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ORIGINAL ARTICLE

Split-mouth evaluation of connective tissue graft with or without enamel matrix derivative for the treatment of isolated gingival recession defects in dogs

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Abstract

Objectives The potential additive effect of an enamel matrix derivative (EMD) to a subepithelial connective tissue graft (CTG) for recession coverage is still controversially discussed. Therefore, the aim of this study was to histologically evaluate the healing of gingival recessions treated with coronally advanced flap (CAF) and CTG with or without EMD in dogs.

Materials and methods Gingival recession defects (5 mm wide and 7 mm deep) were surgically created on the labial side of bilateral maxillary canines in 7 dogs. After 8 weeks of plaque accumulation and subsequent 2 weeks of chemical plaque control, the 14 chronic defects were randomized to receive either CAF with CTG (CAF/CTG) or CAF with CTG and EMD (CAF/CTG/EMD). The animals were sacrificed 10 weeks after reconstructive surgery for histologic evaluation.

Results Treatment with CAF/CTG/EMD demonstrated statistically significantly better results in terms of probing pocket depth reduction ($P < 0.05$) and clinical attachment level gain ($P < 0.001$). The length of the epithelium was statistically significantly shorter in the CAF/CTG/EMD group than in the CAF/CTG group (1.00 ± 0.75 mm vs. 2.38 ± 1.48 mm, respectively, $P < 0.01$). Cementum formation was statistically significantly greater in the CAF/CTG/EMD group than following treatment with the CAF/CTG group (3.20 ± 0.89 mm vs. 1.88 ± 1.58 mm, respectively, $P < 0.01$). The CAF/CTG/EMD group showed statistically significantly greater complete periodontal regeneration (i.e., new cementum, new periodontal ligament, and new bone) than treatment with CAF/CTG (0.54 ± 0.73 mm vs. 0.07 ± 0.27 mm, respectively, $P < 0.05$).

Conclusion Within their limits, the present findings indicate that the additional use of EMD in conjunction with CAF + CTG favors periodontal regeneration in gingival recession defects.

Clinical relevance The present findings support the use of EMD combined with CTG and CAF for promoting periodontal regeneration in isolated gingival recession defects.

Keywords Coronally advanced flap · Subepithelial connective tissue graft · Enamel matrix derivative · Gingival recession · Histological investigation · Animal study

Introduction

Buccal gingival recession results in several problems, such as impaired esthetics, plaque accumulation and gingivitis and/or root caries, or dentin hypersensitivity [1, 2]. During the last decades, a plethora of surgical techniques have been proposed to predictably obtain root coverage of gingival recession defects [3]. For the time being, due to its favorable outcomes for root coverage, the subepithelial connective tissue graft (CTG) in conjunction with a coronally advanced flap (CAF) is still considered as gold standard [2–5].

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Ideally, recession treatment should result not only in root coverage with soft tissue but also in periodontal regeneration (i.e., formation of root cementum, periodontal ligament, and alveolar bone) for ensuring physiological probing depth and long-term stability. Enamel matrix derivative (EMD) was first documented in the literature in 1997 as a tissue healing modulator to mimic events that occur during root development [6]. It is an effective agent for stimulating periodontal regeneration characterized by formation of cementum, periodontal ligament, and alveolar bone [6–8]. Moreover, it has been demonstrated that EMD has positive effects on the early healing of periodontal soft tissue wounds [9–11] or on the increase in gingival thickness in dehiscence-type defects in dogs [12]. The use of EMD combined with CTG in conjunction with CAF has been extensively evaluated for the treatment of gingival recessions [13–20]. However, most studies have only reported clinical data [13–18]. While some studies have shown more favorable results following the combination of EMD and CTG in terms of clinical outcomes (e.g., higher probing depth reductions and clinical attachment level gains) when compared with CTG [15, 16], other studies have failed to reveal adjunctive effects of a combination therapy [17, 18]. Thus, there are yet no systemic or narrative reviews for supporting additive clinical benefit of EMD in combination to CAF and CTG treatment. Moreover, there is still limited histological data on the healing following the combination of EMD + CTG [19, 20], while, to the best of our knowledge, there are no split-mouth comparative studies evaluating healing of gingival recessions after CAF + CTG and CAF + EMD + CTG.

Therefore, the present study aimed to evaluate histologically the healing of gingival recession defects treated with CAF and CTG with or without EMD in dogs.

Materials and methods

Animals

Seven healthy male beagle dogs, approximately 2 years old and with a mean body weight of 12 kg, were used in this study. All experimental procedures were approved by the ethical committee of the Animal Research Center of Kagoshima University, Japan (approval no. D16023).

Surgical protocol

One experienced surgeon (Y.S.) performed all surgical procedures under general and local anesthesia using aseptic routines. Before the surgical procedures, analgesics (buprenorphine hydrochloride, 0.1 ml/kg; Leptan, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) and antibiotics (Procaine penicillin G and dihydrostreptomycin sulfate aqueous suspension for injection, 0.05 ml/kg; Mycillin Sol Meiji for veterinary use, Meiji Seika Pharma Co., Ltd., Tokyo, Japan) were administered intramuscularly. General anesthesia was achieved with pentobarbital sodium (Somunopenchiru, 0.2 ml/kg IV; Kyoritsu Seiyaku Corporation, Tokyo, Japan)/medetomidine hydrochloride (Domitor, 0.08 ml/kg IM; Orion Corporation, Espoo, Finland) combination maintaining spontaneous breathing. Local anesthesia was performed using lidocaine HCl/epinephrine (2%, 1:80,000; Xylocaine; Fujisawa Inc., Osaka, Japan). Dehiscence-type gingival recession defects were surgically fabricated bilaterally in the maxillary canines. Two vertical incisions separated by a distance of 5 mm were made from the gingival margin and extending 7 mm apically. These incisions were connected apically by a horizontal incision and coronally by an intrasulcular incision. The gingival tissue limited by the incisions was removed using a periosteal elevator. The exposed bone was removed by means of bone chisels, and the root surfaces were carefully scaled using curettes. A coronal notch extending 5 mm in the mesio-distal direction was placed on the root surface at the level of the cemento-enamel junction (CEJ) (Fig. 1a,b). The created defects were exposed for a period of 8 weeks to plaque accumulation (Fig. 1c). After 8 weeks of plaque accumulation, scaling and root planing were performed. A regimen of plaque control using a 2% solution of chlorhexidine gluconate (5% HIBITANE®, 25 ml of a 2% solution; Sumitomo Dainippon Pharma Co., Ltd., Osaka, Japan) was instituted for 14 days prior to the reconstructive surgeries (Figs. 1d, 3a,b). Full-thickness flaps were then raised, and the root surfaces were once again scaled to remove the residual inflamed granulation tissue. Additional reference notches were made using a #1 round bur on the root surface at the base of the defects and on the crown surface, to indicate the precise center plane of the dehiscence defects and to aid in optimal histologic processing (Fig. 1e). Bilateral defects were assigned randomly as CAF with CTG (CAF/CTG) and CAF with CTG and EMD (Emdogain®-Gel Straumann, Basel, Switzerland) (CAF/CTG/EMD) sites by flipping a coin. CTGs consisting of deep connective tissue and periosteum were removed from the palate. The grafts were trimmed to cover the recession defects on root surfaces and the surrounding bone. In the CAF/CTG/EMD group, defect root surfaces that received EMD were conditioned with a 24% EDTA gel (PrefGel®, Straumann AG, Basel, Switzerland) for 2 min and, then, along with the adjacent mucoperiosteal flaps, thoroughly rinsed with sterile saline. The EMD gel was applied to the root surfaces, and the defects were filled up to the adjacent alveolar crest (Fig. 1f). Prior to the placement of CTG/EMD on the recession defect, the CTG was fully saturated with the EMD gel for 10 min (Fig. 1g). Subsequently, the CTGs were placed over the denuded root surfaces in both groups

