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Periodontal healing after application of enamel matrix derivative in surgical supra/infrabony periodontal defects in rats with streptozotocin-induced diabetes

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Background and Objective: Epidemiologic and clinical studies have indicated that diabetes is a risk factor for periodontal disease progression and healing. The aim of the present study was to evaluate short-term healing after enamel matrix derivative (EMD) application in combined supra/infrabony periodontal defects in diabetic rats.

Material and Methods: Thirty male Wistar rats were initially divided into two groups, one with streptozotocin-induced diabetes and another one with healthy (non-diabetic) animals. Bony defects were surgically created on the mesial root of the first maxillary molars. After root surface planing and EDTA conditioning, EMD was applied to the roots at one side of the maxillae, while those on the contralateral sides were left untreated. Animals were killed 3 wk after surgery, and block sections were prepared for histologic and histomorphometric analysis.

Results: There was statistically significant more gingival recession in diabetic animals than in non-diabetic animals. The length of the junctional epithelium was significantly shorter in the EMD-treated sites in both diabetic and normoglycemic rats. Sulcus depth and length of supracrestal soft connective tissue showed no statistically significant differences between groups. In all animals, new bone formation was observed. Although new bone occurred more frequently in healthy animals, the extent of new bone was not significantly different between groups. In none of the teeth, a layer of new cementum was detectable. EMD had no influence on bone or cementum regeneration. Adverse reactions such as excessive inflammation due to bacterial root colonization, ankylosis and bone fractures were exclusively observed in diabetic animals, irrespective of EMD treatment.

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Conclusion: Within the limits of the present study, it can be concluded that periodontal healing was impaired in streptozotocin-induced diabetic rats. EMD had no beneficial effects on new bone and cementum formation during short-term healing in this defect model and could not ameliorate the adverse effects in the systemically compromised animals.

Diabetes mellitus (DM) is a metabolic disorder manifested by abnormally high levels of glucose. The hyperglycemic state developed from either a deficiency in insulin secretion or an impaired cellular resistance to the action of insulin is associated with a number of complications, leading to retinopathy, nephropathy, peripheral neuropathy, angiopathy and impaired wound healing (1,2).

Alteration in bone metabolism is another common observation among long-term complications found in diabetic patients (3). Impaired bone formation and reduced bone volume were observed in rats and humans with type 1 diabetes characterized by an absolute deficiency in insulin secretion (4–6). Furthermore, patients with type 2 diabetes, including many who are insulin resistant and have relative insulin deficiency, exhibited dominant bone fractures or bone loss in clinical trials (7,8).

Moreover, it has been reported that periodontitis is the sixth complication of DM (9). When diabetes type 1 and type 2 are directly compared, both increase periodontal disease prevalence to a similar extent (10). Several epidemiological studies have shown a higher prevalence and severity of periodontal diseases in patients with DM when compared to non-diabetic individuals (11–13). These studies have indicated that diabetics with poor metabolic control had more severe periodontitis than diabetics considered to be well-controlled. Chronic hyperglycemia associated with diabetes not only decreases bone metabolism but also increases secretion of proinflammatory cytokines such as interleukin 1 (14,15), tumor necrosis factor-α (15–17) and prostaglandin E_2 (18). Furthermore, it is also reported that hyperglycemia can induce periodontal tissue destruction associated with the formation of advanced glycation end products (15,19,20), vascular dysfunction (21), altered immune function (22) and decreased collagen synthesis (23–25).

Various studies reported that non-surgical (scaling and root planing) periodontal therapy with or without antibiotics not only achieved significant improvements in periodontal clinical parameters, but also had positive effects on metabolic control in diabetic patients, although there were no statistically significant differences (26–29). Moreover, it has been demonstrated that the response to conventional periodontal treatment in diabetic patients was comparable to that in healthy subjects (30). Westfelt *et al.* (31) reported that periodontal clinical parameters were maintained at healthy levels after non-surgical or surgical therapies for 5 years in diabetic (type 1 or type 2) and non-diabetic patients with moderate to advanced periodontal disease alike. Because of compromised wound healing and altered immune function of diabetic patients, periodontal surgical therapy may be indicated for diabetic patients, as this treatment results in a more effective elimination of etiological factors than non-surgical therapy. It has been reported that the postsurgical results were acceptable in a type 2 diabetic patient who was well controlled after guided tissue regeneration (32).

