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ORIGINAL ARTICLE

Histological evaluation of human intrabony periodontal defects treated with an unsintered nanocrystalline hydroxyapatite paste

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Abstract
Objectives The aim of the study was to clinically and histologically evaluate the healing of human intrabony defects treated with open flap surgery (OFD) and application of a new, resorbable, fully synthetic, unsintered, nanocrystalline, phase-pure hydroxyapatite (nano-HA).
Materials and methods Six patients, each of them displaying very advanced intrabony defects around teeth scheduled for extraction due to advanced chronic periodontitis and further prosthodontic considerations, were included in the study. Following local anaesthesia, mucoperiosteal flaps were reflected; the granulation tissue was removed, and the roots were meticulously debrided by hand and ultrasonic instruments. A notch was placed at the most apical extent of the calculus present on the root surface or at the most apical part of the defect (if no calculus was present) in order to serve as a reference for the histological evaluation. Following defect fill with nano-HA, the flaps were sutured by means of mattress sutures to allow primary intention healing. At 7 months after regenerative surgery, the teeth were extracted together with some of their surrounding soft and hard tissues and processed for histological analysis.
Results The postoperative healing was uneventful in all cases. At 7 months following surgery, mean PPD reduction and mean CAL gain measured 4.0±0.8 and 2.5±0.8 mm, respectively. The histological analysis revealed a healing predominantly characterized by epithelial downgrowth. Limited formation of new cementum with inserting connective tissue fibers and bone regeneration occurred in three out of the six biopsies (i.e. 0–0.86 and 0–1.33 mm, respectively). Complete resorption of the nano-HA was found in four out of the six biopsies. A few remnants of the graft particles (either surrounded by newly formed mineralized tissue or encapsulated in connective tissue) were found in two out of the six biopsies.
Conclusion Within their limits, the present results indicate that nano-HA has limited potential to promote periodontal regeneration in human intrabony defects.
Clinical relevance The clinical outcomes obtained following surgery with OFD+nano-HA may not reflect true periodontal regeneration.

Keywords Chronic periodontitis · Intrabony defects · Periodontal regeneration · Grafting materials · Hydroxyapatite · Human histology

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Introduction

Filling of periodontal defects with various types of bone grafts is a widely employed technique aiming to restore the lost periodontal attachment apparatus [1]. The use of certain types of grafting materials such as intra- or extraoral autogenous bone grafts, demineralized freeze dried bone allograft (DFDBA) or a natural bone mineral (NBM) has been shown to result not only in substantial clinical improvements evidenced by probing depth reduction, defect fill and

clinical attachment gain but also to promote, at least to some extent, formation of a new connective tissue attachment (i.e. new cementum with inserting collagen fibres) and of new alveolar bone [2–7]. The use of autogenous bone is limited by its source, unpredictable resorption and increase in patient morbidity, while concerns have been expressed related to the use of DFDBA and NBM related to their origin (i.e. human and bovine) and the theoretical risk for immune-mediated rejection of the graft and for transmission of infectious diseases [8–10]. It was anticipated that the use of synthetically produced bone substitute materials (i.e. alloplasts) may circumvent the mentioned disadvantages of autogenous, allogeneic or xenogeneic bone grafts [11]. The currently available data indeed suggest that in intrabony defects, the implantation of various types of alloplastic grafts in conjunction with open flap debridement may lead to significant improvements in the investigated clinical parameters [12]. On the other hand, the available histological evidence indicates that in human intrabony defects, the healing following grafting with alloplastic grafts is predominantly characterized by epithelial proliferation, connective tissue encapsulation of the graft particles and limited periodontal regeneration [13–19].

Recently, a new, fully synthetic, nanocrystalline, unsintered, phase-pure hydroxyapatite (nano-HA) has been suggested as a potential material for enhancing periodontal and bone regeneration since its chemical composition and crystalline structure correspond to the calcium phosphate component of natural bone and may have greater potential for resorption compared with sintered hydroxyapatite [20–23]. Case series in the field of orthopaedic reconstructive surgery have shown promising outcomes following nano-HA implantation [22]. In the dental field, case reports have demonstrated substantial clinical improvements following the use of nano-HA for sinus and ridge augmentation, and filling of peri-implant defects [23–25]. Furthermore, the results of two randomized controlled clinical studies evaluating the performance of nano-HA in intrabony periodontal defects have indicated statistically significantly higher clinical improvements following open flap debridement (OFD) and subsequent defect fill with nano-HA compared to OFD alone [26, 27].