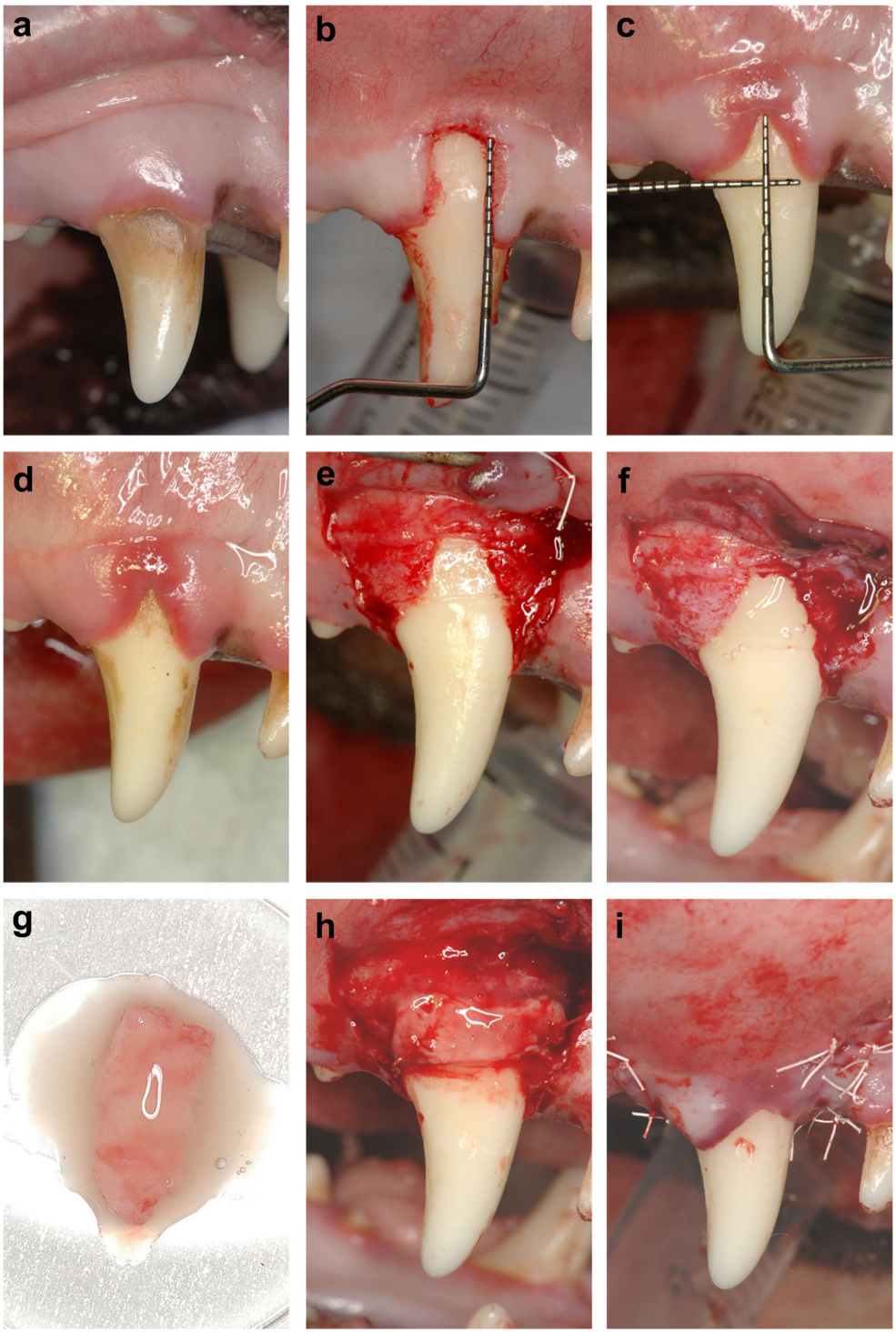

Fig. 1 Clinical overview. **a** Initial clinical aspect. **b** Fabricated dehiscence type of gingival recession defect. A coronal notch was placed at the cemento-enamel junction. **c** Chronic defect after 8 weeks of plaque accumulation. **d** Gingival clinical aspect, 2 weeks after chemical plaque control. Defect before reconstructive surgery. **e** Defect on the root after debridement. **f** EMD was applied onto the denuded root surface after root conditioning. **g** CTG was fully saturated with the EMD for 10 min. **h** The CTG/EMD construct was placed to cover the entire defect and the adjacent bone and sutured. **i** A coronally advanced flap totally covered the CTG/EMD construct

to completely cover the gingival recession defects and fixed to the residual periosteum using resorbable 5–0 sutures (Vicryl, Johnson & Johanson Pty Ltd., Tokyo, Japan) (Fig. 1h). A periosteal-releasing incision was made to allow tension-free coronal advancement of the flaps and sutured using non-resorbable monofilament suture material (Gore-Tex Suture CV-6, W. L., Gore and Associates Inc., AZ, USA) with sling and interrupted sutures (Fig. 1i).

The animals were fed a soft diet for 2 weeks. An analgesic of ketoprofen (Capisten IM 50 mg, 2 mg/kg, 0.1 ml/kg; Kissei Pharmaceutical Co., Ltd., Matsumoto, Japan) and an antibiotic (Mycillin Sol) were administered daily for 2 days. Plaque control was maintained by routine (3 times a week) flushing of the oral cavity with 2% solution of chlorhexidine gluconate for 10 weeks after the reconstructive surgery. Sutures were removed at 2 weeks after the surgery.

Clinical measurements

Before the root coverage surgeries and before sacrifice, measurements of probing pocket depth (PPD), clinical attachment level (CAL), mid-facial gingival recession from the CEJ to the most apical gingival margin (GR1) and from the mesio-distal line at the most coronal gingival level to the most apical gingival margin (GR2), width of the recession (WR) (measured mesio-distally at the most coronal level of gingival recession), and width of keratinized tissue (KT) were recorded using an NC15 periodontal probe (PCP-UNC 15, Hu-Friedy Manufacturing Co., Chicago, IL, USA). All measurements were performed by one experienced, calibrated, and masked examiner (N.T.).