Enamel matrix derivative (EMD) was documented first in the literature (33,34) in 1997 as a tissue healing modulator mimicking events that take place during embryonic root development and as an alternative approach to guided tissue regeneration (35,36). Local application of EMD for the resolution of intraosseous periodontal defects has been shown to result in clinical improvements in terms of clinical attachment gain and pocket depth reduction when compared to treatment with access flap surgery only (37,38). Moreover, it also has been reported that the histologic and clinical results following EMD application were comparable to those obtained with guided tissue regeneration, a more technically demanding regenerative therapy (37–39).

In our previous study, EMD enhanced periodontal healing in combined supra/infrabony defects in healthy rats by reducing gingival recession and junctional epithelium along the root surface and enhancing the formation of new cementum (40). However, it still remains unclear whether EMD has some positive effects on periodontal wound healing and tissue regeneration in diabetic conditions. The aim of the present study was, therefore, to evaluate periodontal healing after EMD application in a diabetic rat model. Our hypothesis was that application of Emdogain® compensates for the compromised wound healing occurring in diabetic conditions.

Material and methods

Experimental animals

Thirty male Wistar rats (*Rattus norvegicus albinus*, 3 mo old, weighing approximately 300 g) were used. The rats were monitored in the animal facilities for at least 1 wk preoperatively and were kept at a constant temperature of 22°C. They were maintained with a light cycle of 12 h (06:00–18:00), had access to drinking water and to a standard laboratory diet *ad libitum* and they were monitored weekly for body weight. The study procedures and protocol design were approved by the Ethical Committee for Animal Care Unit of

the Faculty of Medicine, Tel-Aviv University, Israel.

Diabetes induction

The animals were initially divided into two experimental groups as follows: (a) H group: group with healthy controls (15 animals) (b) D group: group with uncontrolled diabetes (15 animals). Experimental diabetes was induced in the diabetic group via a single intraperitoneal injection of streptozotocin (STZ; Sigma-Aldrich, Dorset, UK) dissolved in citrate buffer (0.01 M, pH 4.3) at a dose of 65 mg/L per kg of body weight. Diabetes onset was confirmed 4 d following STZ delivery via testing of the serum glucose concentration. Rats with serum glucose concentrations greater than 240 mg/dL were regarded as diabetic.

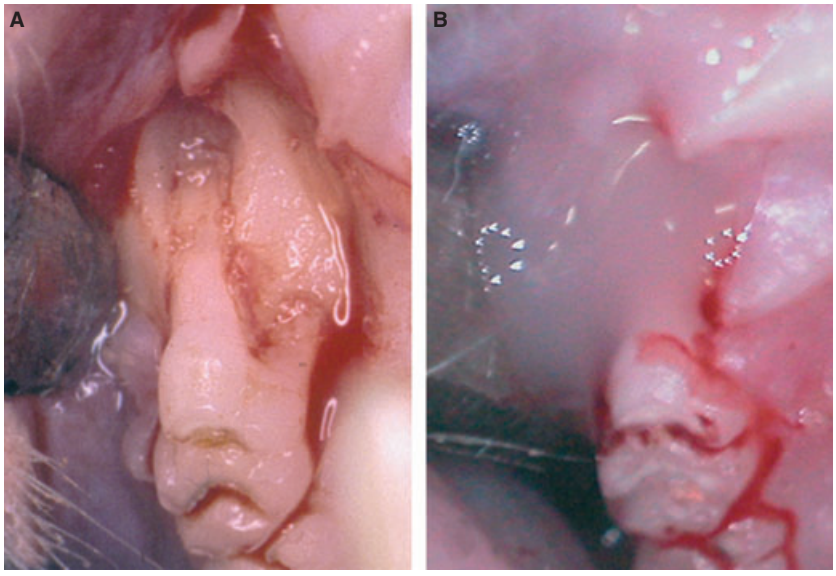

Fig. 1. (A) Surgical defect produced on the mesial aspect of the first maxillary molar. Note supra- and infrabony components of the defect. The root was planed following defect preparation. (B) The Emdogain® gel was then applied to the root surfaces, and the defects were filled up to the adjacent alveolar crest.