Despite these promising results, to the best of our knowledge, no data from human histological studies are available on the healing of intrabony defects following regenerative surgery with nano-HA. Thus, at the time being, it is virtually unknown to what extent this grafting material may promote periodontal wound healing/regeneration in humans.

Therefore, the aim of the present study was to evaluate histologically the healing of human intrabony periodontal defects treated with OFD and nano-HA.

Materials and methods

Subject population

The study protocol was designed and performed according to the latest amendment of the Declaration of Helsinki [http://www.wma.net/en/30publications/10policies/b3/] and was approved by the Ethical Committee of Semmelweis University, Budapest, Hungary (TUKEB 12/2005). Each enrolled patient received verbal and written explanations of the research protocol prior to signing the informed consent form. Patients were recruited in the Department of Periodontology, Semmelweis University, Budapest, Hungary.

Six non-smoker patients (three females) with advanced chronic periodontitis were included in the study. Each of them participated with one advanced intrabony defect around teeth scheduled for extraction due to advanced destruction of the periodontal attachment apparatus and further prosthetic considerations. Each defect presented a probing depth of at least 6 mm and an intrabony component of at least 3 mm as visualized on the intraoral radiographs.

The selected teeth had some potential for periodontal regeneration as diagnosed clinically and radiographically. In every case, the decision to include the tooth in the study was based upon agreement between two clinicians who were not involved in the study.

All patients volunteered for the study and received verbal and written information about its purpose and possible risks and about the possibility to withdraw at any time. In every case written informed consent was obtained prior to the start of the study. The patients met the following inclusion criteria: a) age between 20 and 70 years, b) completed initial phase of periodontal therapy at least 6 weeks prior to surgery, c) full mouth plaque scores (FMPS)≤20 % [28], d) full mouth bleeding scores (FMBS)≤20 % [29], e) good compliance to follow-up visits and self-performed oral hygiene, f) legal ability to sign informed consent form, g) absence of untreated endodontic lesion, and e) absence of hypermobility and occlusal overload. The exclusion criteria were a) general medical history that contraindicates elective surgery and may affect treatment outcome (e.g. uncontrolled diabetes, osteoporosis, immunodeficiency), b) medication that may affect treatment outcome (e.g. high dose steroid, hormone replacement therapy, bisphosphonate, chemotherapy, immunosuppressant), c) systemic antibiotic treatment within 3 months prior to the current study, e) pregnancy during the experimental period, f) smoking within the past 5 years, g) history of irradiation in head and neck region, and h) previous periodontal surgery at the selected site.

Three months before surgery, all patients received oral hygiene instructions and full mouth supra- and subgingival

scaling in order to reduce the soft tissue inflammation. In order to reduce mobility when needed, the teeth were included in temporary bridge reconstructions.

The following clinical parameters were assessed prior to and 7 months after the surgical procedure using the same type of periodontal probe (PCPUNC 15, Hu-Friedy, Chicago, IL, USA): probing pocket depth (PPD) and clinical attachment level (CAL) (Fig. 1a, e). Measurements were rounded up to the nearest millimetre and were made at six sites per tooth: mesiobuccal (mb), midbuccal (b), distobuccal (db), mesiolingual (ml), midlingual (l) and distolingual (dl) by a calibrated investigator (LL) who was not the same as the surgeon. The cemento-enamel junction (CEJ) was used as the reference point. In cases where the CEJ was not visible, a restoration margin was used for these measurements. The site presenting the central part of the defect was included in the calculations. Calibration included CAL and PPD measurements on five periodontal patients with similar disease severity, but other than the patients enrolled in the study. Data were captured from six sites per tooth from all quadrants by the same way and same type of probe as described above. Measurements were repeated alike, 90 min apart. Calibration was accepted if at least 90 % of the collected figures were reproduced within a millimetre difference.

