. The bone volume to be regenerated

and the amount of vital bone surrounding the defect are both critical prognostic factors concerning bone augmentation [3].

Both bone augmentation and osseointegration of titanium implants are based on biological processes such as the formation of new blood vessels, osteoblast differentiation, osteoblast and osteoclast adhesion, viability and activity, as well as active bone remodelling [4]. Many natural and synthetic organic and inorganic molecules, including small amino acid chains [5], entire molecules such as DNA [6], bone morphogenetic proteins (BMPs) [7], vitamins [8] and other factors have been tested, not only as bone stimulating factors, but also as additives for bone grafts or as active coatings for dental implants.

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Extracellular matrix (ECM) proteins have been regarded as candidates for effective stimulation of bone regeneration and as bioactive coatings given their crucial role in regulating cellular bone behaviour in terms of adhesion, proliferation, migration, morphology, gene expression, cell survival and functions. Type I collagen is one of the major constituents of ECM and has been shown to be both the physical substrate for mineralization [9] and a positive promoter of many biological processes that lead to bone regeneration such as cell adhesion, differentiation and growth factor co-activation, among others [10, 11].

Type I collagen from animal derma or skin, including denatured forms, has been applied as a coating to dental fixtures and has shown promising results in experiments both *in vitro* and *in vivo*. *In vitro*, denatured collagen-coated dental implants stimulated osteoblast-like differentiation and gene expression in human mesenchymal cell lines [12]. In *in vivo* models, this coating has been shown to increase bone-to-implant contact (BIC) area over short and long-term periods [13, 14]. Theoretically, collagen extracted under non-denaturing conditions should produce superior effects, since the protein would be in its native tridimensional conformation, and the regular collagen fibre structure would be preserved.

Among inorganic molecules, hydroxyapatite (HA) is widely used as a scaffold for bone regeneration given its osteoconductive properties. Further, coating titanium implants with synthetic HA, which mimics the main inorganic component of bone ECM, improved the mechanical strength of bone bonding in experimental and clinical settings [15]. Potential drawbacks for the use of HA include the *in vitro* inhibition of osteoblasts, the *in vivo* induction of a foreign body reaction by HA particles encapsulated within the soft tissues [16, 17], and the persistence, after placement, of a HA layer around the implant that could favour peri-implantitis [18].

Recently, new advances in bone processing technology have made it possible to extract a natural bone derivative (NBD) containing both natural bone HA and native type I collagen from animal (mammal) bone. Coating of implants with this material has been shown to promote bone formation and osseointegration in an experimental animal model [19]. However, the cell mechanisms causing the increased bone deposition and osteointegration observed *in vivo* are still unknown.

The present study was therefore designed to investigate NBD in an *in vitro* setting with the aim of dissecting its biological activity at a cellular level, both alone and as a coating over titanium implants. Results are discussed in comparison with available published data.

2 Materials and methods

2.1 Natural bone derivative (NBD)

According to the manufacturer's reports (Bioteck S.p.A.), the NBD employed in the study consists of natural hydroxyapatite and type I bone collagen powder (0.12-mm) obtained by partial demineralization of equine bone. The enzymatic removal of equine antigens (Zymo-Teck, Bioteck, Vicenza, Italy) preserves collagen in its native state, as the selected enzymes do not target the structural part of collagen molecule but only its N- and C-telopeptides, and the process occurs at non-denaturing temperatures (37°C). Demineralization is performed at a mild acid concentration (HCl < 0.1 M). Upon request, the company provided the following additional information. NBD mineral phase exhibits the diffraction pattern of carbonate apatite $\text{Ca}_{9.55}(\text{PO}_4)_{5.52}(\text{CO}_3)_{0.48}(\text{CO}_3)_{1.157}$, as shown by an X-ray diffraction analysis (Department of Chemical Sciences, University of Padua, Italy). To assess NBD protein composition, SDS polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed, allowing the identification of type I collagen trimers, dimers, α -1 and α -2 chains only. Type I collagen is therefore the only protein identified in NBD; the available data suggest that its native structure is preserved. The manufacturer agrees to provide the above-mentioned raw data if requested.