Histologic and histometric analysis

Ten weeks after the reconstructive surgery, the animals were euthanized by an overdose injection of sodium thiopental. All the defects were dissected along with the surrounding soft and hard tissues. The tissue blocks were fixed in 10% buffered formalin and trimmed. The samples were dehydrated and embedded in polyester resin. The resin blocks were cut bucco-lingually to a thickness of 100 to 150 μm with a low-speed diamond saw. Slides were ground and polished to a final thickness of 35 to 45 μm using a microgrinding system with non-adhesive abrasive discs and stained with toluidine blue. All the specimens were analyzed histometrically under a light microscope (BX51, Olympus Optical Co., Ltd., Tokyo, Japan) equipped with a computerized image system (cellSens, Olympus Corp., Tokyo, Japan). For the histometric analysis, two sections were selected from the most central area of each gingival recession defect, identified by the coronal and apical notches on the root and the reference notch on the crown. The mean value of each histometric parameter was calculated for each site. The following parameters (Fig. 2) were measured by the same experienced, calibrated, and masked examiner (T.I.). Intra-examiner reproducibility was ensured by reading 28 sections from all sites by the examiner and repeating the same procedure 48 h later. Calibration was accepted, if 90% of the measurements were reproduced within 0.1 mm difference. (1) Gingival recession (GR): distance from the gingival margin to the coronal notch (CEJ) at the sites where the gingival margin was located apically to the coronal notch (Fig. 2a). Negative values were assigned to these measurements. If the gingival margin was located at the level of the coronal notch or coronal to this notch (Fig. 2b), a "0" value was applied [21]. (2) Epithelial length (EL): distance between the apical extent of junctional epithelium and the coronal notch when the gingival margin was coronal to the coronal notch (Fig. 2b). If gingival recession was present, this measurement was recorded from the gingival margin on the denuded root surface to the apical extent of junctional epithelium (Fig. 2a). (3) Connective tissue adhesion (CT; without cementum): distance between apical extent of junctional epithelium and coronal extent of newly formed cementum. (4) New cementum formation (NC): distance between apical extent of root planing and coronal extent of newly formed cementum on denuded root surface. (5) New bone formation (NB): distance between apical extent of root planing and coronal extent of newly formed alveolar bone. (6) Complete periodontal regeneration (CR): linear length of the root surface covered by NC adjacent to the newly formed bone, with functionally oriented collagen fibers. (7) Soft tissue thickness (STT): distance from the buccal outermost gingival/mucosal surface to the tooth surface at three different levels: STT-1, at the top of the coronal notch (CEJ); STT-2, at the middle between the coronal and apical notches; and STT-3, at the base of the apical notch (defect). (8) Soft tissue height (STH): distance between apical extent of root planing and gingival margin. (9) Defect height (DH): distance between apical notch and coronal notch.

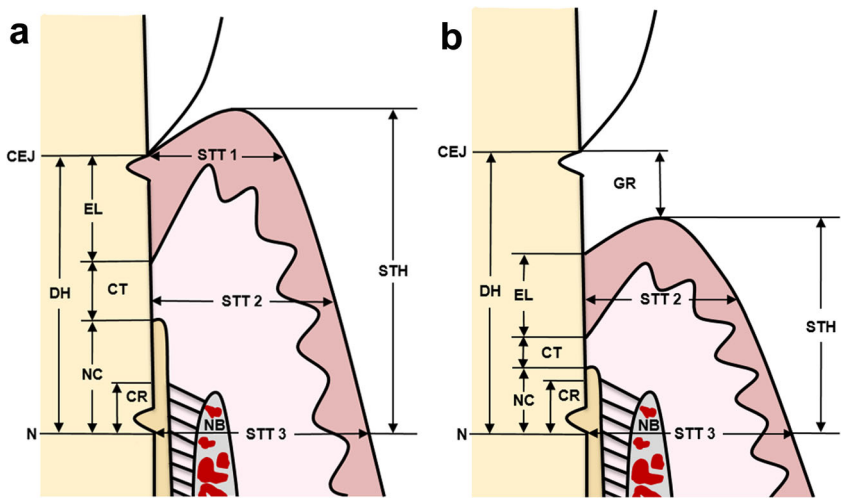

Fig. 2 Schematic drawing of the histometric analysis. In the absence (**a**) or presence (**b**) of gingival recession (GR). CEJ, cemento-enamel junction; EL, epithelial length; CT, connective tissue adhesion; NC, new cementum formation; DH, defect height; NB, new bone formation; CR, complete periodontal regeneration; STT, soft tissue thickness; STH, soft tissue height; N, apical notch

Sample size calculation

Due to the very limited number of preclinical studies in larger animals (e.g., dogs) with a comparable design, no specific power analysis for sample calculation was performed. However, in order to increase the statistical power, a split-mouth design was adopted in all 7 animals.

Statistical analysis

Mean values for each histometric parameter were obtained per defect. The mean values for the CAF/CTG and CAF/CTG/EMD groups were determined by using the individual means from the 7 animals. After confirming a normal distribution, the paired *t* test was utilized to analyze treatment effect (between treatment) as well as time effect (baseline versus 10 weeks) within and between groups, both clinically and histologically. The primary outcome variable of this study was the histometric outcome measured for the CAF/CTG and CAF/CTG/EMD groups at 10 weeks. The clinical parameters were the secondary outcome.

A P value of < 0.05 was considered statistically significant. All calculations were performed using statistical software (BellCurve for Excel, Social Survey Research Information Co., Ltd., Tokyo, Japan).

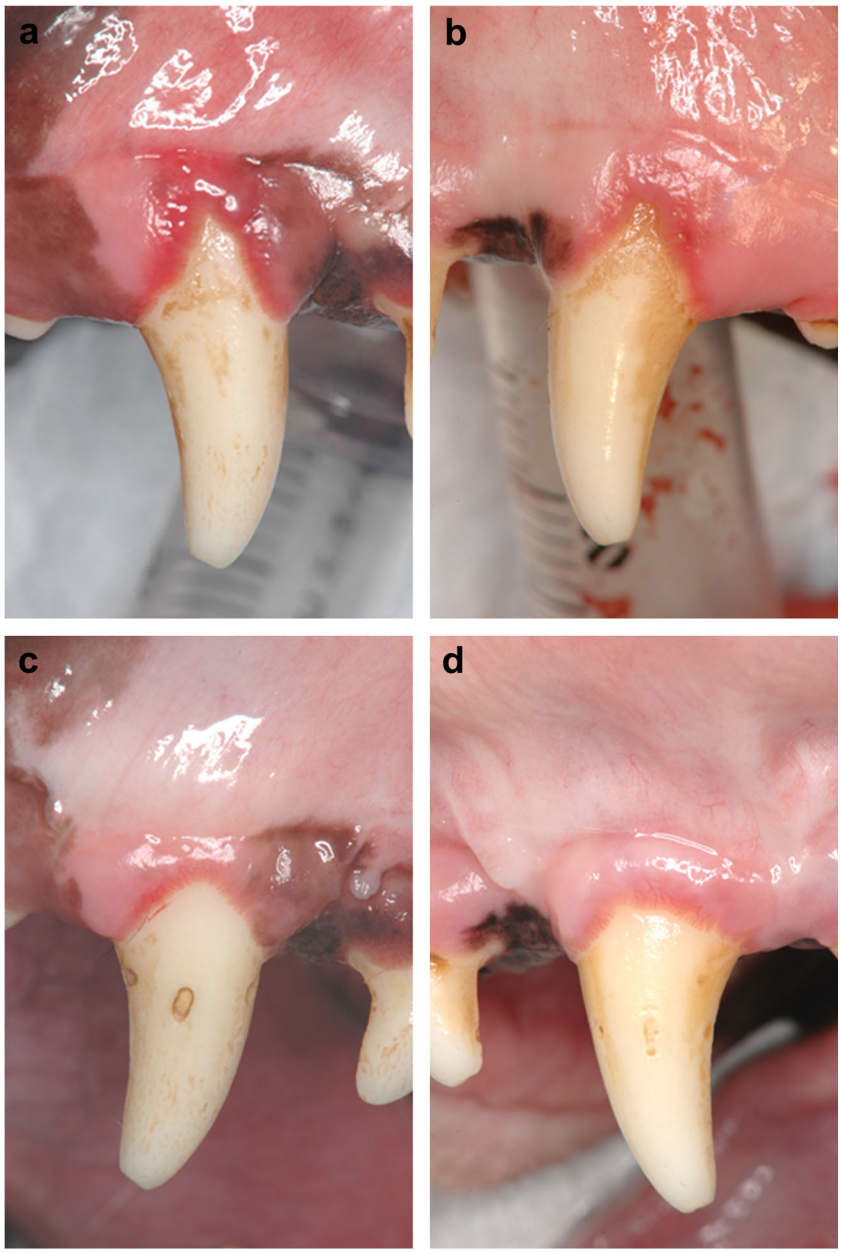

Fig. 3 Clinical photographs. **a** CAF with CTG group at baseline. **b** CAF with CTG and EMD groups at baseline. **c** CAF with CTG group at 10 weeks. **d** CAF with CTG and EMD groups at 10 weeks