Standardized long cone radiographs were taken at baseline and at biopsy removal, utilizing commercial plastic film holder individualized by silicone putty impression material (Fig. 1b, f) [30].

Reconstructive periodontal surgery

All surgeries were performed by the same experienced periodontist (AH). Patients were asked to rinse with 0.2 % chlorhexidine (Curasept ADS 220, Curaden, Kriens, Switzerland) for 2 min just before perioral disinfection. In local anaesthesia (articain 80 mg+epinephrine 0.024 mg; Ultracain D-S forte, Aventis Pharma, Frankfurt am Main, Germany) mucoperiosteal flaps were reflected bucally and orally following intracrevicular incisions at the experimental site plus one to two teeth apart. No releasing incisions were deemed necessary. Granulation tissue was removed, and the roots were meticulously debrided by means of hand and ultrasonic instruments (Fig. 1c).

In all six defects, a notch was placed at the most apical part of the defects using a small round bur (1-mm diameter). In the presence of calculus, the notch would have been placed at the most apical part of it as per study protocol. Thus, any periodontal ligament tissue which later may develop coronally to this notch on the root surface will be de novo formed connective tissue and clearly distinguishable in histological sections of biopsies. No root conditioning or any other surface modifications were used.

During surgery, defect characteristics such as number of bony walls and depth of the intrabony component were recorded.

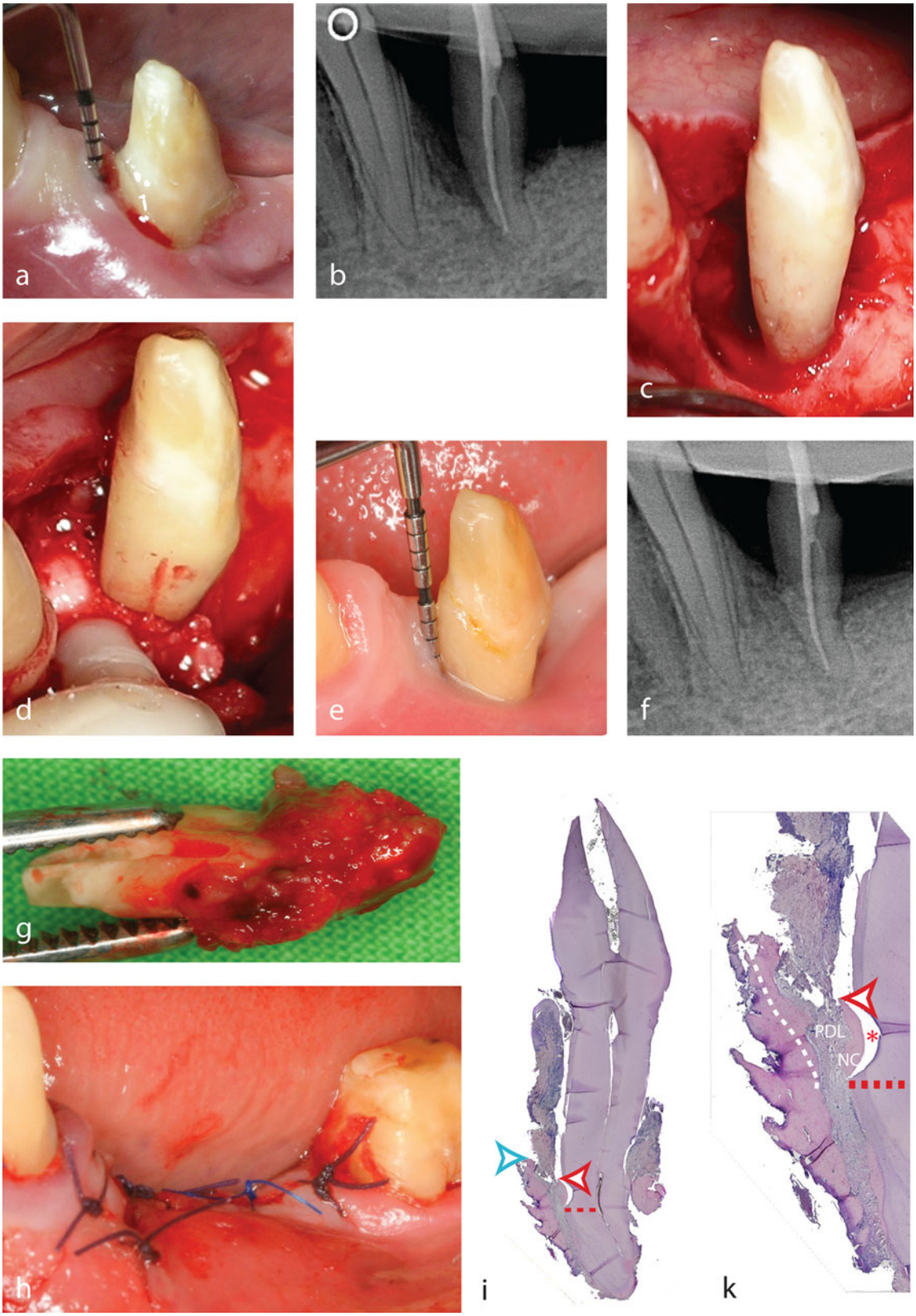
The intrabony defect was subsequently filled with the nano-HA (Ostim®, Heraeus Kulzer, Hanau, Germany) paste until the level of the alveolar crest, according to the manufacturer's instruction (Fig. 1d). The mucoperiosteal flaps were then repositioned and secured with suspended vertical mattress sutures (non-resorbable, monofilament; Dafilon 5/0, Braun Aesculap, Tuttlingen, Germany) in order to achieve tension free flap closure.