2.2 Implants and coating

Screw-shaped titanium implants, featuring a sand-blasted, double-etched surface, were used. Fixtures were 4 mm wide and 8.5 mm long and were coated with NBD by a proprietary coating process (Enhanta Bone Mimic Technology, Akimex, Vicenza, Italy). The bone powder was suspended in physiological solution to saturation (1 mg/ml) and applied on implant surfaces using a brush. Complete adhesion of the NBD was achieved by a lyophilisation stage. Implants were finally sterilized with beta-irradiation (25 kGy, Bioster, Bergamo, Italy). This proprietary process allows for effective coating of dental implants, resulting in stable NBD on the implant surface under non-denaturing conditions.

2.3 Human bone marrow stromal cell culture

Small mandibular bone samples were collected from four donor patients who were undergoing periodontal surgery. The patients signed a written informed consent form. Cells from the mandibular bone samples were dispersed, and bone marrow mononuclear cells (BMMCs) were isolated on a Ficoll-Hypaque density gradient. Human bone marrow stromal cells (BMSCs) were obtained from plastic-adherent

BMSCs and expanded at 37°C, 5% CO₂ in Iscove's modified Dulbecco's medium supplemented with 10% Fetal Calf Serum (FCS, Sigma Aldrich, St. Louis, USA) (complete medium). *Ex-vivo* study procedures complied with the Helsinki Declaration.

2.4 BMSC growth/survival assay

BMSCs from two different donors were seeded at an initial concentration of 1×10^4 cells/well in 96-well plates in complete medium. After 24 h, the culture medium was replaced with a fresh one, and cells were exposed to different concentrations (0, 0.5, 5.0, 50.0, and 500 mg/ml) of NBD for 24, 48 or 72 h. Cells cultured without NBD were used as negative control. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to evaluate cell metabolic activity as follows. During the last 4 h of incubation, 10 µl of a 5 mg/ml stock of MTT (Sigma Aldrich) were added to allow MTT formazan formation. Medium and MTT were replaced with 100 µl of acid isopropanol (0.04 N HCl in isopropanol) to dissolve the formazan crystals. After 30 minutes, the optical density (OD) at a wavelength of 570 nm was determined using a microplate reader. For each BMSC preparation, three different experiments were performed in triplicate. Dose-response curves were determined using the mean OD $\pm$ standard deviation (SD).

2.5 Release of transforming growth factor (TGF)- β 1, TGF- β 2, interleukin (IL)-1 α and IL-6 from BMSCs

Cell-free supernatants from four separate BMSC preparations, cultured in duplicate for 48 h with or without NBD (50 mg/ml), were assayed for TGF- β 1, TGF- β 2, IL-1 α and IL-6 using commercially available ELISA kits (R&D Systems, Abingdon, UK and Orgenium Laboratories, Vantaa, Finland). The minimum concentrations of TGF- β 1, TGF- β 2, IL-1 α and IL-6 detectable were 50 pg/ml, 7 pg/ml, 4 pg/ml and 2 pg/ml, respectively. Results are shown as mean $\pm$ SD.

2.6 Flow cytometric analysis

The surface expression of the hyaluronic acid receptor (CD44) and the C-X-C chemokine receptor type 4 (CXCR4, CD184) was assessed on BMSC preparations after 3 days of culture in complete medium with or without NBD (50 ng/ml). Cells were stained for 30 min at 4°C with Phycoerythrin (PE)-conjugated anti-CD44 and anti-CXCR4 mouse monoclonal antibodies (BD Biosciences, San Diego, CA, USA). The corresponding isotype control antibody served as negative control. After washing with

ice-cold phosphate buffered saline (PBS, Sigma Aldrich) supplemented with 1% bovine serum albumin, cells were analysed in a Coulter Epics XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA) for a minimum of 10,000 total events. Data were analysed using EXPO 32 Analysis software (Beckman Coulter). The percentage of positive cells was calculated. Results are given as the mean $\pm$ SD from three independent experiments.

2.7 In vitro scratch assay

BMSCs from three different donors were seeded in 24-wells plates (Nunc, Roskilde, Denmark; 3×10^5 cells per well) and grown until they formed a confluent cell monolayer. A straight line approximately 0.5 mm wide was scratched in the monolayer using a 100 µl pipet tip, and the cells were rinsed with PBS to remove the debris and smooth the edge of the scratch. 500 µl of complete medium was added to each plate with or without NBD (50 mg/ml); the plates were incubated at 37°C and 5% CO₂ for 48 h. Experiments were performed in triplicate. Cells were monitored by collecting digitized images at various time intervals (0, 24, 48 h) after the wound scratch using an inverted microscope and a digital camera (Leica Microsystems, Wetzlar, Germany). To obtain the same field during the image acquisition, a mark was created close to the scratch and used as a reference point. Distances between one side and the other of the scratch were measured using Photoshop CS3 software (Adobe Systems, Inc., San Jose, USA).

