

Article

Ridge Preservation Using a Novel Enzyme-Treated Xenograft. A Preliminary Retrospective Histomorphometric Investigation

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Abstract: The use of xenografts to preserve the post-extraction alveolar ridge is an established and effective procedure. Recently, a novel freeze-dried, enzyme-deantigenic equine bone (EDEB) particulate combined with a hydrogel carrier (Exur[®]) containing ascorbic acid has been developed (EDEBEX). The aim of this study was to preliminarily investigate histomorphometric and early implant survival outcomes following the graft of EDEBEX in post-extractive sockets. Records of patients who underwent ridge preservation using EDEBEX followed by two-step implant placement were retrospectively collected and analyzed. Newly Formed Bone (NFB) and Residual Biomaterial (RB) at the implant placement site were measured through histomorphometric analysis, and early Marginal Bone Loss (MBL) for implants was calculated at the final follow-up. Records concerned 13 patients (nine women and four men, average age 54.1 ± 9.5 years). The 13 sockets were considered healed 4.5 ± 2.6 months (mean \pm SD) after grafting, with NFB and RB values of $43.2 \pm 22.1\%$ and $8.8 \pm 5.9\%$, respectively. 8.4 ± 5.8 months after implant placement, the median MBL was 0.20 [0.00–0.45] mm. No correlation was observed between MBL and NFB. EDEBEX grafted in post-extractive sockets for ridge preservation seems to allow for new bone formation with satisfactory implant outcomes. Future prospective studies are necessary to confirm these preliminary findings.

Keywords: enzyme-treated equine bone; enzyme deantigenic equine bone; equine-derived bone paste; histomorphometry; newly formed bone; bone substitute; biomaterial; post-extractive socket; ridge preservation

1. Introduction

Tooth extraction triggers a set of biological events that lead to unpreventable alveolar ridge resorption [1]. Resorption may occur to such an extent that implant placement becomes unfeasible unless bone augmentation procedures are undertaken [1]. Grafting post-extractive sockets, that is, applying ridge preservation procedures, has been shown to reduce or even minimize alveolar bone loss, in comparison to natural socket healing [2–5]. The outcomes of these procedures are affected by the morphology of extraction sockets, the type of wound closure, the type of grafting materials, the use of barrier membranes, and the use of growth factors [4,6,7]. Autogenous bone is seldomly used as a ridge preservation bone graft, because of morbidity associated with its collection [8]. Xenografts seem a viable alternative [5,7,9] based on the morphological and composition similarities that can be observed between the mineral bone portions of humans and other mammals. Xenografts are produced by processing heterologous bone to make it non-antigenic [10,11]. Among xenografts, recent studies have proposed an enzyme-deantigenic equine bone (EDEB) as an alternative to anorganic bovine

bone (ABB). While the production of ABB involves high temperatures (>300°) that remove the organic bone component, EDEB is made by processing equine bone with antigen-degrading enzymes at a significantly lower temperature (less than 60 °C), thus allowing for the preservation of type I bone collagen unaltered within the graft [12]. Its clinical use is documented both in oral and maxillofacial surgeries [12–21] as well as in orthopedic interventions [22,23]. Recently, EDEB has been further developed by creating a freeze-dried mixture of EDEB granules (0.5–1 mm in diameter) combined with a polyethylene glycol/hydroxyl-propyl methyl cellulose-based hydrogel (PEG/HPMC), namely Exur[®], and added with type I collagen from the equine tendon (EDEBEX). This gel contains also a subsidiary amount of vitamin C (ascorbic acid) acting as a visco-modulator [24]. EDEBEX, thanks to this new carrier, is plastic and easy to handle when rehydrated. Histomorphometric clinical investigations showed that the amount of newly-formed bone observed after grafting EDEB is higher compared to ABB, both when performing sinus augmentation procedures [21] and post-extractive socket grafting [12]. In these investigations, EDEB was used without the carrier; at present, therefore, no clinical histomorphometric data on EDEBEX exist. This paper aims to preliminarily investigate the histomorphometric and short-term clinical outcome of EDEBEX when used to graft post-extractive sockets for the purpose of ridge preservation.