Postoperative care

All patients were postoperatively administered antibiotics (amoxicillin 500 mg+clavulanic acid 125 mg; Augmentin 675 mg, GlaxoSmithKline, Brentford, Middlesex, UK) three times daily for 7 days and painkiller (Diclofenac 75 mg; Diclofenac Duo, Pharmavit, Veresegyház, Hungary) according to individual need. Subjects were advised not to brush the surgical area but rinse with 0.2 % chlorhexidine two times daily for 90 s during the following period of 4 weeks. Sutures were removed at 14 days after surgery. The patients resumed tooth cleaning with the use of a soft brush at 4 weeks post-surgery. Additional appointments including oral hygiene instructions and with professional supragingival tooth cleaning were performed fortnightly during the first 12 postoperative weeks. After this period and until biopsy removal, recall appointments were scheduled monthly. Neither subgingival instrumentation nor periodontal probing was performed during the entire experimental period of 7 months.

Biopsy removal and histological processing

After a healing period of 7 months, the teeth were removed together with some of their surrounding periodontal tissues and immediately placed in 10 % buffered formalin for fixation (Fig. 1g). Subsequently, the flaps were closed with horizontal or vertical mattress sutures (Fig. 1h). The block biopsies were fixed in 10 % buffered formalin, decalcified in EDTA for a period of 4–6 weeks (depending on tooth/root volume) and dehydrated in graded series of ethanol. Immediately prior to embedding in paraffin, the roots/teeth were split in two along their long axis either in buccooral or mesiodistal direction (depending on the location of the deepest site of the defect) exactly at the notch indicating the deepest aspect of the intrabony defect (i.e. site of interest). Thus, each biopsy provided two specimen blocks while histological sections representing the deepest aspect of the defect (prior to treatment) were obtained without the need for extensive cutting. Twenty sections from each of the two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◀ **Fig. 1** **a** Mesial aspect of tooth 34 prior to surgery with OFD+nano-HA depicting a probing depth of 10 mm (defect No. 2 from Table 1). **b** Preoperative radiograph demonstrating the presence of a deep intrabony defect. **c** Following removal of granulation tissue and thorough scaling and root planing, the intraoperative situation revealed a deep one- and two-wall intrabony defect. **d** The intrabony component was filled with nano-HA. **e** At 7 months following surgery, a substantial reduction of probing depth was measured. **f** At 7 months, the intraoral radiograph revealed a hard tissue fill of the intrabony component. **g** Removed biopsy. **h** Complete flap closure, immediately after biopsy removal. **i** The histological evaluation revealed a healing predominantly characterized by a long junctional epithelium and limited regeneration of cementum and bone. *Red arrowhead*: coronal extension of new cementum, *blue arrowhead*: coronal extension of new bone, *red dotted line*: apical extension of the notch. Original magnification×5. **k** Higher magnification of the defect shown in **i**. Formation of new cementum (*NC*) and new periodontal ligament (*PDL*) was confined to the area of the notch. *Red arrowhead*: coronal extension of new cementum, *red dotted line*: apical extension of the notch, *white dotted line*: margin between the newly formed bone and old bone, *red asterisk*: artifact. Original magnification×25