2.8 Human osteoblast culture

Bone samples, placed in 35-mm tissue-culture Petri dishes containing α -Minimal Essential Medium (α -MEM, Sigma Aldrich, USA) and supplemented with 10% FCS, L-glutamine, penicillin, streptomycin, and amphotericin (Sigma Aldrich), were cultured at 37°C, 5% CO₂. Osteoblast-like cells started growing after 7–10 days. Cultures were fed weekly by replacing half of the medium until confluence was achieved. The nature of these cultured osteoblast cells was determined by their typical morphology after Giemsa-Wright staining.

2.9 Measurement of alkaline phosphatase (ALP) activity

Osteoblasts were cultured in 35-mm dishes containing NBD-coated or uncoated implants (one per Petri dish) for 5 days as previously described [20]. After incubation, cells were washed with a Ca²⁺ and Mg²⁺-free PBS, suspended in 0.05% Triton X-100 containing 1 mM MgCl₂ and sonicated for 30 seconds. The homogenates were centrifuged

at 1,000 × g for 15 minutes, and the supernatants were used to assay ALP activity with p-nitrophenyl phosphate (pNPP) as substrate, as described by Lowry et al [21]. One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 nmol of the substrate per minute per mg of protein. All conditions were tested in two different experiments in triplicate. Results are shown as mean ± SD.

2.10 Statistical analysis

The statistical significance of the results was evaluated using the paired Student's t-test. Results found in the treated cells were considered significantly different from the untreated controls when $p < 0.05$. Statistical analyses were performed using SPSS for Windows (SPSS Inc, USA) and Excel2007 (Microsoft Corp, USA).

3 Results

3.1 Effect of NBD on BMSC viability

Among the first cells recruited in bone augmentation and at implant placement sites are BMSCs. Therefore, we first evaluated the cytotoxic potential of NBD on these cells. In the dose range of 0.5–500 mg/ml, the NBD did not cause any cytotoxicity at any exposure time (Fig. 1). On the

Fig. 1 NBD activity on *in vitro* growth/viability of BMSCs. Cells (1×10^4 /well) were incubated with increasing concentrations of NBD for 24, 48 and 72 h at 37°C in 5% CO₂. Cell growth/viability was monitored by MTT assay. OD values are expressed as means ± SD of three different experiments performed in triplicate. *** $p < 0.001$

contrary, NBD appeared to promote cell viability/proliferation, based on the capacity of living cells to reduce MTT to formazan. In fact, when BMSCs were treated for 48 h, NBD exhibited a dose-dependent activity, with significant stimulation starting from a concentration of 5 mg/ml (23 ± 12 % increase versus control) and peaking at the 500 mg/ml dose (60 ± 10 % increase versus control). The cell survival/growth-promoting capacity of NBD was partially retained 72 h after treatment at the highest concentration (21 ± 9 % increase versus control).

3.2 Effect of NBD on the release of TGF-β1, TGF-β2, IL-1α and IL-6

Since TGF-β has been shown to exert a pro-mitogenic effect on fibroblasts [20], and both IL-1α and IL-6 seem to play a role in initiating the bone repair cascade and remodelling [22], the effect of NBD treatment on the production of these cytokines was evaluated. As shown in Table 1, cultured BMSCs spontaneously secreted TGF-β1 and, to a lesser extent, TGF-β2, IL-1α and IL-6. After 48 h incubation with NBD, BMSCs released significantly higher amounts of TGF-β1 ($p < 0.001$), whereas the levels of TGF-β2, IL-1α and IL-6 remained unchanged compared to the basal condition ($p = 0.056$, $p = 0.638$, and $p = 0.840$, respectively; Table 1).

3.3 Effect of NBD on CD44 and CXCR4 expression in BMSCs

Homing of BMSCs to regenerating sites is mediated by ECM and inflammatory factors, like chemokines [23]; therefore, the ability of NBD in modulating CD44 and CXCR4 expression was evaluated. Both receptors are expressed on the surface of BMSCs under basal conditions (Table 2). When exposed to NBD for 72 h, the percentage of CXCR4 positive cells significantly increased from an average of 8.42 to 26.03 ($p = 0.032$; Table 2; Fig. 2). Conversely, NBD did not modulate CD44 expression, which remained stable in both of the experimental conditions ($p = 0.204$; Table 2; Fig. 2).

