Preclinical evaluation of the effect of a collagen matrix on periodontal regeneration in two-wall intrabony defects

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Abstract

Objectives: To histologically evaluate the effect of a collagen matrix on periodontal wound healing/regeneration.

Materials & Methods: Acute type, two-wall intrabony defects (6 x 6 x 6 mm) were surgically created bilaterally distally to the maxillary first and third premolars in eight beagle dogs. The defects were randomly allocated to open flap debridement either with (test) or without (control) a collagen matrix. After a healing period of 12 weeks, the dogs were euthanized, and the specimens histologically processed. Descriptive, histomorphometrical, and statistical analyses were then performed.

Results: Healing was uneventful in most cases. Residual collagen matrix was still present and showed integration into new bone, new periodontal ligament, new connective tissue and, in some specimens, into new cementum. Periodontal regeneration occurred to a varying extent in both groups. New cementum and bone formation were statistically significantly greater in the test-group than in the control group ($p=0.009$, $p=0.037$, respectively). The junctional epithelium was longer in the control group than in the test group ($p=0.16$).

Conclusion: The present results have for the first time provided histologic evidence for the potential of this novel CM to facilitate periodontal wound healing/regeneration thus warranting further preclinical and clinical testing.

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Introduction

The rationale to integrate regenerative/reconstructive protocols in the overall treatment concept is supported by findings from clinical studies showing generally larger clinical improvements when compared to conventional treatments (e.g. access flap surgery) [1-5]. Furthermore, since regenerative periodontal surgery is a non-resective approach, it may also offer superior aesthetic outcomes as compared to conventional or pocket resective protocols.

Over the last decades, a plethora of clinical protocols including the use of various surgical techniques in conjunction with root surface demineralization, implantation of bone grafts/bone substitutes, guided tissue regeneration (GTR), growth and differentiation factors, enamel matrix derivative (EMD), or various combinations thereof has been shown to enhance periodontal regeneration and to improve the clinical outcomes in intrabony and in class II furcation defects [1-9].

Findings from preclinical and clinical studies have shown that from a biologic point of view, the following factors are of pivotal importance for obtaining periodontal regeneration: a) wound stability to allow undisturbed blood clot adhesion and maturation on the instrumented root surface, b) space provision to enable formation and maturation of periodontal tissues, and c) uneventful healing (e.g. without bacterial infection) to support formation and maturation of newly formed tissues [1-3, 5, 7, 10]. Therefore, treatment concepts aiming to provide a clinical benefit should be based on a sound biologic rationale incorporating not only the use of regenerative materials, but also taking into consideration the host's innate healing potential. The decision for selecting the appropriate regenerative material or various combinations is made after careful evaluation of defect anatomy (e. g. non-contained or contained defects) in order to ensure space provision and wound stability.

Since space provision and wound stability have been shown to decisively influence periodontal wound healing and regeneration, novel biomaterials should possess the capacity to stabilize the blood clot and prevent flap collapse thus maintaining the space needed for the regeneration process. A stable blood clot may not only maintain a high concentration of autogenous growth factors in the wound but would also restrict epithelium proliferation. Consequently, formation of root cementum, periodontal ligament (PDL) and alveolar bone may occur and would translate clinically into probing depth reduction, gain of clinical attachment level and bone fill [10].

A novel collagen matrix (CM) has shown to possess an excellent biocompatibility in preclinical and human studies [11-14]. Due to its biocompatibility and structural configuration (e.g. high porosity and interconnectivity) the material stabilizes the blood clot while its cross-linked configuration provides and maintains the volume stability [15]. It shows in a preclinical model favourable soft connective tissue (CT) integration and promotion of angiogenesis [16]. Different CMs are applied to replace subepithelial CT and free gingival grafts harvested from the palate [17].

However, until now, no studies have evaluated the biologic potential of this CM to influence periodontal wound healing and regeneration. Therefore, the aim of this study was to investigate the effect of this

CM to promote periodontal regeneration (formation of root cementum, PDL, alveolar bone, and junctional epithelium [JE]) in acute type two-wall defects in dogs.