blocks per specimen were obtained with the microtome set at 8 μm and subsequently stained with hematoxylin–eosin and the oxone-aldehyde-fuchsin-Halmi staining method. On the section with the best technical quality, one experienced examiner (AS) measured the following parameters by means of a computer assisted toolbox, while viewing the biopsies on an LCD flat screen with live streaming of images, captured by a digital camera (Olympus DP 71, Olympus Denmark AS, Ballerup, Denmark) adapted to the light microscope (Olympus DH 50, Olympus Denmark AS, Ballerup, Denmark): a) cementum regeneration height: distance between apical extension of the root planing and the coronal extension of a continuous layer of new cementum or cementum-like deposit on the planed root (millimetres), b) periodontal ligament (PDL) regeneration height: distance between apical extension of the root planing and the coronal extension of a functionally oriented PDL on the planed root (millimetres), c) bone regeneration height: distance between the apical extension of root planing and the coronal extension of regenerated alveolar bone along the planed root (millimetres) (i.e. the coronal extension of regenerated bone was defined as the most coronal level where the periodontal ligament space had an almost normal width), d) root resorption: combined linear heights of distinct resorption lacunae on the planed root (millimetres), e) ankylosis: combined linear heights of ankylotic union between the regenerated alveolar bone and the planed root (millimetres). The histometric evaluation was carried out under×25 magnification. The apical border of the notch or the apical extension of instrumentation (in the absence of a visible notch) was used as the landmark for the histomorphometric measurements.

Results

Baseline characteristics

All six patients completed the study. The mean age was 44.7± 14.2 years. Distribution of treated sites and the recorded parameters are shown in Table 1. All six defects displayed a combined one- and two-wall configuration.

Clinical outcomes

Postoperative healing was uneventful in all cases. Neither adverse events nor complications such as allergic reactions, infections, ulcerations or abscesses were detected. No exfoliation of the nano-HA paste through the sulcus was observed in any of the cases.

At 7 months following surgery, mean PPD reduction and mean CAL gain measured 4.0±0.8 and 2.5±0.8 mm, respectively.

Table 1 Clinical and histological results (expressed in mm) following treatment of intrabony defects with OFD and nano-HA

PPD					GR			CAL		CAL gain	INTRA	New cementum	New bone
Patient No.	Tooth type	Preoperative	After 7 months	Diff.	Preoperative	After 7 months	Diff.	Preoperative	After 7 months				
1	11	11	6	5	2	4	2	13	10	3	4	0	0
2	34	10	6	4	1	3	2	11	9	2	4	0.86	0.86
3	47	10	7	3	5	4	1	15	11	4	4	0	0
4	41	8	3	5	3	6	3	11	9	2	3	0.53	1.02
5	34	6	3	3	5	6	1	11	9	2	3	0.79	1.33
6	11	7	3	4	5	7	2	12	10	2	3	0	0
Mean		8.7	4.7	4.0	3.5	5.0	1.8	12.2	9.7	2.5	3.5	0.4	0.5
SD (±)		1.8	1.7	0.8	1.6	1.4	0.7	1.5	0.7	0.8	0.5	0.4	0.6