3.4 Effect of NBD on BMSC migration

The potential effect of NBD on BMSC migration was assessed using an *in vitro* scratch assay on BMSCs after exposition or no exposition to NBD. Cell behaviour was monitored by measuring the gap distance between the two margins of the scratch over time. As a result of cell migration, this distance decreased in both treated and untreated cells 24 h after the scratch (35 ± 10 vs 22 ± 4.72 pixels/μm, $p = 0.085$). After 48 h, it was further reduced, reaching a significantly shorter distance in the NBD-treated

Table 1 Effect of NBD on the release of TGF- β 1, TGF- β 2, IL-1 α and IL-6 from BMSCs as assessed by ELISA

Experimental conditions	TGF- β 1 (pg/ml)	TGF- β 2 (pg/ml)	IL-1 α (pg/ml)	IL-6 (pg/ml)
BMSCs	164.7 $\pm$ 34 ^a	12.2 $\pm$ 4.0	72.11 $\pm$ 21	14.02 $\pm$ 1.6
NBD-treated BMSCs	223.2 $\pm$ 29.5	19.2 $\pm$ 5.5	66.13 $\pm$ 4.8	14.37 $\pm$ 1.4
	$p < 0.001$	$p = 0.056$	$p = 0.638$	$p = 0.840$

p values versus control (BMSCs) are indicated

^a Mean $\pm$ SD of cytokine amounts measured in four different experiments performed in duplicate

Table 2 Effect of NBD on BMSC CD44 and CXCR4 surface expression as assessed by flow cytometry

Experimental conditions	(Mean $\pm$ SD % of positive cells)	
	CD44	CXCR4
BMSCs	1.30 $\pm$ 0.51 ^a	8.42 $\pm$ 2.10
NBD-treated BMSCs	1.79 $\pm$ 0.55	26.03 $\pm$ 10.2
	$p = 0.204$	$p = 0.032$

p values versus control (BMSC) are indicated

^a Mean $\pm$ SD of three different experiments

cells compared to controls (24 $\pm$ 7 vs 12 $\pm$ 3 pixels/ μ m, $p = 0.041$) (Fig. 3).

3.5 Effect of NBD-coated implants on osteoblastic ALP activity

The potential osteoinductive properties of NBD were investigated by measuring the levels of ALP activity, an early marker of bone deposition. The activity of ALP of primary osteoblasts significantly increased after 5 days of incubation with NBD-coated implants (38.1 $\pm$ 3.7 nmol of pNPP/min/mg vs 22.3 $\pm$ 3.3 nmol of pNPP/min/mg, $p = 0.008$).

4 Discussion

The NBD evaluated in the present study showed neither toxicity on BMSCs at all concentrations tested (from 0.5 to 500 mg/ml) nor on osteoblasts exposed to implants coated with the bone derivative. Conversely, NBD stimulated the viability/growth of BMSCs in a dose-dependent manner, with the maximum stimulatory effect achieved after 48 hours of incubation. This outcome was not observed when the same assays were performed with denatured type I collagen-coated implants [24, 25], even if on a different cell type (osteoblast-like Saos-2 cells). BMSCs are adherent cells of non-hematopoietic origin that are capable of self-renewal and can differentiate into several phenotypes, including bone. A direct involvement of BMSCs in implant osseointegration has been shown in animal models [26, 27]. The positive effect of NBD on BMSC viability and growth

Fig. 2 Exposure to NBD increases BMSC migration in *in vitro* scratch assay. **A** BMSC monolayers were grown in 24-well plates with or without NBD (50 mg/ml) and scraped in the middle of the well. Images were acquired at 0, 24 and 48 h after the scratch using a light microscope (magnification 10X). Representative pictures of one experiment are shown. **B** histogram of the mean gap distances $\pm$ SD measured between one side of scratch and the other. Three measurements were collected and averaged for each experimental condition. p -values vs control (BMSCs) are indicated

are in line with the findings documented when NBD was applied *in vivo*, where it was found to improve both BIC and trabecular thickness [19].