Results

Clinical observations and clinical parameters

All of the experimental sites (14 sites, 7 sites/group) in the CAF/CTG and CAF/CTG/EMD groups exhibited favorable clinical healing without any complications, such as swelling, suppuration, abscess formation, or exposure of the connective tissue grafts (Fig. 3c,d). Root coverage was obtained to a varying degree in this chronic gingival recession model. Basically, the gingival margin was located approximately at the CEJ (Fig. 3c,d) in all sites. Residual gingival recessions (gingival margin was located apically to the CEJ) were found in 2 of the 7 treated sites in the CAF/CTG and CAF/CTG/EMD groups.

At baseline, differences between the CAF/CTG and CAF/CTG/EMD groups for all tested clinical parameters were not statistically significant (Table 1). There was a statistically significant reduction in the PPD for the CAF/CTG/EMD group compared to the CAF/CTG group ($P < 0.05$). The CAL was gained statistically significantly in the CAF/CTG/EMD group, changing from 3.71 ± 1.15 to 1.21 ± 1.15 mm ($P < 0.001$). At 10 weeks, the amount of KT in the CAF/CTG/EMD group was also statistically significantly greater than that in the CAF/CTG group ($P < 0.05$). No statistically significant differences were detected between the CAF/CTG/EMD and the CAF/CTG groups with regard to the following parameters (GR1, GR2, and WR).

Descriptive histology

In all experimental sites, the connective tissue grafts were completely integrated with the adjacent connective tissue with no signs of multinucleated giant cells or granulation tissue (Figs. 4a, 5a, 6a, and 7a). However, apical migration of junctional epithelium was more pronounced in the CAF/

Table 1 Clinical parameters for each surgical treatment at baseline and 10 weeks (mean ± SD; mm)

Clinical parameter	Surgical treatment	
	CAF/CTG ($n = 7$)	CAF/CTG/EMD ($n = 7$)
PPD		
Baseline	2.14 ± 0.24	2.43 ± 0.45
10 weeks	2.14 ± 0.90	1.29 ± 0.49
PPD reduction over 10 weeks	0.00 ± 0.96	1.14 ± 0.85*
CAL		
Baseline	3.21 ± 0.86	3.71 ± 1.15
10 weeks	2.14 ± 1.35	1.21 ± 1.15
CAL gain over 10 weeks	1.07 ± 1.46	2.50 ± 1.23**
GR1 (CEJ～)		
Baseline	1.07 ± 0.79	1.36 ± 0.90
10 weeks	0.00 ± 0.58	0.79 ± 1.63
GR1 reduction over 10 weeks	1.07 ± 1.06	2.14 ± 1.38
GR2 (GM～)		
Baseline	5.71 ± 1.25	5.14 ± 1.35
10 weeks	3.43 ± 0.79	2.86 ± 0.95
GR2 reduction over 10 weeks	2.29 ± 0.76	2.29 ± 0.99
WR		
Baseline	8.00 ± 1.29	8.00 ± 1.29
10 weeks	6.50 ± 1.44	5.71 ± 2.98
WR change over 10 weeks	1.50 ± 2.50	2.29 ± 3.64
KT		
Baseline	3.93 ± 1.27	4.21 ± 0.95
10 weeks	2.93 ± 1.06	3.57 ± 0.93*
KT change over 10 weeks	1.00 ± 1.29	0.64 ± 1.73

PPD, probing pocket depth; CAL, clinical attachment level; GR1, mid-facial gingival recession from the CEJ to the most apical gingival margin; GR2, mid-facial gingival recession from the mesio-distal line at the most coronal gingival level to the most apical gingival margin; WR, width of gingival recession; KT, width of keratinized tissue

*Statistically significantly different from the CAF/CTG group ($P < 0.05$)

**Statistically significantly different from the CAF/CTG group ($P < 0.001$)

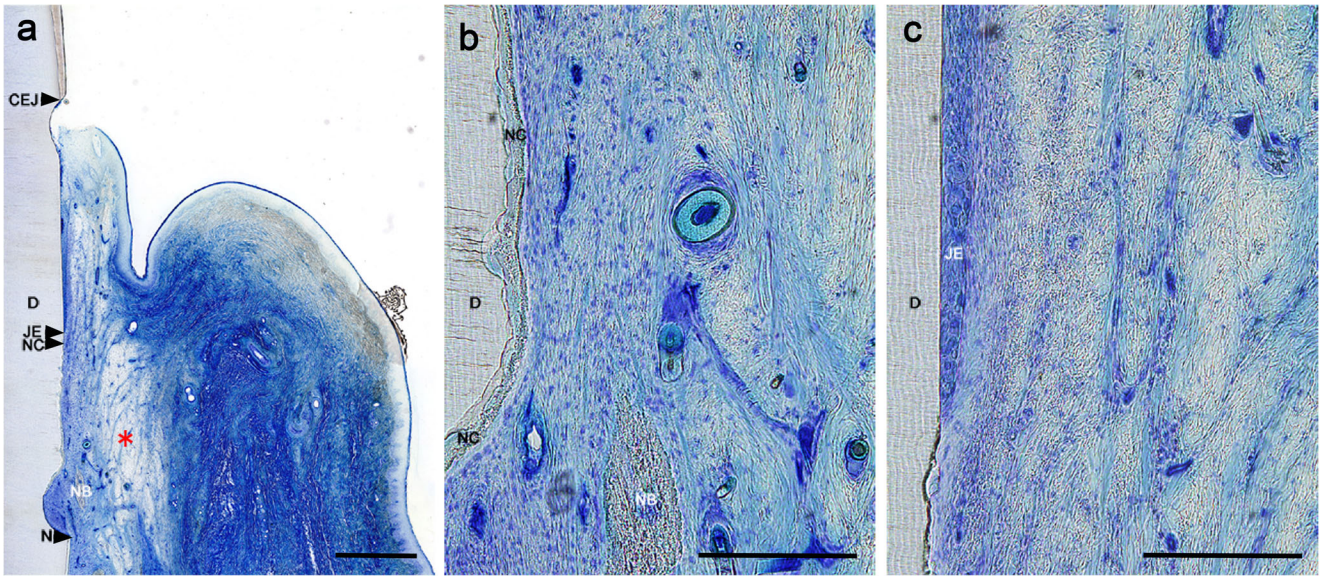

Fig. 4 Representative photomicrographs of a gingival recession defect treated by CAF with CTG. **a** The defect with new bone formation; overview (scale bar, 1 mm; toluidine blue staining). **b** Higher magnification of the apical portion of the defect (scale bar, 200 μm; toluidine blue staining). **c** Higher magnification of the middle portion of the defect (scale bar, 200 μm; toluidine blue staining). CEJ, cemento-enamel junction; D, root dentin; JE, apical end of junctional epithelium; N, apical end of apical notch; NC, new cementum; * CTG