Materials and Methods

Animals

Eight 18-24 months old beagle dogs each weighing 12-15 kg were used. The animals had an intact dentition and a healthy periodontal status. This sample size of eight animals is well suitable based on literature [18]. The animals were kept at the animal facility of the Veterinary Faculty of the University of Santiago de Compostela (Lugo, Spain). The dogs were housed under laboratory conditions, at a room temperature of 15–21 °C and humidity >30 %. They had access to tap water and laboratory diet ad libitum.

The current study was conducted in accordance with the European Communities Council Directive 2010/63/EU. In addition, the Guidelines for Animal Research: Reporting In Vivo Experiments (ARRIVE) [19] have been included as much as possible.

Study design and sample size

The study was designed as a randomized controlled experiment with one test and one negative control group with a randomized assignment to the groups.

Test Group: Open flap debridement (OFD) + CM (Geistlich Fibro-Gide®, Geistlich Pharma AG, Wolhusen, Switzerland)

Control Group: OFD (alone), negative control

With eight animals available and four sites per animal a total of 32 sites were treated. The individual animal was considered as the experimental unit and thus the sample size was eight. Based on literature, the sample size of eight is well suitable for animal experiments on periodontal regeneration [18].

In order to reduce the risk of bias, the following persons were blinded to the experimental allocation: the animal caregivers, the veterinarian responsible for regular check of animals and the histologist.

Surgical procedure

In a first phase, the animals were pre-anesthetized with medetomidine (20 µg/kg/i.m., Domtor; Esteve, Barcelona, Spain) and morphine (0.4 mg/kg/i.m., Morfina Braun 2%; B. Braun Medical, Barcelona, Spain). The anesthesia was initiated by propofol (2 mg/kg/i.v.; Propovet, Abbott Laboratories, Kent, UK) and maintained by inhalation of an O_2 and 2.5–4% isoflurane mixture (Isobavet, Schering-Plough, Madrid, Spain). A local anesthesia composed of lidocaine and adrenaline (Anesvet®, Ovejero, Leon,

Spain) was used to reduce peri-operative pain and bleeding. After the surgical intervention, atipamezol (50 µg/kg/i.m.; Antisedan, Esteve) was administered to revert the effects of the medetomidine.

The second and fourth premolars of both maxillary quadrants were extracted, and the sites are allowed to heal for 12 weeks. The remaining dentition receives oral prophylaxis after the extraction procedure.

In a second phase, the animals were anesthetized like in the first phase. The surgeries were performed by two well trained periodontists with extensive experience in regenerative periodontal surgery (J.-C. I. and A.S.). Mucoperiosteal flaps were elevated and acute "box-type" 2-wall intrabony defects of approximately 5x5x5 mm were surgically created by leaving the palatal bone wall intact (Fig. 1a, 1b). Before bone removal a coronal reference notch (notch A) was created with a round burr (diameter 1mm) into the root at the initial alveolar crest. The defects were created at the distal aspects of the first (P1) and third premolars (P3) of both maxillary quadrants by means of rotating and hand instruments. Subsequently, the roots were thoroughly scaled in order to remove the root cementum and periodontal ligament. Following root planing, a reference notch (notch B) was created at the apical extension of the defect in the same manner as the coronal notch. The notches served as reference point for the histomorphometric measurements. Thus, all periodontal tissues that would form coronally to the notch, is newly formed periodontal tissue. Clinical measurements including the defect depth and width along with intraoral photographs were taken at baseline. The treatment was performed according to the allocated group procedure. Per Quadrant a test site was randomly chosen and a trimmed CM, in shape of the defect, was placed (Fig. 1c). At the other site per quadrant the defect was left empty. After treatment the soft tissues were positioned at the pre-surgical level and the wound was closed (Fig. 1d) tension-free by means of monofilament sutures (Ethilon 6-0 blue, Johnson & Johnson Medical GmbH, Ethicon, Norderstedt, Germany).