Fig. 3 Surface expression of CD44 and CXCR4 on BMSCs. Representative flow cytometric profiles of BMSCs after 72 h incubation with NBD (B and D) and their corresponding untreated control (A and C). M1 indicates positive events. Positivity for CD44 (A and B) or CXCR4 (C and D) is shown as a percentage of the total cell population

The stimulating effect observed on BMSCs was transitory and peaked after 48 h of incubation. This could be interpreted either as a loss of the NBD activity at longer treatment times or, more likely, as a transient response of the biological model (BMSCs) to NBD at the effective concentration. This early response is consistent with the findings already published *in vivo* in which the kinetics of bone trabeculae deposition was investigated through the administration of different dyes at fixed intervals, allowing visualization of the amount and pattern of the deposited bone at each time interval [19]. Cecconi et al. found that most of the effect of NBD on BIC augmentation occurred in the first two weeks after surgery, corresponding to the earliest stages of bone regeneration and implant osseointegration [19].

A number of local and systemic factors have been found to initiate and regulate the cascade of events during bone repair. Such molecules include members of TGF- β superfamily, pro-inflammatory cytokines [IL-1, IL-6 and tumour necrosis factor (TNF)- α], BMPs, as well as other growth and angiogenic factors, forming complex networks with several signalling pathways [28].

In the present study, the ability of NBD to induce TGF- β 1 secretion in BMSCs was demonstrated. TGF- β 1 has been

shown to have pleiotropic effects on several biological pathways concerning bone healing. By inhibiting osteoclast formation and activity and inducing osteoblast differentiation and proliferation, TGF- β 1 acts as a central component of the coupling of bone formation to resorption during bone remodelling [29]. Moreover, TGF- β 1 orchestrates leukocyte recruitment [30], promotes matrix synthesis, and has chemotactic and mitogenic activities on mesenchymal cells [31–33]. There is evidence that TGF- β 1 levels around dental implants are positively correlated to the osseointegration rate of fixtures [34]. Moreover, when experimentally added to implants positioned in animal models, TGF- β 1 induced greater BIC compared to controls, even if it did not accelerate the rate of osseointegration [35].

The proinflammatory cytokines IL-1 and IL-6 are involved in fracture repair. They are secreted by macrophages, inflammatory cells and cells of mesenchymal origin immediately after injury and can induce a downstream response by recruiting other inflammatory cells, enhancing ECM synthesis and stimulating angiogenesis [22]. Our data show that NBD does not affect the production of both of these cytokines by BMSCs *in vitro*.

In light of these findings and of the published literature, the authors speculate that the increased BIC and the higher

thickness of the newly formed bone around the NBD-coated implants positioned in animal models [19] might be mediated by activation of the TGF- β 1 signalling pathway. Furthermore, as collagen-induced platelet activation causes TGF- β 1 release [36], another possible action of NBD *in vivo* might be the stimulation of TGF- β 1 secretion also from these cells. In the clinical setting, this might imply that the use of NBD can promote both faster bone tissue deposition and implant osseointegration, thus accelerating implant stabilization, allowing early or immediate loading of the fixtures and improving the probability of successful rehabilitation.

However, besides promoting osteogenesis, TGF- β 1 could potentially have also an inhibitory effect on osteoblast differentiation by mechanisms depending on its concentration, density and differentiation stage of the target cells [37]. Further complications and side effects possibly associated to TGF- β 1 include the induction of an immunosuppressive condition [38]. Hence, more detailed studies on the possible TGF- β -mediated side effects of NBD seem to be advisable.

The BMSCs involved in tissue reparative processes have the ability to migrate to sites of damaged or inflamed tissues and cross the endothelial barriers to participate in tissue regeneration. Even if molecular mechanisms underlying BMSC homing remain poorly understood, evidence suggests that local up-regulation of chemo-attractants, cytokines, integrins and ECM proteins play critical roles. In particular, the G protein-coupled receptor CXCR4 and its ligand CXCL12 (C-X-C motif chemokine 12, also known as stromal cell-derived factor 1, SDF-1) exert an important homeostatic function by mediating the homing of stem/progenitor cells in the BM and regulating their mobilization into peripheral tissues after injury or stress [23].

The involvement of the CXCL12/CXCR4 axis on BMSCs in bone repair has been elucidated by Kitaori et al. [39]. CXCL12 was found to be overexpressed in the periosteum of murine bone graft models during the early phase of endochondral bone repair. Moreover, CXCL12 inhibition with an anti-CXCL12 neutralizing antibody was followed by reduced new bone formation *in vivo* [39]. Overall, these observations indicate that BMSC recruitment to bone repair sites occurs via the CXCL12/CXCR4 axis and that this step is critical for endochondral bone repair.