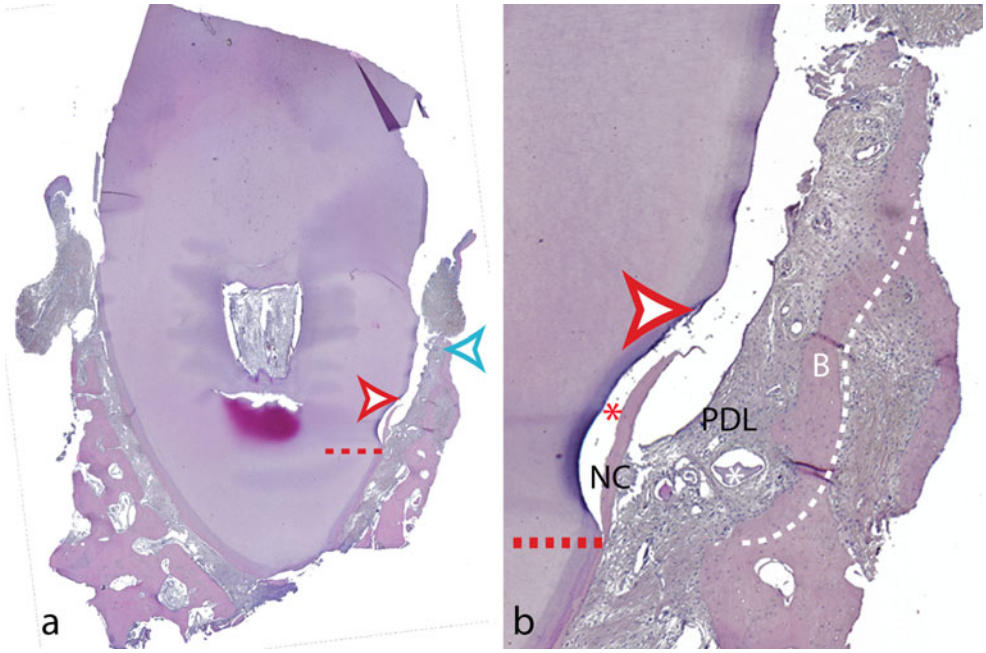

Fig. 2 **a** Representative photomicrograph revealing limited periodontal regeneration (case 5 from Table 1). *Red dotted line*: apical extension of the notch, *red arrowhead*: coronal extension of new cementum, *blue arrowhead*: coronal extension of new bone. Original magnification×25. **b** Higher magnification of the defect shown in **a**. Formation of new cementum (*NC*), new periodontal ligament (*PDL*) and new bone (*B*) was confined to the area of the notch. *Red arrowhead*: coronal extension of new cementum, *red dotted line*: apical extension of the notch, *white dotted line*: margin between the newly formed bone and old bone, *red asterisk*: artefact, *white asterisk*: remnant nano-HA particle encapsulated in connective tissue. Original magnification×50

Histological outcomes

The histological analysis revealed that in three out of the six defects, the healing occurred through formation of a long junctional epithelium along the debrided root surfaces extending until the most apical part of the defects. Neither ankylosis nor root resorption was observed in any of the biopsies. Limited formation of cementum, periodontal ligament and bone was found in three out of the six biopsies and was confined to the apical part of the defects (Figs. 1i, k and 2a, b). In two out of the six biopsies, some remnants of the grafting material were observed (Fig. 2b). The particles were predominantly surrounded by connective tissue, without signs indicating a potential to promote periodontal or bone regeneration.

Discussion

The main focus of the present investigation was to provide histological insight on the potential of nano-HA to promote periodontal wound healing/regeneration in human intrabony defects. In all six cases, a substantial reduction of probing pocket depth and gain of clinical attachment was measured at 7 months following surgery (i.e. mean PPD reduction of 4.0 mm and mean CAL gain of 2.5 mm). The observation that no adverse reactions such as allergies or abscesses occurred in any of the patients indicates that the used grafting material is biocompatible and well tolerated, thus corroborating findings from previous reports [20–27].