2. Materials and Methods

2.1. Retrospective Data Collection

Clinical records of patients who presented to the private practice of one of the authors (DDS) between January 2016 and July 2019 to be rehabilitated through an implant-supported prosthesis were screened. Records were included for analysis if they concerned patients who (1) had one or more elements extracted anywhere in the two arches; (2) had sockets whose walls were intact; (3) had sockets which were not acutely infected; (4) had sockets immediately grafted using EDEBEX (Activabone Putty, Bioteck, Arcugnano, Vicenza, Italy); (5) had implants placed between 4 and 8 months from grafting; (6) had implants subjected to delayed loading. Patients also had to be aged between 18 and 80 years, with no systemic diseases. The exclusion criteria for being subjected to bone regeneration were the presence of one of the following conditions: ongoing bisphosphonate therapy; pregnancy, neoplasia, or psychiatric disease; osteoporosis; coagulation disorders; acute oral infections; immunocompromised status; previous chemotherapy or radiotherapy in the head or neck region; smoking more than 10 cigarettes per day; chronic alcohol or drug abuse. The patients signed an informed consent for the treatment, biopsy collection at the time of implant placement, and for the retrospective collection and analysis of their clinical data. Biopsies were collected when drilling the grafted socket for implant placement; therefore, patients were not subjected to additional interventions other than those involved in standard, routine implant placement. In light of this and of the retrospective nature of the study, an Ethic Committee approval was not sought.

2.2. Extraction, Grafting and Implant Surgery

Patients rinsed with chlorhexidine 0.2% (Corsodyl, GSK, Verona, Italy). Antibiotic prophylaxis consisted of 2 g amoxicillin/clavulanic acid 1 h before surgery (Augmentin, GSK) to be continued every 12 h for 8–10 days post-surgery. Analgesic prophylaxis consisted of 100 mg Nimesulide (Aulin, Roche, Milano, Italy) administered 1 h before surgery and to be taken twice a day for seven days after surgery. Adrenaline 1:100,000 and articaine hydrochloride 40 mg/mL were used to anesthetize the surgical area. The compromised tooth was extracted atraumatically without elevating any flaps. The socket was carefully debrided with manual instruments (Lucas Curettes, Hu-Friedy, Chicago, IL, USA) and then grafted with EDEBEX: EDEBEX was hydrated with sterile saline solution and inserted into the cavity while applying a gentle pressure to ensure proper adhesion to the underlying bone. The socket was filled completely. Once the gingival rim was detached from the bone, a hemostatic porcine gelatin sponge (Spongostan Dental, Ethicon, Johnson and Johnson Medical, Pratica di Mare,

Rome, Italy) was positioned under the gingival margins. The soft tissues were then stabilized by applying a single cross stitch with non-resorbable 5-0 sutures. The patients were instructed to continue chlorhexidine rinses for two weeks after surgery. After 10 days the sutures were removed. After surgery, follow-up visits were performed once a month and an intra-oral digital radiograph was taken at each one. Implant-placement surgery was performed only after observing a significant change in the graft's radio-opacity, which was suggestive of new bone formation. After performing antibiotic prophylaxis and anesthesia as previously described, a full-thickness flap was elevated, and a biopsy sample was collected using a trephine, drilling the occlusal aspect of the alveolar ridge under irrigation. Biopsies were approximately 3 mm wide and 10 mm long. A mark was left on the occlusal side to properly orientate the sample during histological processing. Implants were double-etched, sandblasted fixtures (Xive, Dentsply, USA or Stone/Tiger, IDI Evolution, Concorezzo, Italy) varying from 3.3 to 4.8 mm in diameter and from 8 to 14 mm in length. Three months later, they were uncovered, and the healing screws were positioned. After three weeks, an intraoral radiograph was taken, and a dental impression was made using pick-up impression copings. After 10 days, a provisional prosthesis was manufactured and delivered to the patients, who used it for approximately 40 days. After this period, the definitive abutments and metal-ceramic crowns were positioned. Patients were instructed to follow a maintenance program that included professional oral hygiene every 6 months.