CTG group (Figs. 4a, 4c, 5a, and 5d) than in the CAF/CTG/EMD group (Figs. 6a and 7a). Minimal new bone formation was observed (Fig. 4a,b) in 2 sites in the CAF/CTG group, and new cementum formation was limited (Figs. 4 and 5) in 4 sites in the CAF/CTG group. Formation of new connective tissue attachment (i.e., new cementum with inserting collagen fibers) was not observed, and connective tissue fibers were aligned parallel to the root surface (Figs. 4b, 5b, and 5c). In the CAF/CTG/EMD group, bone formation was noted extending from the apical notches towards the coronal region of the defects (Fig. 6a–c) in 3 sites. In these defects, dense collagen fibers were seen inserting into the newly formed cementum, oriented oblique to the root surface (Fig. 6b,c). New cementum formation with or without new bone was observed in 6 sites. A thin continuous layer of a blend of new cellular, intrinsic or mixed fiber cementum was mostly found at the apical portion (Figs. 6 and 7) and tended to change to acellular extrinsic fiber cementum at the coronal portion on the denuded root surface in the CAF/CTG/EMD group (Figs. 6 and 7). Ankylosis was not observed in any of the defects.

Histometric analysis

The results of the histometric analysis are described in Table 2. No statistically significant differences were detected between

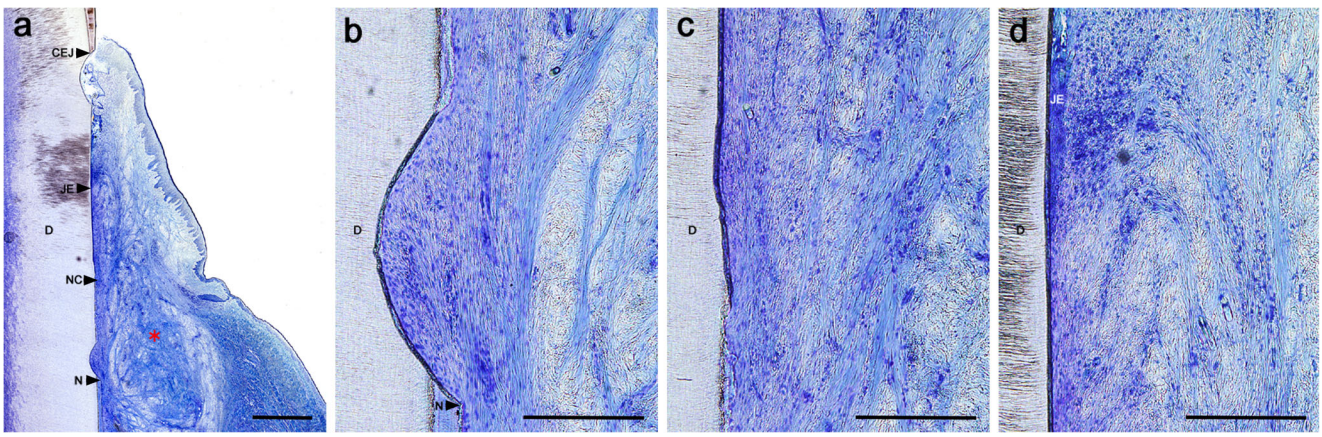

Fig. 5 Representative photomicrographs of a gingival recession defect treated by CAF with CTG. **a** The defect without new bone formation; overview (scale bar, 1 mm; toluidine blue staining). **b** Higher magnification of the apical portion of the defect (scale bar, 200 μm; toluidine blue staining). **c** Higher magnification of the middle portion of the defect (scale bar, 200 μm; toluidine blue staining). **d** Higher magnification of the apical extent of junctional epithelium (scale bar, 200 μm; toluidine blue staining). CEJ, cemento-enamel junction; GM, D, root dentin; JE, apical end of junctional epithelium; N, apical end of apical notch; NC, new cementum; * CTG

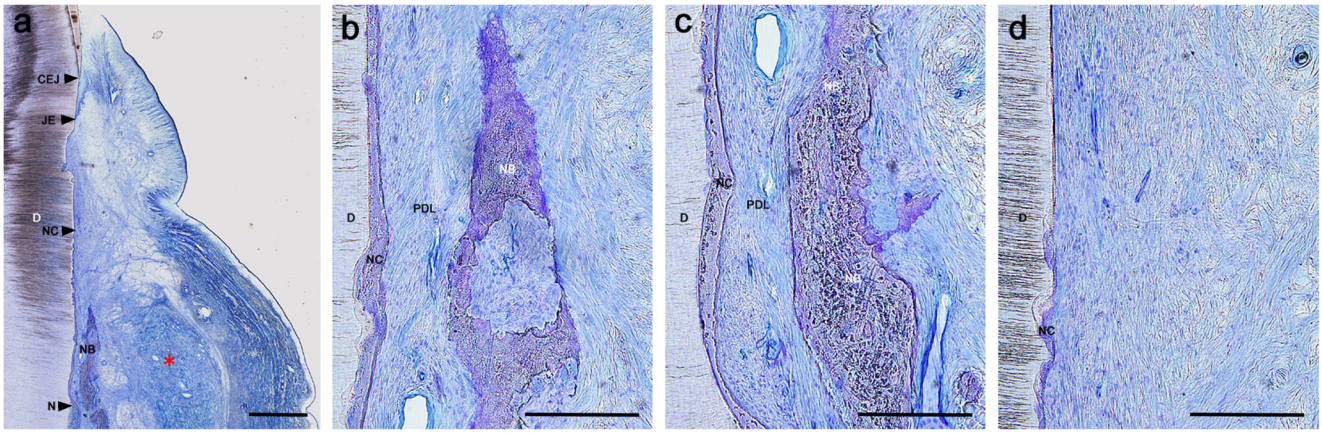

Fig. 6 Representative photomicrograph of a gingival recession defect treated by CAF with CTG and EMD. The defect with new bone formation. **a** Overview (scale bar, 1 mm; toluidine blue staining). **b** Higher magnification of the apical portion of the defect (scale bar, 200 μm; toluidine blue staining). **c** Higher magnification of the coronal extent of newly formed bone (scale bar, 200 μm; toluidine blue staining). **d** Higher magnification of the middle portion of the defect (scale bar, 200 μm; toluidine blue staining). CEJ, cemento-enamel junction; D, root dentin; JE, apical end of junctional epithelium; N, apical end of apical notch; NB, new bone; NC, new cementum; PDL, periodontal ligament; * CTG.

the CAF/CTG/EMD and CAF/CTG groups with regard to the following parameters (GR, CT, NB, STH, STT-1, -2, -3, and DH). In the CAF/CTG/EMD group, EL on the denuded root surface was statistically significantly shorter than that in the CAF/CTG group ($P < 0.01$). New cementum formation (NC, $P < 0.01$) with a continuous periodontal ligament-like tissue (i.e., CR, $P < 0.05$) in the CAF/CTG/EMD group was statistically significantly greater than that in the CAF/CTG group. More new bone was observed in the CAF/CTG/EMD group compared to the CAF/CTG group, although without statistically significant differences.