After the surgeries, pain was controlled with morphine (0.3 mg/kg/i.m./6h) for 24 hours and meloxicam (0.1 mg/kg/s.i.d/p.o.; Metacam, Boehringer Ingelheim, Barcelona, Spain) for 3 days. Antibiotics (amoxicillin 22 mg/kg/s.i.d./s.c.; Amoxoil retard, Syva, Leon, Spain) were administrated for 7 days. The animals were controlled daily for the health symptomatology using standardized score sheets. During the first two postoperative weeks, the oral mucosa and the teeth were disinfected three times a week by using gauzes soaked in a chlorhexidine solution (0.12%, Perio-Aid Tratamiento®, Dentaid, Barcelona, Spain). Subsequently, a toothbrush and a chlorhexidine gel (0.2%; Chlorhexidine Bioadhesive Gel, Lacer, Barcelona, Spain) was used for plaque control three times weekly. The dogs were fed a soft-pellet diet for one week. The sutures were kept in place during the entire healing time of 12 weeks.

The animals were euthanized 12 weeks after the second phase by sedation with medetomidine (30 µg/kg/i.m.; Esteve) and subsequently sacrificed with an overdose of sodium pentobarbital (60 mg/kg/i.v., Dolethal, Vetoquinol, France).

Histological procedures

After euthanization, the maxilla of each animal was removed, and individual bone blocks containing the implanted biomaterials and the surrounding soft and hard tissues were obtained and subsequently fixed in formaldehyde.

Out of 32 defects 24 (12 test and 12 control) were randomly selected for dehydration in an ascending series of ethanol and infiltrated and embedded in methylmethacrylate (MMA). After polymerization, the specimens were sectioned in a mesiodistal plane along their longitudinal axis with a slow-speed diamond saw with a coolant (Varicut® VC-50; Leco, Munich, Germany). Thereafter, the approximal 800 µm-thick ground sections were then mounted on Plexiglas slabs and ground to a final thickness of 150 µm (Knuth-Rotor-3; Struers, Rodovre/ Copenhagen, Denmark). Finally, the sections were superficially stained with toluidine blue/ McNeal combined with basic fuchsin. Staining was done with acid fuchsin and toluidine blue. Furthermore, the remaining 8 defects were decalcified in 10 % ethylene-diaminetetraacetic acid. They were cut in the same direction as the MMA sections with a microtome set at 8 µm. Staining was done using hematoxylin and eosin. Paraffin sections were produced for future immunohistochemical evaluation. For both processing procedures, photography was performed using a digital camera (AxioCam MRc; Carl Zeiss, Oberkochen, Germany) connected to a light microscope (Axio Imager M2; Carl Zeiss).

Histomorphometric analysis

In an Eclipse Ci (Nikon Corporation, Tokyo, Japan), connected to a digital video-camera (Digital Sight DS-2Mv, Nikon) and a computer, landmarks were identified. The best section (visible apical and coronal notches, presumably central position within the defect area) was chosen for histomorphometric analysis. All the histomorphometrical landmarks were identified and discussed by two investigators (D.D. B. and J.-C. I.). Thereafter, the following histomorphometric measurements were performed in the axis through the cementoenamel junction to the apical root using the software NIS-Elements D 4.1 (Laboratory Imaging, Nikon Corporation, Tokyo, Japan):

1. Defect height in mm (apical end of notch A to apical end of notch B)
2. Height of new continuous cementum in % and mm (between notch A and notch B)
3. Height of new interrupted cementum in % and mm (between notch A and notch B)
4. New bone height in % and mm (between apical end of notch B and most coronal point of newly formed bone)
5. JE height with gingival sulcus in mm
6. CT adhesion height in mm (apical end of the JE to most coronal end of newly formed cementum)

Statistical analysis

The number of defects per group was 13 for the test and 10 for the control group. Data analyses were performed using Prism v7 (GraphPad Software, La Jolla, CA, USA). To assess the differences between test and control groups one section per defect was analysed. The measured parameters were calculated as means, standard deviation, medians, minimum, maximum and interquartile ranges. The differences of means between the two groups was analysed based on the nonparametric Wilcoxon signed-rank test as the distribution of the samples was not normally distributed. For the orientation of the newly formed PDL, the paired t-test was used. Significance was set at $p<0.05$. For sample size calculation assuming equal variability and sample size in the two groups, a two-tailed alpha of 0.05, and a power of 80, a minimal number of 10 defects per group was calculated.

Results

Clinical findings

During the entire period of 12 weeks after surgery, the healing was uneventful in all dogs. No swelling or wound dehiscence occurred. During the surgical phase, one tooth of the control group presented an apical pathology. Furthermore, in one PM1 site, the sinus was perforated during surgery.