2.3. Histologic and Histomorphometric Analysis

The biopsies were placed in a test tube with buffered 10% formalin. The sample was anonymized with a code and shipped to the histology lab. Bone cores were placed for 21 days in a solution composed of 0.76 M sodium formate and 1.6 M formic acid (Panreac Quimica, Barcelona, Spain) for decalcification. The samples were then dehydrated in ascending concentrations of ethanol and subsequently embedded in paraffin. Sections with 5 μ m thickness were obtained from the bone cores by cutting them with a microtome. The sections were then mounted on slides and stained with hematoxylin-eosin. The stained samples were analyzed qualitatively to identify any sign of inflammation or immune response. Digital photomicrographs of the whole sample were collected at 10 \times magnification, and morphometrical measurements including the total sample area (TSA), the total bone area (TBA), the newly-formed bone area (NBA) and the residual bone substitute area (RBA) were performed using the Image J 1.33 analysis software (National Institute of Health, Bethesda, USA). The author who performed morphometrical measurements (FO) did not carry out any surgery and was unaware of the correspondence between the sample code and the associated patient. Each assessment was repeated in triplicate. The mean newly formed bone (NFB) and residual biomaterial (RB) were calculated and expressed as the percentage over the total sample area ($\%NFB = NBA \times 100/TSA$; $\%RB = RBA \times 100/TSA$).

2.4. Implant Success and Peri-Implant Marginal Bone Loss

Implant success was assessed according to the criteria set by Albrektsson and Zarb [25] including: (i) no persistent pain, dysesthesia, or paresthesia in the implant area; (ii) no peri-implant infection with or without suppuration; (iii) no perceptible mobility of the implant; (iv) peri-implant bone resorption not greater than 1.5 mm during the first year of loading and 0.2 mm/year in the following years. Implants were considered successful when all the above-mentioned conditions were met, else they were regarded as failed. Implants were considered as failed also if an amount of bone loss greater than half the implant length was observed with radiological analysis or if implant mobility was detected.

Intraoral radiographs were digitally collected and analyzed using an image analysis software (Image J, NIH, Bethesda, MD, USA). This was calibrated using the known implant length from the implant-abutment interface to the apex. A single peri-implant marginal bone level was calculated by averaging the distances, both at the mesial and distal implant sides, between the implant-abutment interface and the most apical point of crestal bone observed to be in intimate contact with the implant (Figure 1).

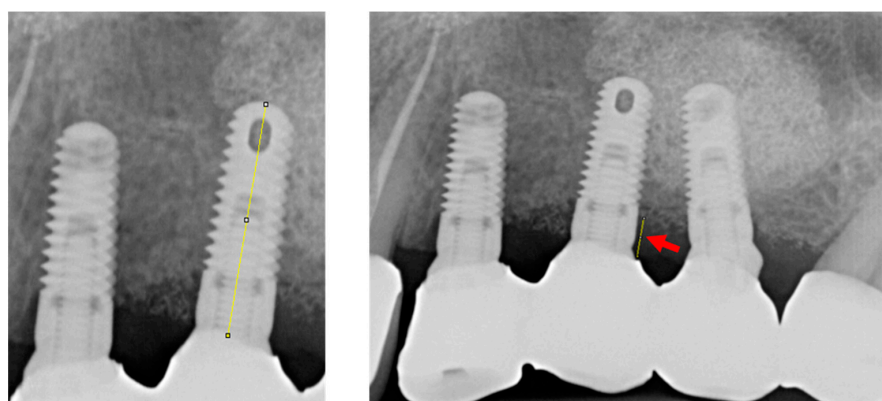


Figure 1. Measurement of peri-implant bone levels from representative intraoral radiographs. The known implant diameter length from implant-abutment interface to the apex was used for calibration (left panel). Then, the distance between the implant-abutment interface and the most apical point of crestal bone observed to be in intimate contact with the implant was measured (right panel).