Discussion

In the present study, CAF/CTG/EMD treatment was clinically effective in improving clinical parameters as shown in a number of previous studies [13–19]. In other words, the CAF/CTG/EMD group showed statistically significantly better results in PPD reduction and CAL gain at 10 weeks. However, other studies have failed to show a beneficial effect on the clinical outcomes when combining EMD with CTG compared to the use of CTG alone [17, 18]. The discrepancy in PPD reduction and CAL gain between our study and the mentioned ones may be attributed to the

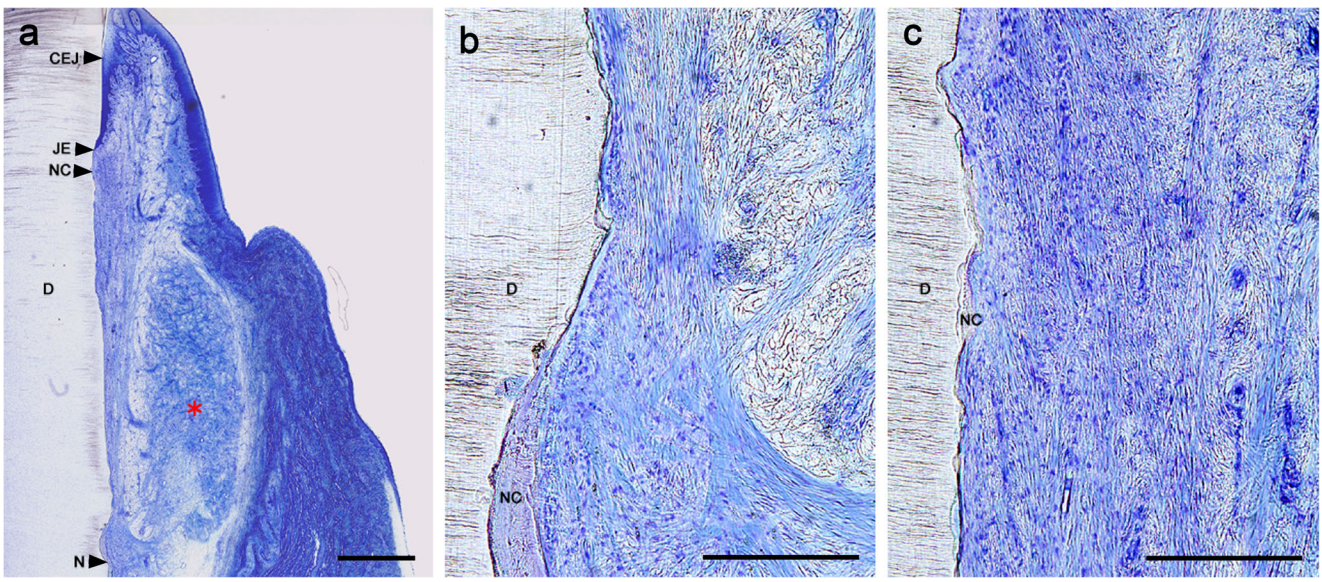

Fig. 7 Representative photomicrograph of a gingival recession defect treated by CAF with CTG and EMD. The defect without new bone formation. **a** Overview (scale bar, 1 mm; toluidine blue staining). **b** Higher magnification of the apical portion of the defect (scale bar, 200 μm; toluidine blue staining). **c** Higher magnification of the coronal extent of newly formed cementum (scale bar, 200 μm; toluidine blue staining). CEJ, cemento-enamel junction; D, root dentin; JE, apical end of junctional epithelium; N, apical end of apical notch; NB, new bone; NC, new cementum, * CTG

Table 2 Histomorphometric parameters for each surgical treatment at 10 weeks (mean ± SD; mm)

Histometric parameter	Surgical treatment	
	CAF/CTG ($n = 7$)	CAF/CTG/EMD ($n = 7$)
DH	4.69 ± 0.97	4.62 ± 0.92
GR	− 0.10 ± 0.17	− 0.17 ± 0.35
EL	2.38 ± 1.48	1.00 ± 0.75**
CT	0.38 ± 0.55	0.26 ± 0.26
NC	1.88 ± 1.58	3.20 ± 0.89**
NB	0.18 ± 0.30	0.45 ± 0.68
CR	0.07 ± 0.27	0.54 ± 0.73*
STH	5.34 ± 1.20	5.14 ± 1.58
STT-1	0.70 ± 0.44	0.73 ± 0.52
STT-2	2.62 ± 1.10	2.61 ± 1.20
STT-3	3.61 ± 1.03	3.75 ± 1.13

DH, defect height; GR, gingival recession; EL, epithelial length; CT, connective tissue adhesion (without cementum); NC, new cementum formation; NB, new bone formation; CR, complete periodontal regeneration; STH, soft tissue height; STT, soft tissue thickness (STT-1) at the top of the coronal notch, (STT-2) at the middle between the coronal and apical notches, and (STT-3) at the base of the apical notch

*Statistically significantly different from the CAF/CTG group ($P < 0.05$)

**Statistically significantly different from the CAF/CTG group ($P < 0.01$)

differences in the level of oral hygiene, width of the gingival recessions, thickness of the flap, position of the CTG, and the used surgical techniques [18, 22, 23].

Histologically, the amounts of STT and STH obtained in the CAF/CTG/EMD group were comparable to those in the CAF/CTG group, without statistically significant differences. However, STT and STH were greater in the CTG-applied groups than in the CAF group (STT-1: 0.13 ± 0.31 mm; STT-2: 1.24 ± 0.58 mm; and STH: 4.39 ± 0.90 mm) in the same design of our previous study [24]. These results may be explained by the fact that connective tissue grafts were completely integrated with the proximal tissue in the CAF/CTG and CAF/CTG/EMD groups and indicate that CTG may increase and maintain the volume of soft tissue in root coverage treatment [25, 26]. However, the amount of EL was statistically significantly shorter in the CAF/CTG/EMD group than in the CAF/CTG group. This finding is in agreement with previous reports showing restricted migration of junctional epithelium after EMD application in gingival recession or periodontal defects [20, 27, 28] and is supported by in vitro studies showing that EMD inhibits the proliferation of oral squamous cell carcinoma-derived (SCC23) [29] as well as normal human gingival epithelial cells [30]. Moreover, new bone formation was minimal and connective tissue adaptation without new cementum was dominant in the CAF/CTG group. These findings are similar to those of previous studies demonstrating limited periodontal regeneration characterized by long junctional epithelium and connective tissue adhesion on the root surfaces treated by CAF alone [24] or CAF with CTG [31–33].