Descriptive histology

All 32 defects were available for descriptive analysis either embedded in MMA (24 defects) or paraffin (eight defects). Processing artefacts were very seldom and never compromised the analysis. Because the sutures were kept in place for the entire healing time, they could be observed in all tissue sections. Around the sutures close to the gingival epithelium, inflammatory cells were present. For sutures deeper in the soft tissues less or no inflammation could be seen, and the extent of inflammation never compromised regeneration in the defect region. In almost all defects, varying amounts of new cementum, new bone, and new PDL had been formed (Fig. 2), only one tooth in the control group presented no cementum formation at all. Furthermore, newly formed cementum was either continuous from the apical end of the defect until it ends or was interrupted. Old cementum removal was either complete or incomplete. Interestingly, in some test sites, residual CM could be detected integrated into cementum. In some sites, cementum removal was incomplete, and the cementum remnants were either superficially removed during instrumentation or not.

The CM was still present in all test sites with a varying degree of degradation. In regions of regenerated alveolar process up to the root surface the CM showed always integrated in newly formed bone and PDL (Fig. 3). Moreover, the newly formed PDL had always normal anatomical dimensions, comparable to the pristine tooth site of the same tooth. In addition, the collagen fibres from the newly

formed PDL were perpendicularly inserting into the newly formed cementum in nine cases of the test and 5 sites of the control group without any statistically significant differences between the groups (p: 0.12). In the CT adjacent to the alveolar process, the CM was always visible in short distance to the root surface.

Moreover, in distance of the alveolar process, the residual CM was infiltrated with blood vessels, fibroblasts, and the pores were filled with soft or hart CT (Fig. 3). In four sites, almost the complete CM was interspersed with new woven bone with ongoing bone formation (Fig. 4).

Two test sites in one animal showed a CM with a mass of inflammatory cells which could account for an infection during surgery. The surgically already visible apical pathology in one control site was histologically verified. Multinucleated giant cells were never observed.

Histomorphometry

Out of 32 defects, nine defects were not suitable for histomorphometrical analysis because of not visible landmarks (three control sites), sinus perforation (one test site), inflammation (two test sites in one animal), apical lesion (one control site), and not removed old cementum on the root surface (two control sites). Therefore, 13 defects of the test (nine MMA and four paraffin) and 10 defects of the control group (eight MMA and two paraffin) were eligible for histomorphometry. The mean values and standard deviations for all sites are presented in Table 1 and visualized for each defect in Table 2. Minimum and maximum values, median and interquartile range are demonstrated in Table 3. No statistically significant differences were observed between test and control sites for defect height (p: 0.10). The formation of cementum and bone was analysed in mm and in percentage of the defect height. For each defect, the values are visualized in Table 3. New formation of continuous and maximum cementum height was statistically significant greater in the test compared to the control group (p: 0.0098 / p: 0.0391). Moreover, vertical bone gain was significant greater in the test group compared to the control (p: 0.0371). Furthermore, the length of the CT adhesion tended to be longer in the control group without statistical significance (p: 0.1055). The mean value for the length of the JE was higher in the control group but did not reach statistical significance (p: 0.1602).

Discussion

The present study has investigated the regenerative potential of a cross-linked, volume stable CM on the healing of acute-type two-wall intrabony defect in dogs. To the best of our knowledge, this is the first study where this novel biomaterial scaffold was tested for periodontal regeneration. The findings of this study demonstrate favourable regenerative outcomes as shown by statistically significantly higher vertical formation of both new cementum and new bone when the CM was used. Although the height of the junctional epithelium was smaller in the test group than in the control group, this difference was not statistically significant. Furthermore, the CM showed excellent biocompatibility as demonstrated by extensive ingrowth of bone and soft CT and absence of inflammatory and foreign body giant cells.

However, one limitation of the present study is that a positive control with a proven treatment modality to promote periodontal regeneration is missing. Nevertheless, this aspect is negligible, since there are enough data available in the literature with comparable defect anatomies and healing periods in the same species. Furthermore, the dog is still one of the most well-established animal models for periodontal research [20, 21]. Even though, translation of results from animal studies into the human situation is problematic because of different anatomical and physiological environments and different healing rates.