Accuracy of measurements was 0.01 mm. Marginal bone loss (MBL) for each implant at a given time-point was calculated by subtracting the peri-implant bone level at that time point to peri-implant bone level at implant insertion.

2.5. Statistical Analysis

Normally distributed quantitative variables are presented as mean ± standard deviation (SD), whereas non-normal quantitative variables are reported as median [interquartile range]. Data normality was assessed by means of Shapiro-Wilk tests. To investigate if MBL was correlated with the amount of NFB at implant insertion, the relation between MBL and NFB was investigated by means of regression analysis calculating the corresponding Spearman’s r coefficients. A dedicated software (Origin 2020, Microcal, Northampton, MA, USA) was used for all statistical analyses. Statistical significance was set at the 5% level.

3. Results

Records concerned 13 patients, nine (69.2%) women and four (30.8%) men; their average age was 54.1 ± 9.5 years and they were followed for 5.4 [4.2–13.9] (mean, 8.4 ± 5.8) months. The median healing time was 3.1 [2.6–6.1] (mean, 4.5 ± 2.6) months. Overall, 13 sockets were grafted, and the same amount of implants were placed. Data concerning patient demographics, site of extraction, healing time, newly formed bone (NFB), residual bone (RB), type of implant placed, follow-up time and MBL are reported in Table 1.

Table 1. Data concerning patient demographics, site of extraction, healing time, newly formed bone (NFB), residual bone (RB), placed implant, follow-up time, and marginal bone loss (MBL).

Patient#	Age (Yrs.)	Gender	Tooth	Healing Time (Mo.)	NFB(%)	RB(%)	Implant#	Implant Diameter (mm)	Implant Length (mm)	Follow-Up (Mo.)	MBL at Final Follow-Up (mm)
1	55	M	26	3.1	69.33	18.63	1	4.5	8.0	21.6	0.3
2	48	M	25	2.3	32.61	2.53	2	3.3	12.0	14.3	0.0
3	45	F	36	3.0	18.68	4.33	3	3.8	13.0	13.9	0.7
4	52	F	46	2.5	39.16	0.87	4	4.1	10.0	8.4	0.0
5	43	F	36	1.9	18.83	4.40	5	4.6	11.5	5.4	1.5 (failed)
6	75	F	27	7.3	25.11	8.15	6	4.3	8.0	5.3	0.5
7	58	F	35	3.1	31.90	13.49	7	3.4	11.0	3.6	0.0
8	50	F	26	3.7	16.93	14.59	8	5.5	9.5	14.3	0.0
9	59	M	37	2.7	38.89	8.46	9	4.0	10.0	5.7	0.2
10	52	M	46	5.0	53.62	16.91	10	3.8	9.5	5.1	0.2
11	45	F	46	8.3	71.92	3.81	11	4.5	11.0	4.0	0.3
12	52	F	37	10.5	78.07	4.84	12	3.8	8.0	3.2	0.4
13	70	F	36	4.7	66.47	12.78	13	4.0	8.0	4.2	0.2

A case is shown in Figure 2 for illustrative purposes.