The present study has demonstrated that the additional application of EMD to CTG in conjunction with CAF resulted to a greater extent in periodontal regeneration than CAF with CTG. These findings are similar to the results of our previous animal study showing more periodontal regeneration in gingival recession defects treated by CAF with EMD than CAF alone [24]. The amount of newly formed cementum was statistically significantly greater in the CAF/CTG/EMD group than in the CAF/CTG group. These findings are in agreement with previous reports demonstrating that the deposition of EMD on root surfaces potently affects cementum formation [6, 8, 19, 20, 24, 28, 34]. Moreover, dense collagen fibers inserted into the newly formed cementum were oriented obliquely to the root surface. Highly vascularized new periodontal ligament-like tissue was found in the area between new cementum and new bone in the CAF/CTG/EMD group. As a result, the amounts of NC and CR in the CAF/CTG/EMD group were statistically significantly greater than those in the CAF/CTG group, although substantial differences between NC and CR were detected. The discrepancy between NC and CR may be explained by the fact that CR was defined as the linear length of the root surface covered by NC adjacent to newly formed bone, with functionally oriented collagen fibers. Since in this buccal model, the alveolar bone plate was very thin (generally < 1 mm) and the space between the flap and the root surface was minimal, the amounts of newly formed bone were more limited [24, 35–37]. Nevertheless, the CAF/CTG/EMD group appeared to yield greater bone formation with narrow bone growth along the root surface compared to the CAF/CTG group, although the differences were not statistically significant. These observations are supported by the findings that new bone formation occurs moderately along the root surface following formation of cementum and periodontal ligament [24, 34, 38, 39] and also points to the adjunctive effects of EMD to improve bone formation in gingival recession defects treated by CAF, CAF/GTR, and CAF/CTG [24, 35, 40]. The present results may be explained by the results of in vitro studies demonstrating that EMD possesses a growth factor-like activity depending on TGF-β, BMP-like, and connective tissue growth factor inducing also mineral deposition [8, 41, 42]. It thus appears that EMD not only induces proliferation of gingiva-derived mesenchymal stem cells but also enhances their osteogenic differentiation [43]. Furthermore, EMD significantly promotes both periodontal ligament and bone cell attachment [8, 44], stimulates angiogenesis directly through endothelial cells and indirectly through production of vascular endothelial growth factor by periodontal ligament cells [8, 10, 45], and increases extracellular matrix protein production [8, 46] and the level of TGF-β, which has been reported to facilitate tissue repair and regeneration [8, 47, 48] of gingival and periodontal ligament fibroblasts [49, 50].

Taken together, the favorable results obtained in the CAF/CTG/EMD group appear to justify the use of EMD in combination with CTG for the treatment of gingival recession defects.

However, since the encouraging results were obtained in isolated gingival recession defects in dogs with substantial differences compared to humans regarding healing potential and morphology of tooth/gingiva, further studies are warranted to determine whether the results of this study are in agreement with the reported clinical findings and whether the additive use of EMD to CTG and CAF treatment has beneficial effects on more challenging (e.g., class II to III, multiple) gingival recession defects.

Conclusion and clinical relevance

Within the limitations of the present study, it can be concluded that CAF/CTG/EMD treatment may promote periodontal regeneration in isolated gingival recession defects.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed, and all procedures performed in studies involving animals were in accordance with the ethical standards of the ethical committee of the Animal Research Center of Kagoshima University, Japan (Approval No. D16023).

Informed consent For this type of study, formal consent was not required.

References

1. Löe H, Anerud A, Boysen H (1992) The natural history of periodontal disease in man: prevalence, severity, and extent of gingival recession. J Periodontol 63:489–495
2. Cheng GL, Fu E, Tu YK, Shen EC, Chiu HC, Huang RY, Yuh DY, Chiang CY (2015) Root coverage by coronally advanced flap with connective tissue graft and/or enamel matrix derivative: a meta-analysis. J Periodontal Res 50:220–230
3. Hofmänner P, Alessandri R, Laugisch O, Aroca S, Salvi GE, Stavropoulos A, Sculean A (2012) Predictability of surgical techniques used for coverage of multiple adjacent gingival recessions—a systematic review. Quintessence Int 43:545–554
4. Griffin TJ, Cheung WS, Zavras AI, Damoulis PD (2006) Postoperative complications following gingival augmentation procedures. J Periodontol 77:2070–2079
5. Aroca S, Molnár B, Windisch P, Gera I, Salvi GE, Nikolidakis D, Sculean A (2013) Treatment of multiple adjacent Miller class I and II gingival recessions with a modified coronally advanced tunnel (MCAT) technique and a collagen matrix or palatal connective tissue graft: a randomized, controlled clinical trial. J Clin Periodontol 40:713–720
6. Hammarström L (1997) Enamel matrix, cementum development and regeneration. J Clin Periodontol 24:658–668
7. Heijl L, Heden G, Svardström G, Östgren A (1997) Enamel matrix derivative (Emdogain®) in the treatment of intrabony periodontal defects. J Clin Periodontol 24:705–714
8. Miron RJ, Sculean A, Cochran DL, Froum S, Zucchelli G, Nemcovsky C, Donos N, Lyngstadaas SP, Deschner J, Dard M, Stavropoulos A, Zhang Y, Trombelli L, Kasaj A, Shirakata Y, Cortellini P, Tonetti M, Rasperini G, Jepsen S, Bosshardt DD (2016) 20 years of enamel matrix derivative: the past, the present and the future. J Clin Periodontol 43:668–683
9. Wennström JL, Lindhe J (2002) Some effects of enamel matrix proteins on wound healing in the dento-gingival region. J Clin Periodontol 29:9–14
10. Miron RJ, Dard M, Weinreb M (2015) Enamel matrix derivative, inflammation and soft tissue wound healing. J Periodontal Res 50: 555–569
11. Maymon-Gil T, Weinberg E, Nemcovsky C, Weinreb M (2016) Enamel matrix derivative promotes healing of a surgical wound in the rat oral mucosa. J Periodontol 87:601–609
12. Al-Hezaimi K, Al-Fahad H, O'Neill R, Shuman L, Griffin T (2012) The effect of enamel matrix protein on gingival tissue thickness in vivo. Odontology 100:61–66
13. Berlucchi I, Francetti L, Del Fabbro M, Testori T, Weinstein RL (2002) Enamel matrix proteins (Emdogain) in combination with coronally advanced flap or subepithelial connective tissue graft in the treatment of shallow gingival recessions. Int J Periodontics Restorative Dent 22:583–593
14. Sato S, Yamada K, Kato T, Haryu K, Ito K (2006) Treatment of Miller class III recessions with enamel matrix derivative (Emdogain) in combination with subepithelial connective tissue grafting. Int J Periodontics Restorative Dent 26:71–77
15. Henriques PS, Pelegrine AA, Nogueira AA, Borghi MM (2010) Application of subepithelial connective tissue graft with or without enamel matrix derivative for root coverage: a split-mouth randomized study. J Oral Sci 52:463–471
16. Rasperini G, Roccuzzo M, Francetti L, Acunzo R, Consonni D, Silvestri M (2011) Subepithelial connective tissue graft for treatment of gingival recessions with and without enamel matrix derivative: a multicenter, randomized controlled clinical trial. Int J Periodontics Restorative Dent 31:133–139
17. Aroca S, Keglevich T, Nikolidakis D, Gera I, Nagy K, Azzi R, Etienne D (2010) Treatment of class III multiple gingival recessions: a randomized-clinical trial. J Clin Periodontol 37:88–97
18. Roman A, Soancă A, Kasaj A, Stratul SI (2013) Subepithelial connective tissue graft with or without enamel matrix derivative for the treatment of Miller class I and II gingival recessions: a controlled randomized clinical trial. J Periodontal Res 48:563–572
19. Rasperini G, Silvestri M, Schenk RK, Nevins ML (2000) Clinical and histologic evaluation of human gingival recession treated with a subepithelial connective tissue graft and enamel matrix derivative (Emdogain): a case report. Int J Periodontics Restorative Dent 20:269–275
20. Carnio J, Camargo PM, Kenney EB, Schenk RK (2002) Histological evaluation of 4 cases of root coverage following a connective tissue graft combined with an enamel matrix derivative preparation. J Periodontol 71:31–43
21. Casati MZ, Sallum EA, Caffesse RG, Nociti FH Jr, Sallum AW, Pereira SL (2000) Guided tissue regeneration with a bioabsorbable polylactic acid membrane in gingival recessions. A histometric study in dogs. J Periodontol 71:238–248
22. Harris RJ (1997) Creeping attachment associated with the connective tissue with partial-thickness double pedicle grafts. J Periodontol 68:890–899