Surgical protocol

Before each procedure, all animals were weighed and anesthetized with an intramuscular injection of ketamine chlorhydrate (Rhone Merieux, Lion, France), 90 mg/L per kg body weight and 2% xylazine (Vitamed, Bat-Yam, Israel), 10 mg/L per kg body weight. During all treatment procedures, animals were placed in a custom-made head-restraining device to allow proper access to the maxillary first molar region.

A 3-mm long crestal incision was made on the alveolar ridge mesial to the first maxillary molar on both sides of the maxilla in all animals. Minimal buccal and palatal flaps were raised mesially to expose the mesial root of the first maxillary molars. Bony defects were surgically created using a water-cooled high-speed turbine with a 1 mm diameter round diamond bur. The defects involved the mesial aspect of the mesial root and the adjacent proximal bone. Root planing was carried out on the exposed root surface using hand curettes to remove cementum. The defects were partially suprabony, as the bone crest was reduced. The most apical part of the defects was infrabony, apical to the bony crest (Fig. 1A). Similar defects were created in all animals in both groups. The root surfaces on both sides of the maxilla were conditioned with 24% EDTA gel (Prefgel®, Straumann AG, Basel, Switzerland) for 2 min to remove the smear layer and to obtain a surface devoid of organic debris and then copiously rinsed with water. The root surfaces were slightly dried with gauze sponge. On the experimental root surfaces, Emdogain® (Straumann AG, Basel, Switzerland), which consists of EMD and a carrier, was applied on the entire root surface (Fig. 1B). The control side received no Emdogain®. Owing to the extremely delicate gingival tissues in the surgical area in this small animal model, all experimental surgical procedures were completed by the approximation of the gingival flap and use of surgical cyanoacrylate glue without suturing. Three weeks after surgery, the animals were killed by an overdose of pentobarbital sodium.

Histologic processing

The maxillae were removed and block biopsies of each surgical site were fixed in 4% buffered formalin. The block biopsies were histologically processed. Forty-five defects were processed for the production of undecalcified ground sections, whereas 15 defects were used for paraffin histology. Briefly, the specimens were trimmed, rinsed in running tap water, dehydrated in ascending concentrations of ethanol and embedded in methylmethacrylate. The embedded tissue blocks were cut in a mesio-distal direction into approximately 500 μm thick ground sections using a slow-speed diamond saw (Varicut® VC-50; Leco, Munich, Germany). The most central section of each site was used for morphologic and morphometric evaluation. The sections were ground and polished to a final thickness of about 100 μm (Knuth-Rotor-3; Struers, Rodovre/Copenhagen, Denmark) and surface stained with basic fuchsin and toluidine blue/McNeal. The remaining samples were decalcified with 10% EDTA, and embedded in paraffin. Blocks were sectioned serially into 6 μm sections and stained with hematoxylin/eosin. The reason for using paraffin histology in addition to undecalcified ground sections was to keep some samples available for a future immunohistochemical evaluation. Digital photography of all of the central samples of the defects was performed using a ProgRes® C5 digital camera

(Jenoptik Laser, Optik; Systeme GmbH, Jena, Germany) connected to a Zeiss Axioplan microscope (Carl Zeiss, Göttingen, Germany).