Surgeries aiming to restore the periodontal tissues after periodontal diseases are frequently performed and many different types of biomaterials have been widely used [7]. Regenerative / reconstructive periodontal surgeries with some of these biomaterials have shown to provide better clinical outcomes in terms of pocket depth reduction, clinical attachment level gain and hart tissue fill compared to conventional open flap debridement [3]. Furthermore, in access flap procedures often residual periodontal pockets persist and resective techniques are associated with increases in gingival recessions and attachment loss [22, 23]. Biologics like EMD (Enamel matrix derivative) or rh-PDGF (recombinant human platelet derived growth factor) plus β-tricalcium phosphate are comparable with DFDBA (demineralized freeze-dried bone allograft) and GTR (guided tissue regeneration) and superior to open flap debridement [3].

A systematic review about periodontal regeneration on intrabony defects in preclinical studies is showing new cementum formation of 33 - 75% and new bone formation ranging from 12 - 75% of the original defect height with the use of autografts, xenografts, allografts, alloplastics, GTR, growth factors and combination therapies [24]. GTR for example, one of the best documented methods to obtain periodontal regeneration, showed 66 % new cementum and 58 % new bone formation. Therefore, treatment of intrabony defects with a CM with 71% new cementum and 57% new bone formation represents a promising treatment option with comparable results to well established treatment modalities.

The histological observations in this study are clearly showing that the bone can growth into the CM and a new attachment apparatus can form with normal anatomical features. Bone ingrowth was shown in a smaller extent in another preclinical study where bone formation towards a ridge defect was enhanced with the use of this CM [25]. Furthermore, in an animal study with gingival recession defects, grafting with a CM attained more tissue regeneration characterized by a shorter JE and more vertical new cementum formation compared to a coronally advanced flap alone [26].

New concepts to simultaneously regenerate the entire bone-PDL-cementum complex are exploring the effects of stem cells, bio-printing, gene therapy and layered bio-mimetic technologies alone or in combination [27]. Nowadays, biomaterial design with the use of stem cells and transplantation into patients is a promising field in medicine but difficult to transfer into daily practice [28]. Therefore, biomaterials that act as a scaffold to encourage (or support) the innate regenerative capabilities of the host's own cells from the periodontium could be a valuable option in the field of periodontal reconstructive/regenerative surgery. Endogenous cell homing with the use of biomaterials can be regarded as a more economic, effective and safe method for treating patients [29]. That the tested volume stable, cross-linked CM did not only stabilize the blood clot but was additionally acting as a scaffold for progenitor cell invasion is a possible explanation for the observed CM integration in the newly formed periodontal tissues. This view would support the cell homing concept of biomaterials in the form of scaffolds.

The present findings are in line with the results of an animal study in rat calvaria defects suggesting that collagen membrane may not exclusively serve as an occlusive barrier but may also support the ingrowth of bone tissue, thus acting like an osteoconductive biomaterials [30].

The results of the present study open up a wide field for future investigations to answer many important questions around the potential use of collagenous scaffolds in regenerative periodontal therapy. For instance, we have only evaluated one single healing period and thus, knowledge about the early wound healing events and sequence of healing and tissue regeneration showing the dynamics of cell migration and invasion into the pores of the CM is currently missing. Furthermore, the question on combining growth and differential factors with CM to further enhance periodontal regeneration should be addressed in future studies. With more biological data from preclinical studies available, the last step may include evaluation of safety and efficacy of this biomaterial in human intrabony or even suprabony defects.

In conclusion, the results of the present study have for the first time provided histologic evidence for the potential of this novel CM to facilitate periodontal wound healing/regeneration thus warranting further preclinical and clinical testing.