23. Al-Zahrani MS, Bissada NF (2005) Predictability of connective tissue grafts for root coverage: clinical perspectives and a review of the literature. Quintessence Int 36:609–616
24. Shirakata Y, Sculean A, Shinohara Y, Sena K, Takeuchi N, Bosshardt DD, Noguchi K (2016) Healing of localized gingival recessions treated with a coronally advanced flap alone or combined with an enamel matrix derivative and a porcine acellular dermal matrix: a preclinical study. Clin Oral Investig 20:1791–1800
25. Cortellini P, Tonetti MS, Baldo C, Francetti L, Rasperini G, Rotundo R, Nieri M, Franceschi D, Labriola A, Prato GP (2009) Does placement of a connective tissue graft improve the outcomes of coronally advanced flap for coverage of single gingival recessions in upper anterior teeth? A multi-centre, randomized, double-blind, clinical trial. J Clin Periodontol 36:68–79
26. Cairo F, Cortellini P, Pilloni A, Nieri M, Cincinelli S, Amunni F, Pagavino G, Tonetti MS (2016) Clinical efficacy of coronally advanced flap with or without connective tissue graft for the treatment of multiple adjacent gingival recessions in the aesthetic area: a randomized-controlled clinical trial. J Clin Periodontol 43:849–856
27. Hammarström L, Heijl L, Gestrelius S (1997) Periodontal regeneration in a buccal dehiscence model in monkeys after application of enamel matrix proteins. J Clin Periodontol 24:669–677
28. Nemcovsky CE, Zahavi S, Moses O, Kebudi E, Artzi Z, Beny L, Weinreb M (2006) Effect of enamel matrix protein derivative on healing of surgical supra-infrabony periodontal defects in rat molar. J Periodontol 77:996–1002
29. Kawase T, Okuda K, Yoshie H, Burns DM (2000) Cytostatic action of enamel matrix derivative (EMDOGAIN®) on human oral squamous cell carcinoma-derived SCC25 epithelial cells. J Periodontal Res 35:291–300
30. Weinberg E, Topaz M, Dard M, Lyngstadaas P, Nemcovsky C, Weinreb M (2010) Differential effects of prostaglandin E_2 and enamel matrix derivative on the proliferation of human gingival and dermal fibroblasts and gingival keratinocytes. J Periodontal Res 45:731–740
31. Bruno JF, Bowers GM (2000) Histology of a human biopsy section following the placement of a subepithelial connective tissue graft. Int J Periodontics Restorative Dent 20:225–231
32. McGuire MK, Cochran DL (2003) Evaluation of human recession defects treated with coronally advanced flaps and either enamel matrix derivative or connective tissue. Part 2: histological evaluation. J Periodontol 74:1126–1135
33. McGuire MK, Scheyer ET, Schupbach P (2016) A prospective, case-controlled study evaluating the use of enamel matrix derivative on human buccal recession defects: a human histologic examination. J Periodontol 87:645–653
34. Mellonig JT (1999) Enamel matrix derivative for periodontal reconstructive surgery: technique and clinical and histologic case report. Int J Periodontics Restorative Dent 19:8–19
35. Sallum EA, Casati MZ, Caffesse RG, Funis LP, Nociti Júnior FH, Sallum AW (2003) Coronally positioned flap with or without enamel matrix protein derivative for the treatment of gingival recessions. Am J Dent 16:287–291
36. de Oliveira CA, Spolidório LC, Cirelli JA, Marcantonio RA (2005) Acellular dermal matrix allograft used alone and in combination with enamel matrix protein in gingival recession: histologic study in dogs. Int J Periodontics Restorative Dent 25:595–603
37. Suaid FF, Carvalho MD, Santamaria MP, Casati MZ, Nociti FH Jr, Sallum AW, Sallum EA (2008) Platelet-rich plasma and connective tissue grafts in the treatment of gingival recessions: a histometric study in dogs. J Periodontol 79:888–895
38. Shirakata Y, Taniyama K, Yoshimoto T, Miyamoto M, Takeuchi N, Matsuyama T, Noguchi K (2010) Regenerative effect of basic fibroblast growth factor on periodontal healing in two-wall intrabony defects in dogs. J Clin Periodontol 37:374–381
39. Windisch P, Sculean A, Klein F, Tóth V, Gera I, Reich E, Eickholz P (2002) Comparison of clinical, radiographic, and histometric measurements following treatment with guided tissue regeneration or enamel matrix proteins in human periodontal defects. J Periodontol 73:409–417
40. Fujita T, Yamamoto S, Ota M, Shibukawa Y, Yamada S (2011) Coverage of gingival recession defects using guided tissue regeneration with and without adjunctive enamel matrix derivative in a dog model. Int J Periodontics Restorative Dent 31:247–253
41. Suzuki S, Nagano T, Yamakoshi Y, Gomi K, Arai T, Fukae M, Katagiri T, Oida S (2005) Enamel matrix derivative gel stimulates signal transduction of BMP and TGF-β. J Dent Res 84:510–514
42. Heng NH, N'Guessan PD, Kleber BM, Bernimoulin JP, Pischon N (2007) Enamel matrix derivative induces connective tissue growth factor expression in human osteoblastic cells. J Periodontol 78:2369–2379
43. Wu SM, Chiu HC, Chin YT, Lin HY, Chiang CY, Tu HP, Fu MM, Fu E (2014) Effects of enamel matrix derivative on the proliferation and osteogenic differentiation of human gingival mesenchymal stem cells. Stem Cell Res Ther 5:52
44. Hoang AM, Klebe RG, Steffensen B, Ryu OH, Simmer JP, Cochran DL (2002) Amelogenin is a cell adhesion protein. J Dent Res 81:497–500
45. Schlueter SR, Carnes DL, Cochran DL (2007) In vitro effects of enamel matrix derivative on microvascular cells. J Periodontol 78:141–151
46. Haase HR, Bartold PM (2001) Enamel matrix derivative induces matrix synthesis by cultured human periodontal fibroblast cells. J Periodontol 72:341–348
47. Faler BJ, Macsata RA, Plummer D, Mishra L, Sidawy AN (2006) Transforming growth factor-beta and wound healing. Perspect Vasc Surg Endovasc Ther 18:55–62
48. Rodrigues TL, Marchesan JT, Coletta RD, Novaes AB Jr, Grisi MF, Souza SL, Taba M Jr, Palioto DB (2007) Effects of enamel matrix derivative and transforming growth factor-beta 1 on human periodontal ligament fibroblasts. J Clin Periodontol 34:514–522
49. Van der Pauw MT, Van den Bos T, Everts V, Beertsen W (2000) Enamel matrix-derived protein stimulates attachment of periodontal ligament fibroblasts and enhances alkaline phosphatase activity and transforming growth factor beta1 release of periodontal ligament and gingival fibroblasts. J Periodontol 71:31–43
50. Okubo K, Kobayashi M, Takiguchi T, Takada T, Ohazama A, Okamatsu Y, Hasegawa K (2003) Participation of endogenous IGF-I and TGF-beta 1 with enamel matrix derivative-stimulated cell growth in human periodontal ligament cells. J Periodontal Res 38:1–9