The histological evaluation has, however, indicated that in all six biopsies the healing was predominantly characterized by formation of a long junctional epithelium along the debrided root surfaces, while some limited periodontal regeneration was only observed in three out of the six defects. In those three cases, formation of cementum, periodontal ligament and bone was confined to the apical portion of the defects. Remnants of the grafting material encapsulated in connective tissue without apparent signs of bone formation were detected in two out of the six biopsies, thus indicating that the material is resorbable but has no visible effect on enhancing periodontal regeneration.

The clinical findings are comparable to those reported in two randomized controlled studies [26, 27]. In a first randomized controlled clinical study comparing treatment of intrabony defects with OFD and nano-HA to OFD alone, the additional application of the grafting material yielded a mean PPD reduction of 3.9 mm and a mean CAL gain of 3.6 mm compared to 2.6 mm and 1.8 mm following OFD alone [26]. A subsequent study has evaluated in intrabony defects the clinical performance of papilla preservation flap surgery with or without the application of nano-HA [27]. At

6 months after surgery, both treatments yielded significant improvements compared to the baseline defects filled with nano-HA yielding statistically significantly higher mean PPD reduction (4.3±1.6 mm) and mean probing bone level gain (4.3±1.4 mm) compared to the non-filled ones (i.e. 2.9±1.1 and 2.6±1.4 mm, respectively).

The PPD changes reported in the two aforementioned studies compare well to those obtained in the present one (i.e. 4.0 mm versus 3.9 and 4.3 mm, respectively). A possible explanation for the slight discrepancies in terms of CAL gain may be related to differences in defect configuration (i.e. in the present study the majority of the defects displayed a predominantly one- and two-wall configuration while in the mentioned ones the great majority of the defects had either a two-wall or a three-wall configuration. It is well known that three-walled defects have a significantly higher healing potential compared to the more complicated one-walled ones [31–33]. Nevertheless, it should be kept in mind that neither clinical nor radiographical evaluation is adequate means to demonstrate periodontal regeneration [34]. It has been extensively demonstrated that positive clinical outcomes such as PPD reduction, CAL gain or defect fill may not necessarily represent a regenerative type of healing [13–18, 34]. This is especially evident when analyzing the literature on human histological studies on intrabony defects filled with various types of alloplastic materials suggesting that in the great majority of the defects grafted with these materials, the healing occurred through a long junctional epithelium and connective tissue encapsulation of the graft particles, and limited to no periodontal regeneration [13–19]. Thus, the present findings corroborate the results from the mentioned human histological studies and provide further evidence on the limited biological value of using alloplastic materials alone to promote periodontal regeneration.

Another important aspect which needs to be addressed when interpreting the present findings is related to one of the inherent weaknesses associated with human histology studies and namely that, for obvious ethical reasons, only the less successful cases can be removed as biopsy specimens. Typically teeth that have been saved (based on clinical outcome) by a periodontal regenerative procedure are usually retained according to the patient's wishes [35]. Therefore, the sometimes inconsistent findings of periodontal regeneration may be also related to the histological evaluation of "hopeless" cases. All teeth selected for this study displayed very advanced destruction of the periodontal supporting apparatus, which may have limited their regenerative potential. In the present study, only a limited number of defects have been evaluated, and a control group is lacking. Undoubtedly, it would have been better to include more defects and also a control group treated with open flap alone. However, it should be kept in mind that due to the aforementioned selection criteria, only certain carefully selected defects, can be considered as potential candidates for a human histological study, thus minimizing the possibility to find sufficient patients serving as test and control groups allowing for an adequate statistical analysis. On the other hand, previous studies performed in similarly advanced cases have provided evidence for periodontal regeneration following the use of an enamel matrix derivative or guided tissue regeneration with and without the use of various types of grating materials [19, 35–40]. Therefore, it is important to emphasize that histological evidence of periodontal regeneration provides strong proof of principle, even if inconsistency is observed in the outcomes.

In conclusion, within their limits, the present results indicate that the used nano-HA has limited potential to promote periodontal regeneration in human intrabony defects.

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Conflict of interest The authors declare that they have no personal financial interest in any association with the investigated material or its manufacturer.

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