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Tables:

Table 1:

Parameter	*Test Group (mean ± SD)*	*Control Group (mean ± SD)*	*p-value*
Defect height			
In mm	*5.75 ± 0.74*	*5.37 ± 0.72*	*0.10*
New continuous cementum			
In mm	*4.12 ± 1.22*	*1.54 ± 1.45*	*0.0098*
In % of the defect	*71.14 ± 17.46*	*29.09 ± 26.83*	*0.0078*
New interrupted cementum			
In mm	*5.08 ± 0.94*	*3.20 ± 2.31*	*0.0391*
In % of the defect	*89.85 ± 20.92*	*59.23 ± 43.14*	*0.1055*
Height of new bone			
In mm	*3.28 ± 0.69*	*2.47 ± 0.87*	*0.0371*
In % of the defect	*57.39 ± 11.20*	*45.39 ± 13.48*	*0.0137*
Length of the JE			
In mm	*1.49 ± 0.61*	*2.21 ± 1.43*	*0.1602*
Length of the CT-adhesion			
In mm	*1.91 ± 1.03*	*3.36 ± 2.16*	*0.1055*

SD, standard deviation; mm, millimetre; JE, junctional epithelium; CT, connective tissue

Table 2:

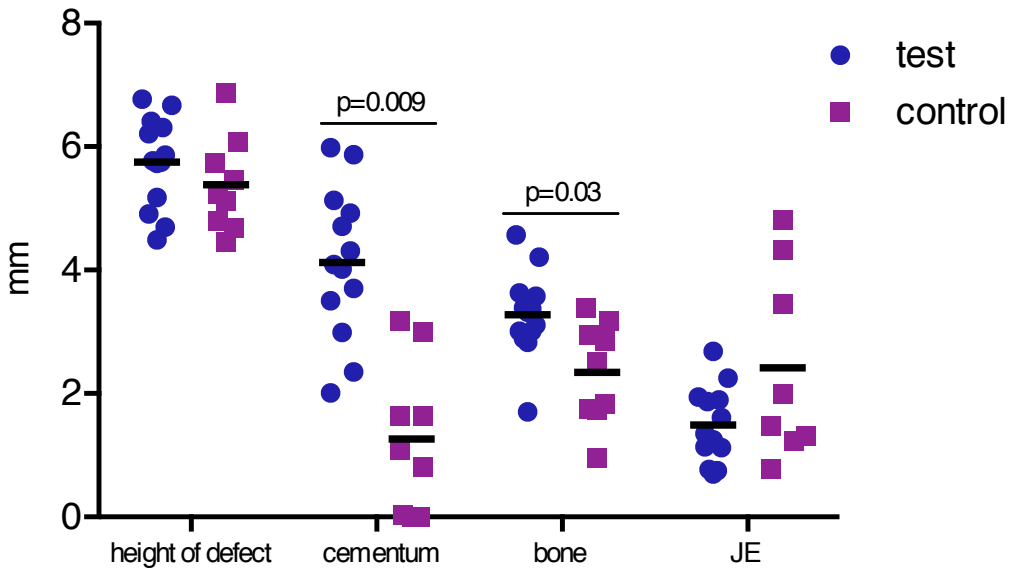

Table 3:

Parameter	*Test (Min/Max)*	*Control (Min/Max)*	*Test (median)*	*Control (median)*	*Test (IQR)*	*Control (IQR)*
Defect height						
in mm	*4.49/6.77*	*4.46/6.87*	*5.77*	*5.25*	*1.14*	*0.79*
New continuous cementum						
in mm	*2.01/5.98*	*0/4.04*	*4.09*	*1.36*	*1.42*	*2.43*
in % of the defect	*40.92/101.83*	*0/76.82*	*74.64*	*25.07*	*18.71*	*42.06*
New interrupted cementum						
in mm	*2.99/6.28*	*0/6.33*	*5.21*	*3.11*	*1.40*	*3.35*
in % of the defect	*52.22/125.79*	*0/88.28*	*88.25*	*56.47*	*48.01*	*58.42*
Height of new bone						
in mm	*1.70/4.57*	*0.95/3.60*	*3.32*	*2.68*	*0.57*	*1.35*
in % of the defect	*29.64/71.83*	*19.87/68.34*	*58.56*	*13.45*	*47.12*	*13.55*
Length of the JE						
in mm	*0.70/2.68*	*0.77/4.81*	*1.35*	*1.47*	*0.78*	*1.78*
Length of the CT-adhesion	*0.12/3.75*	*0.44/6.49*	*1.81*	*3.17*	*1.55*	*3.33*
in mm						

Min, minimum; Max, maximum; IQR, interquartile range; mm, millimetre; JE, junctional epithelium; CT, connective tissue

Figures:

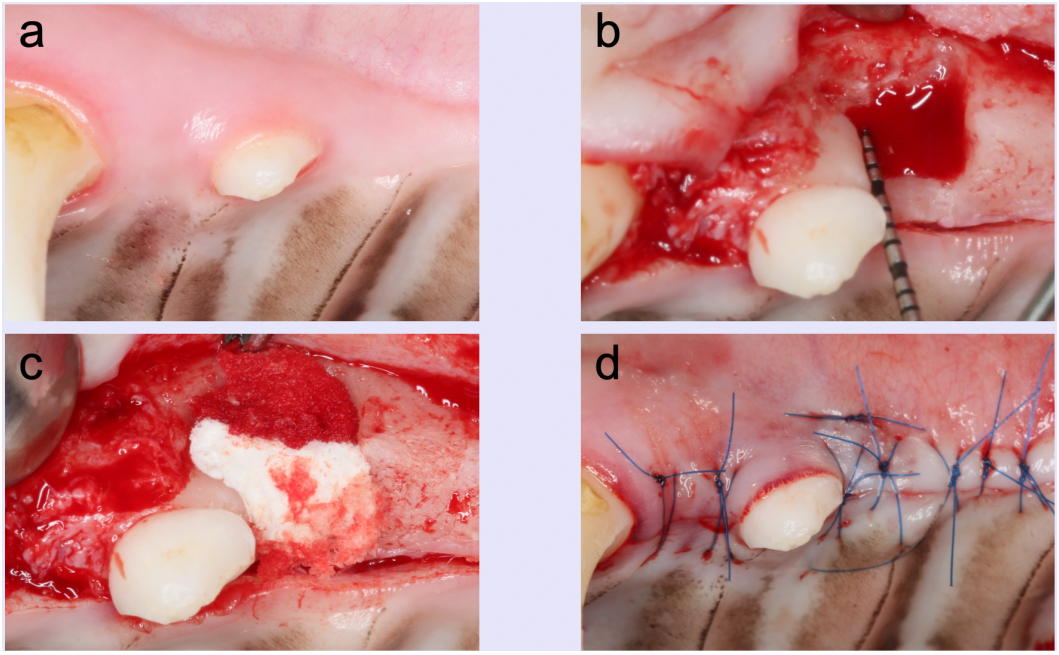

Fig. 1: *Surgical procedure in the test group; a, presurgical; b, acute type, two-wall defect; c, insertion of a trimmed CM; d, wound closure*

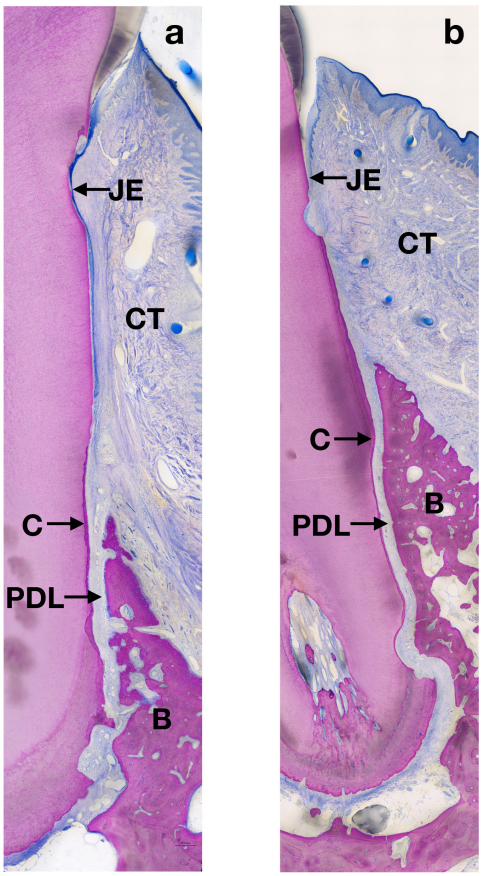

Fig. 2: *a, control-group; b, test-group; JE, junctional epithelium; CT, connective tissue; C, cementum; PDL, periodontal ligament; B, bone*