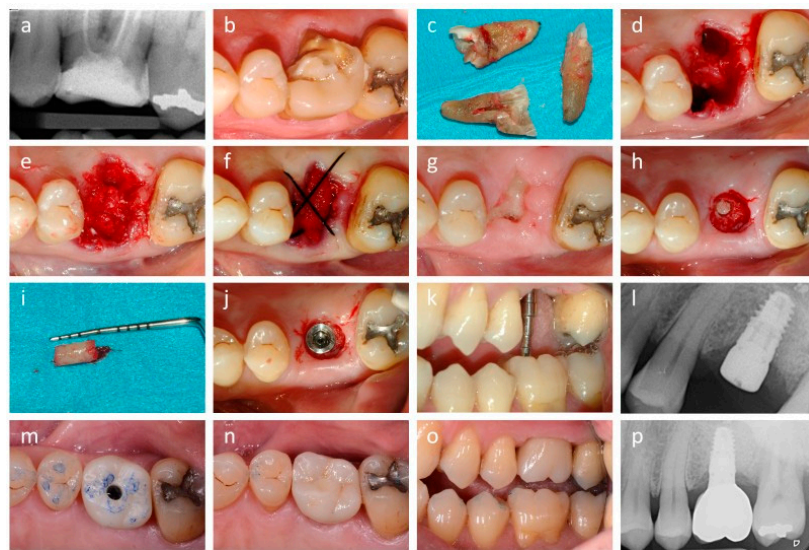


Figure 2. An illustrative case of a patient (patient #1) treated with enzyme-deantigenic equine bone particulate combined with a hydrogel carrier (EDEBEX). (a) Intra-oral X-ray at presentation; (b) clinical appearance at presentation; (c) the tooth after been cut to be extracted atraumatically; (d) the post-extractive socket; (e) the socket grafted with EDEBEX; (f) the gingival rims are stabilized with a cross stitch; (g) soft tissue healing at implant insertion; (h) and (i) a bone biopsy is collected and (j,k) an implant placed. (l) intra-oral X-ray following implant placement; (m), (n), (o) the final prosthesis delivery; (p) intra-oral X-ray recorded at the last control visit (21.6 months after implant placement).

A representative image of a histologic slide is shown in Figure 3.

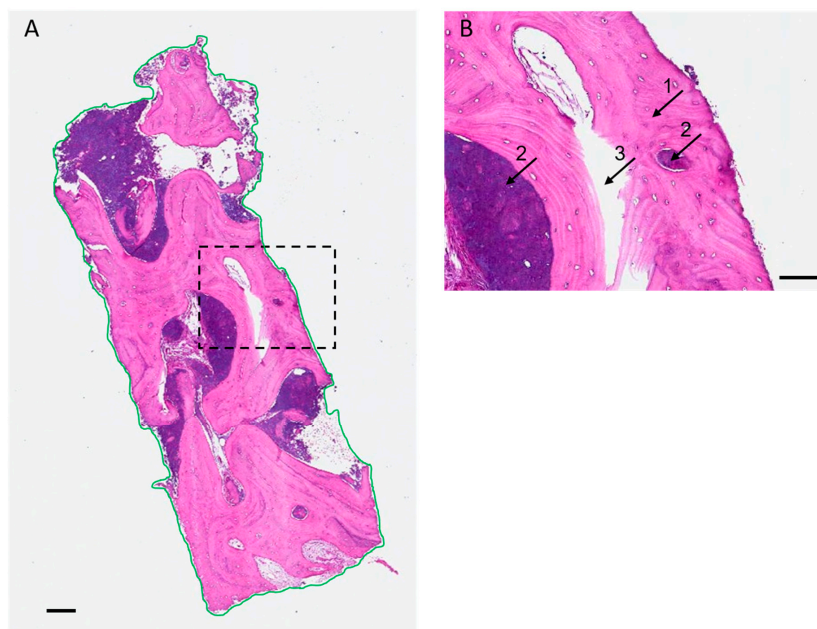


Figure 3. Image of a representative histologic sample taken from a socket grafted with EDEBEX and stained with hematoxylin-eosin. The biopsy was collected from the patient whose surgery is shown in Figure 2. Panel A shows the sample at 2.5× magnification, and the green line highlights how the total area of the sample was calculated for the histomorphometric analysis. Scale bar, 200 μm. Panel B shows the area bounded by the dashed line in panel A at higher magnification (12×). Arrow 1 indicates an area of newly formed bone (NBA), arrow 2 indicates residual biomaterial (RB), and arrow 3 indicates marrow space. Scale bar, 100 μm.