Systemic BMSCs were found to home also to the root canal and participate in dental-pulp-like tissue regeneration in an *ex vivo* study performed on human roots implanted in mice [40]. Compared to a control group, intracanal application of CXCL12 was found to enhance both BMSC homing and angiogenesis, suggesting it may recruit progenitor cells in this specific district *in vivo*. In the present

study, exposure of BMSCs to NBD resulted in a robust up-regulation of CXCR4 but not of CD44 cell surface expression. This suggests that NBD might directly enhance or sensitize the responsiveness of BMSCs to CXCL12 gradient formation following acute bone tissue injury [41] without interfering with hyaluronic acid and its receptor, which are known to interact with CXCL12/ CXCR4 signalling in the regulation of cell motility [42]. Accordingly, the NBD-stimulated BMSCs exhibited better migration properties in an *in vitro* scratch assay. These observations are in line with the report showing that enhanced migratory capacity of BMSCs is associated to an enhanced ability to form new bone *in vivo* [43]. Overall, our findings provide a theoretical basis for NBD-dependent recruitment of BMSCs in which NBD enhances BMSC recruitment through CXCR4 upregulation. This could be potentially useful to accelerate bone formation and/or osteointegration.

Finally, NBD spread over dental implants as a coating significantly increased the ALP activity in human primary osteoblasts by 70.4% at 5 days of incubation. This effect was not observed when denatured type I collagen-coated implants were placed in contact with osteoblast-like cells (Saos-2) [24]. However, if denatured collagen solutions were given enough time to form collagen fibres before implant coating, ALP activity of Saos-2 cells increased by 44.4% at 3 days and by 35.3% at 7 days [25]. The greater stimulating effect observed in our experiments could be attributable to the conformation of the collagen, preserved in its native form in NBD.

In conclusion, the present study indicates that xenogenous NBD has no cytotoxic effects; on the contrary, it promotes BMSC viability. Moreover, NBD stimulates processes underlying bone regeneration and osseointegration such as osteoblastic ALP activity, TGF- β 1 secretion, BMSC migration and possibly homing to the NBD-enriched injured sites. We believe that these actions might be, in turn, some of the cellular mechanisms underlying its activity *in vivo*, where it was found to promote bone deposition and increase BIC area around NBD-coated implants. The cellular actions investigated here provide evidence that NBD can act as an adjuvant for bone regeneration procedures and, if applied on dental fixtures, as an enhancer of osseointegration.

The comparison of these data with the relevant published literature indicate that the native conformation of type I collagen might be a critical factor affecting the efficacy on the biological processes investigated. The potential impact of NBD activity in the clinical setting should be explored to confirm and further analyse the outcomes observed in animal experimental data.

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Compliance with ethical standards

Conflicts of interest The authors have no financial conflicts of interest.

Ethical statement Mandibular bone samples were collected from the lower jaw of two donor patients undergoing periodontal surgery. Because of the small sizes of the collected samples, sampling procedures did not cause any additional trauma to the operated jaw. Both of the donor patients gave their written informed consent. *Ex-vivo* study procedures complied with the Helsinki Declaration.