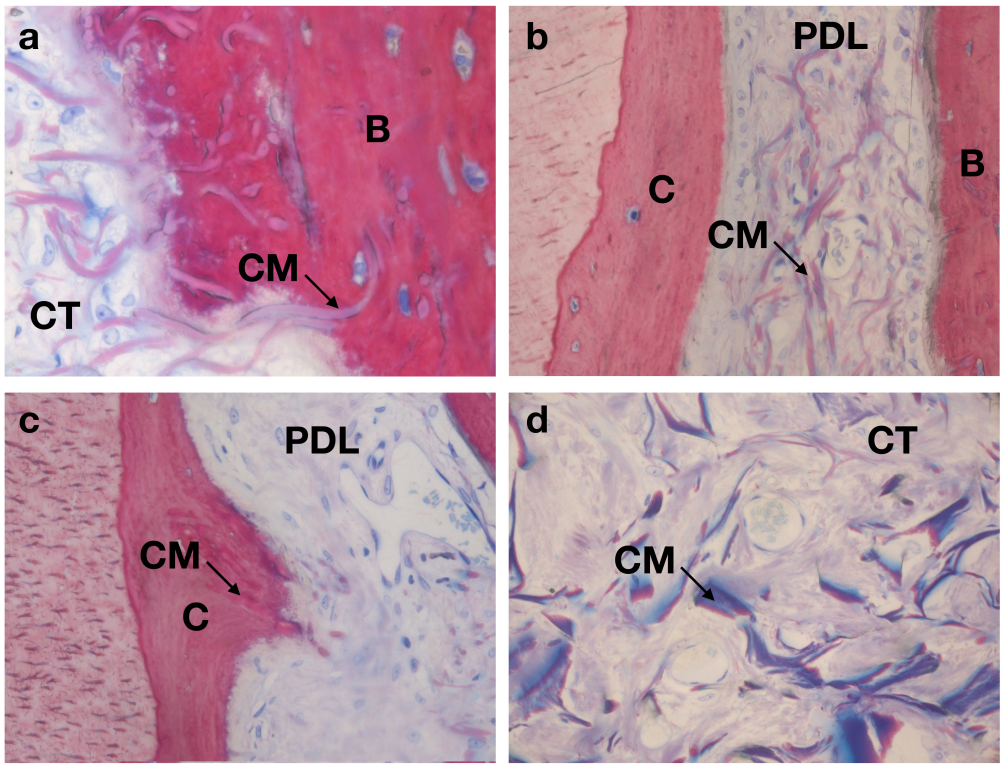

Fig. 3: *Integration of a collagen matrix (CM); a, into bone (B); b, into periodontal ligament (PDL); c, into cementum (C); d, into connective tissue (CT)*

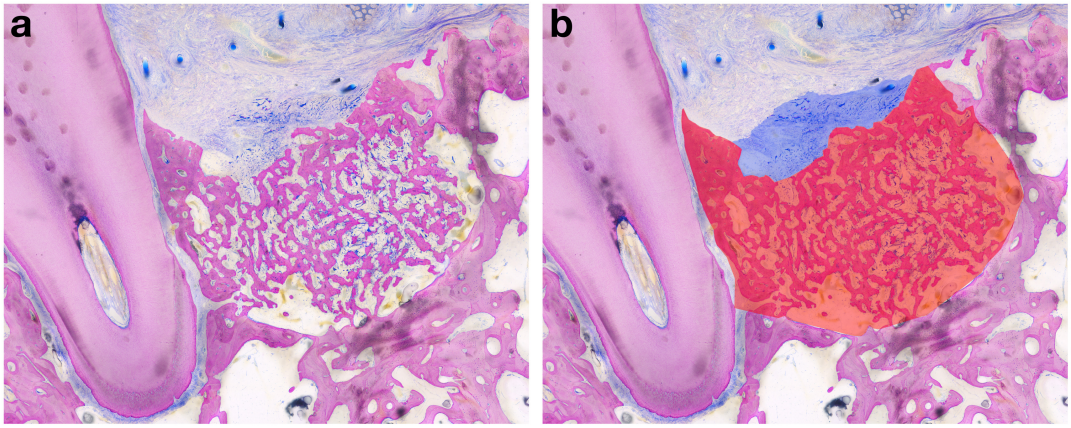

Fig. 4: *a+b: same test site; b, highlighted new bone (red) and connective tissue (blue) with integrated CM*