The qualitative histological analysis of the samples revealed no signs of inflammatory reactions. Considering the socket as the statistical unit of analysis, NFB was $43.2 \pm 22.1\%$, and RB was $8.8 \pm 5.9\%$ (Figure 4). At the final follow-up, the implant success rate was 92.3% (one implant showed a MBL > 1.5 mm on the first year, Table 2).

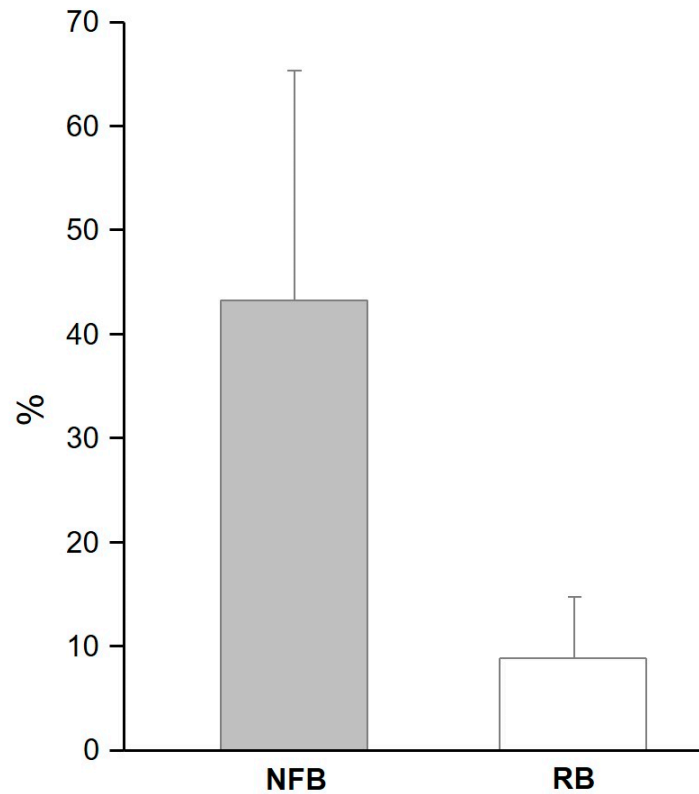


Figure 4. Newly Formed Bone (NFB, %) and Residual Biomaterial (RB %) in sockets grafted with EDEBEX. Data are presented as mean \pm SD.

Table 2. Outcome at final follow-up according to the Albrektsson and Zarb [25] criteria. In particular, these criteria were: (1) absence of persistent pain, dysesthesia, or paresthesia in the implant area; (2) absence of peri-implant infection with or without suppuration; (3) absence of perceptible mobility of the implant; (4) absence of peri-implant bone resorption greater than 1.5 mm during the first year of loading and 0.2 mm/year in the following years. Implants were considered successful when all the above-mentioned conditions were met, else they were regarded as failed. Green = satisfied criterium, red = non-satisfied criterium

# Implant	A&Z criterium 1	A&Z criterium 2	A&Z criterium 3	A&Z criterium 4	Outcome at Final Follow-Up
1	Green	Green	Green	Green	Success
2	Green	Green	Green	Green	Success
3	Green	Green	Green	Green	Success
4	Green	Green	Green	Green	Success
5	Green	Green	Green	Red	Failure
6	Green	Green	Green	Green	Success
7	Green	Green	Green	Green	Success
8	Green	Green	Green	Green	Success
9	Green	Green	Green	Green	Success
10	Green	Green	Green	Green	Success
11	Green	Green	Green	Green	Success
12	Green	Green	Green	Green	Success
13	Green	Green	Green	Green	Success

At the implant level, median MBL was 0.20 [0.00–0.45] mm, with four implants showing MBL = 0 (30.8%), eight implants showing an MBL < 1 mm (61.5%), and one implant with MBL > 1.5 mm (7.7%)

at the final follow-up. No correlation was observed between MBL and NFB (Spearman's $r = 0.00842$, $p = 0.98$, Figure 5).