References

- Belser UC, Mericske-Stern R, Bernard JP, Taylor TD. Prosthetic management of the partially dentate patient with fixed implant restorations. *Clin Oral Implants Res.* 2000;11(Suppl 1):126–415.
- Jang HW, Kang JK, Lee K, Lee YS, Park PK. A retrospective study on related factors affecting the survival rate of dental implants. *J Adv Prosthodont.* 2011;3:204–15.
- Esposito M, Grusovin MG, Felice P, Karatzopoulos G, Worthington HV, Coulthard P. The efficacy of horizontal and vertical bone augmentation procedures for dental implants—a Cochrane systematic review. *Eur J Oral Implantol.* 2009;2:167–84.
- Mavrogenis AF, Dimitriou R, Parvizi J, Babis GC. Biology of implant osseointegration. *J Musculoskelet Neuronal Interact.* 2009;9:61–71.
- Kämmerer PW, Heller M, Brieger J, Klein MO, Al-Nawas B, Gabriel M. Immobilisation of linear and cyclic RGD-peptides on titanium surfaces and their impact on endothelial cell adhesion and proliferation. *Eur Cell Mater.* 2011;21:364–72.
- van den Beucken JJ, Walboomers XF, Leeuwenburgh SC, Vos MR, Sommerdijk NA, Nolte RJ, et al. Multilayered DNA coatings: in vitro bioactivity studies and effects on osteoblast-like cell behavior. *Acta Biomater.* 2007;3:587–96.
- Huh JB, Kim SE, Kim HE, Kang SS, Choi KH, Jeong CM, et al. Effects of anodized implants coated with *Escherichia coli*-derived rhBMP-2 in beagle dogs. *Int J Oral Maxillofac Surg.* 2012;41:1577–84.
- Cho YJ, Heo SJ, Koak JY, Kim SK, Lee SJ, Lee JH. Promotion of osseointegration of anodized titanium implants with a 1 α ,25-dihydroxyvitamin D3 submicron particle coating. *Int J Oral Maxillofac Implants.* 2011;26:1225–32.
- Marino AA, Becker RO. Evidence for epitaxy in the formation of collagen and apatite. *Nature.* 1970;226:652–3.
- Gungormus M. The effect on osteogenesis of type I collagen applied to experimental bone defects. *Dent Traumatol.* 2004;20:334–7.
- Liu G, Hu YY, Zhao JN, Wu SJ, Xiong Z, Lu R. Effect of type I collagen on the adhesion, proliferation, and osteoblastic gene expression of bone marrow-derived mesenchymal stem cells. *Chin J Traumatol.* 2004;7:358–62.
- Morra M, Cassinelli C, Cascardo G, Bollati D, Baena RR. Gene expression of markers of osteogenic differentiation of human mesenchymal cells on collagen I-modified microrough titanium surfaces. *J Biomed Mater Res A.* 2011;96:449–55.
- De Barros RR, Novaes AB Jr, Korn P, Queiroz A, de Almeida AL, Hintze V, et al. Bone formation in a local defect around dental implants coated with extracellular matrix components. *Clin Implant Dent Relat Res.* 2015;17:742–57.
- Sartori M, Giavaresi G, Parrilli A, Ferrari A, Aldini NN, Morra M, et al. Collagen type I coating stimulates bone regeneration and osteointegration of titanium implants in the osteopenic rat. *Int Orthop.* 2015;39:2041–52.
- Moroni A, Cadossi M, Romagnoli M, Faldini C, Giannini S. A biomechanical and histological analysis of standard versus hydroxyapatite-coated pins for external fixation. *J Biomed Mater Res B Appl Biomater.* 2008;86:417–21.
- Bloebaum RD, Beeks D, Dorr LD, Savory CG, DuPont JA, Hofmann AA. Complications with hydroxyapatite particulate separation in total hip arthroplasty. *Clin Orthop Relat Res.* 1994;298:19–26.
- Sun JS, Lin FH, Hung TY, Tsuang YH, Chang WH, Liu HC. The influence of hydroxyapatite particles on osteoclast cell activities. *J Biomed Mater Res.* 1999;45:311–21.
- Hanisch O, Cortella CA, Boskovic MM, James RA, Slots J, Wikesjö UM. Experimental peri-implant tissue breakdown around hydroxyapatite-coated implants. *J Periodontol.* 1997;68:59–66.
- Cecconi S, Mattioli-Belmonte M, Manzotti S, Orciani M, Piccioli A, Gigante A. Bone-derived titanium coating improves in vivo implant osseointegration in an experimental animal model. *J Biomed Mater Res B Appl Biomater.* 2014;102:303–10.
- Schierano G, Bellone G, Manzella C, Preti G, Emanuelli G. In vitro effect of transforming growth factor-beta on adhesion molecule expression by human gingival fibroblasts cultured in the presence of a titanium abutment. *J Periodontol.* 2001;72:1658–65.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem.* 1951;193:265–75.
- Mountziaris PM, Mikos AG. Modulation of the inflammatory response for enhanced bone tissue regeneration. *Tissue Eng B Rev.* 2008;14:179–86.
- Lataillade JJ, Clay D, Dupuy C, Rigal S, Jasmin C, Bourin P, et al. Chemokine SDF-1 enhances circulating CD34⁺ cell proliferation in synergy with cytokines: possible role in progenitor survival. *Blood.* 2000;95:756–68.
- Morra M, Cassinelli C, Cascardo G, Cahalan P, Cahalan L, Fini M, et al. Surface engineering of titanium by collagen immobilization. Surface characterization and in vitro and in vivo studies. *Biomaterials.* 2003;24:4639–54.
- Morra M, Cassinelli C, Cascardo G, Bollati D, Rodriguez Y, Baena R. Multifunctional implant surfaces: surface characterization and bone response to acid-etched Ti implants surface-modified by fibrillar collagen I. *J Biomed Mater Res A.* 2010;94:271–9.
- Xu B, Zhang J, Brewer E, Tu Q, Yu L, Tang J, et al. Osterix enhances BMSC-associated osseointegration of implants. *J Dent Res.* 2009;88:1003–7.
- Tsuchiya S, Hara K, Ikeno M, Okamoto Y, Hibi H, Ueda M. Rat bone marrow stromal cell-conditioned medium promotes early osseointegration of titanium implants. *Int J Oral Maxillofac Implants.* 2013;28:1360–9.
- Dimitriou R, Tsiridis E, Giannoudis PV. Current concepts of molecular aspects of bone healing. *Injury.* 2005;36:1392–404.
- Sun Y, Zhang W, Lu Y, Hu Y, Ma F, Cheng W. Role of transforming growth factor beta (TGF-beta) in repairing of bone defects. *Chin Med Sci J.* 1996;11:209–14.
- Bessa X, Elizalde JJ, Mitjans F, Piñol V, Miquel R, Panés J, et al. Leukocyte recruitment in colon cancer: role of cell adhesion molecules, nitric oxide, and transforming growth factor beta1. *Gastroenterology.* 2002;122:1122–32.
- Wan M, Li C, Zhen G, Jiao K, He W, Jia X, et al. Injury-activated transforming growth factor β controls mobilization of

- mesenchymal stem cells for tissue remodeling. *Stem Cells*. 2012;30:2498–511.
32. Anitua E, Tejero R, Zalduendo MM, Orive G. Plasma rich in growth factors promotes bone tissue regeneration by stimulating proliferation, migration, and autocrine secretion in primary human osteoblasts. *J Periodontol*. 2013;84:1180–90.
 33. Ota K, Quint P, Weivoda MM, Ruan M, Pederson L, Westendorf JJ, et al. Transforming growth factor beta 1 induces CXCL16 and leukemia inhibitory factor expression in osteoclasts to modulate migration of osteoblast progenitors. *Bone*. 2013;57:68–75.
 34. Schierano G, Canuto RA, Navone R, Peirone B, Martinasso G, Pagano M, et al. Biological factors involved in the osseointegration of oral titanium implants with different surfaces: a pilot study in minipigs. *J Periodontol*. 2005;76:1710–20.
 35. Clokie CM, Bell RC. Recombinant human transforming growth factor beta-1 and its effects on osseointegration. *J Craniofac Surg*. 2003;14:268–77.
 36. Baumgartner HR. Platelet interaction with collagen fibrils in flowing blood. I: reaction of human platelets with alpha chymotrypsin-digested subendothelium. *Thromb Haemost*. 1977;37:1–16.
 37. Kaji H, Naito J, Sowa H, Sugimoto T, Chihara K. Smad3 differently affects osteoblast differentiation depending upon its differentiation stage. *Horm Metab Res*. 2006;38:740–5.
 38. Kasagi S, Chen W. TGF-beta1 on osteoimmunology and the bone component cells. *Cell Biosci*. 2013;3:4.
 39. Kitaori T, Ito H, Schwarz EM, Tsutsumi R, Yoshitomi H, Oishi S, et al. Stromal cell-derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model. *Arthritis Rheum*. 2009;60:813–23.
 40. Zhang L-X, Shen L-L, Ge S-H, Wang L-M, Yu X-J, Xu Q-C, et al. Systemic BMSC homing in the regeneration of pulp-like tissue and the enhancing effect of stromal cell-derived factor-1 on BMSC homing. *Int J Clin Exp Pathol*. 2015;8:10261–71.
 41. Yellowley C. CXCL12/CXCR4 signaling and other recruitment and homing pathways in fracture repair. *Bonekey Rep*. 2013;2:300.
 42. Fuchs K, Hippe A, Schmaus A, Homey B, Sleeman JP, Orian-Rousseau V. Opposing effects of high- and low-molecular weight hyaluronan on CXCL12-induced CXCR4 signaling depend on CD44. *Cell Death Dis*. 2013;4:e819.
 43. Andersen RK, Zaher W, Larsen KH, Ditzel N, Drews K, Wruck W, et al. Association between in vivo bone formation and ex vivo migratory capacity of human bone marrow stromal cells. *Stem Cell Res Ther*. 2015;6:196.