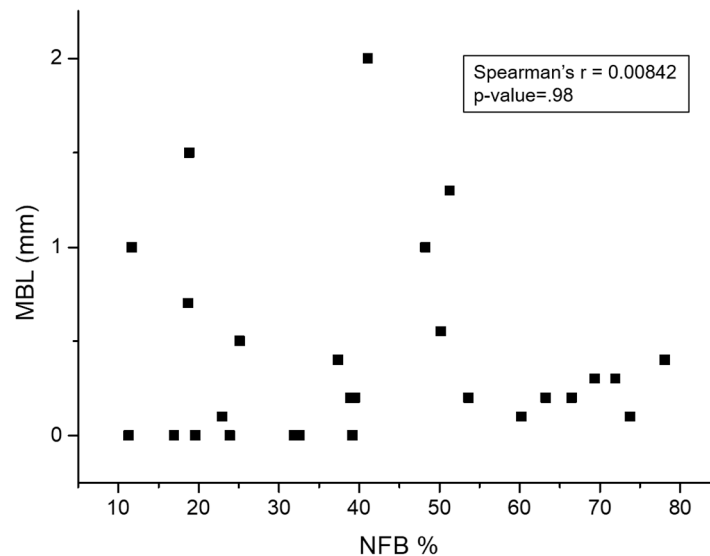


Figure 5. Regression analysis between Marginal Bone Loss (MBL, mm) and Newly Formed Bone (NFB, %).

4. Discussion

Results of this preliminary study suggest that the use of EDEBEX for ridge preservation surgeries allows for the formation of new bone tissue as early as two months after surgery, with an average of 4.5 ± 2.6 months. The amount of NFB found in the present study (NFB, $43.2 \pm 22.1\%$; RB, $8.8 \pm 5.9\%$) is in line with another study that compared the histomorphometric outcome of particulate EDEB with anorganic bovine bone (ABB) used for post-extractive socket grafting [12]. In this latter study, NFB was observed to be significantly higher with EDEB than with ABB ($45.12 \pm 10.54\%$ vs. $33.61 \pm 9.71\%$), and RB lower ($10.91 \pm 4.27\%$ vs. $18.47 \pm 5.62\%$), with no difference in the healing time (4.1 ± 1.2 vs. 4.4 ± 1.2 months) [12]. It is worth noting that the percentage of bioptic samples collected at an early stage of healing (<3.5 months) in the present study was almost double (53.85% vs. 27.27%) compared to that reported in the study comparing EDEB and AB [12]. Altogether, these results suggest that the presence of type I bone collagen in its native conformation in EDEB, which is absent in ABB, allows for EDEB to undergo fast remodeling [12]. Indeed, the presence of type I collagen is known to be associated with active osteoclastic remodeling, as demonstrated by in vitro studies [26,27]. A non-freeze-dried, mouldable version of EDEBEX combined with a demineralized bone matrix (DBM) was shown to stimulate the expression of known modulator of bone regeneration, such as the runt-related transcription factor 2 (RUNX2), bone sialoprotein (BSP) and osteocalcin in an in vitro study on human bone marrow stem cells [24]. The authors compared the results obtained with those achieved by EDEB + DBM mixed in a water-based carrier, showing that the latter did not exhibit such a pro-regenerative effect. This observation suggests that the Exur[®] carrier itself may contribute to the regenerative action of EDEBEX. The same study also investigated in vivo the effects of this bone paste when grafted in artificially induced femoral defects in rabbits. The histological analysis of the grafted sites at one and two months showed an interesting re-organization of the regenerating tissue within the lesion boundaries [24]. In one patient, this mouldable EDEBEX + DBM formulation, when grafted in post-extractive sockets, was observed to allow for considerable new bone formation already at three months from grafting (NFB, 60.12% ; RB = 20.53%) [28], a timing consistent with the observations reported in this study. Moreover, it can be also speculated that the pro-regenerative potential of EDEBEX may be partially due to the presence of ascorbic acid (vitamin C) in the hydrogel carrier, which may favor collagen deposition throughout the entire lesion volume. Indeed, ascorbic acid is known to

be a co-factor for the activity of prolyl- and lysyl-hydroxylases, essential enzymes for collagen fibril assembly [29]. From the operative perspective, the malleability of the EDEBEX bone paste granted by the hydrogel carrier ensures a more intimate contact between the graft and the underlying bone tissue, which can potentially favor bone regeneration. Even if a better performance of EDEBEX in terms of NFB was not observed when comparing the percentage obtained in the present study with that obtained in another study employing EDEB [12], it must be highlighted that the majority of the samples analyzed in the present study were collected after less than 3.5 months from the grafting surgery, suggesting a possible role of EDEBEX in further enhancing the regenerative performance showed by EDEB.

The present study also highlights a satisfactory clinical outcome, with a good success rate (92.3%, only one implant failed) for implants placed in the grafted post-extractive sockets after an average of 4.5 months after grafting. This result is in line with another study on a sinus augmentation procedure using particulate EDEB as grafting material, reporting an overall success rate of 98% with no difference between implants placed at earlier and later times after grafting [20]. It is interesting to note that when particulate EDEB was used as graft material for maxillary sinus augmentation procedures, new bone formation was observed [20,21], with the period of bone formation pinpointed to the first three months after grafting [20]. In particular, a randomized-controlled clinical trial [21] comparing particulate EDEB and ABB in sinus augmentation reported that six months after grafting NFB was higher and RB was lower in sinuses grafted with EDEB compared to ABB (NFB, $46.86\% \pm 12.81\%$ vs. $25.12\% \pm 7.25\%$; RB, $11.05\% \pm 9.27\%$ vs. $28.65\% \pm 9.70\%$). A major limitation of the present study is the limited number of patients treated with EDEBEX—great care, therefore, must be taken when interpreting these results. Another important limitation is the lack of a control group treated with a different bone substitute. Further comparative clinical investigations on larger cohorts of patients shall be conducted to gather more reliable data. Other major limitations of the present study concern its retrospective nature, and the analysis of any post-extractive socket, irrespective of the tooth being extracted (incisor, canine, premolar or molar) and the thickness of the buccal bone plate. Finally, results concerning MBL are purely indicative also considering they relate to a very short implant functioning time and to different implant brands. Future studies should definitively address these limitations and investigate if differences exist between EDEBEX and ABB freeze-dried formulations in terms of their histomorphometric and short- and long-term clinical outcomes.

5. Conclusions

EDEBEX, a novel freeze-dried bone substitute composed of EDEB granules (0.5–1 mm in diameter) combined with a polyethylene glycol/hydroxyl-propyl methyl cellulose-based hydrogel (PEG/HPMC) containing vitamin C, and complemented with type I collagen from the equine tendon, when grafted in post-extractive sockets for ridge preservation, seem to allow for the formation of a substantial amount of new bone at an early time after grafting (four months). The rapid formation of new bone in the sockets grafted with EDEBEX allowed for early implant placement, with satisfactory success and survival rates. Future, long-term prospective and comparative studies on a larger number of patients and bone samples are needed to further validate these preliminary results in terms of histomorphometric and clinical outcomes.

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Conflicts of Interest: Danilo Alessio Di Stefano has a consultancy relationship with Bioteck S.p.A. Francesco Orlando declares that he has no conflicts of interest.

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