Histomorphometric analysis

The following parameters were measured in the four groups (healthy control without EMD = HE− and with EMD = HE+; uncontrolled diabetes without EMD = DE− and with EMD = DE+) by a single experienced blinded examiner: (i) root length: distance from cemento-enamel junction (CEJ) to root apex; (ii) defect depth: distance from CEJ to bottom of infrabony defect; (iii) sulcus depth: distance from the most coronal portion of junctional epithelium on the root surface to the gingival margin; (iv) gingival recession: distance between CEJ and gingival margin, so that positive values indicate no recession and negative values indicate recession; (v) length of junctional epithelium: distance between the most coronal and most apical aspects of the junctional epithelium on the root surface; (vi) length of supracrestal connective tissue: distance from the most apical portion of junctional epithelium to the level of alveolar bone crest; (vii) length of new bone: vertical distance from bottom of intrabony defect to the most coronal extension of newly formed bone along the root surface; (viii) length of new cementum: distance from the apical extension of root planing to the most coronal extension of newly formed cementum on the denuded root surface; (ix) length of ankylosis: distance of an ankylotic union between the newly formed bone and root surface; and (x) area of newly formed bone. Linear measurements, except for root length and defect depth, were calculated as the percentage of the defect depth within each defect, whereas area measurements of new bone were recorded as absolute values.

Statistical analysis

The differences in the measured histomorphometric parameters were tested with a mixed-design two-way ANOVA with repeated measures, where the health status (healthy control or uncontrolled diabetes) was the between-subject factor and application of EMD or no application of EMD was the within-subject factor.

Results

Because of technical problems during tissue processing (two sites in HE–, one site in DE−, and one site in DE+ groups), massive inflammation and bacterial contamination (three sites in DE− and two sites in DE+ groups), bone fractures (two sites in DE− and two sites in DE+ groups) and root fracture (one site in DE+ group), 14 teeth had to be excluded from the histomorphometric analyses. Owing to the lack of counterpart from the other side among the groups, six teeth were additionally excluded from the histomorphometric analysis. Thus, a total of 40 teeth were available for histomorphometry (Table 1) and this lower number might have compromised the statistical significance of differences between treatments. Furthermore, the parameters of NC and ANK were excluded from histomorphometric analysis, as no measurable new cementum and inconsistent occurrence of ankylosis were observed.

The root lengths (HE−, 2550.8 ± 175.2 μm; HE+, 2657.8 ± 272.4 μm; DE−, 2527.7 ± 347.0 μm and DE+, 2458.6 ± 284.8 μm) and defect depths (HE−, 2147.7.8 ± 445.9 μm; HE+, 2387.8 ± 164.8 μm; DE−, 2443.1 ± 210.4 μm and DE+, 2238.8 ± 299.9 μm) were very similar in all groups with no statistically significant differences between groups (Fig. 2). The differences in gingival recession between the two diabetes groups and the two non-diabetic groups were statistically significant (Table 1). Diabetes significantly increased gingival recession ($F(1,18) = 8.04$, $p < 0.01$), while application of EMD had no significant effect. However, the length of the junctional epithelium was significantly shorter in the EMD-treated sites of both diabetic and normoglycemic rats ($F(1,18) = 4.50$, $p < 0.05$). The differences in sulcus depth and length of supracrestal soft connective tissue did not reach statistical significance (Table 1). There was no new cementum layer visible on all planed root surfaces in all groups (Fig. 3). However, the superficial layer of the scaled dentin surface was sometimes more intensely stained. Spotty resorption cavities were seen on some roots

Table 1. Histomorphometric linear measurements expressed as a percentage of the defect depth (%) and area measurements (μm^2) in each group (Mean ± SD)

	Experimental condition				
Group number	1 HE(−) $n = 12$	2 HE(+) $n = 12$	3 DE(−) $n = 8$	4 DE(+) $n = 8$	Statistically significant differences
SD (%)	17.3 ± 7.0	11.5 ± 5.9	12.6 ± 6.2	13.1±7.4	NS
GR (%)	4.3 ± 7.6	0.6 ± 5.5	−3.6 ± 8.0	−3.1 ± 4.2	Significant effect of diabetes (3 and 4 vs. 1 and 2) $p < 0.01$
JE (%)	33.1 ± 12.5	27.1 ± 10.5	31.9 ± 9.0	24.7 ± 7.7	Significant effect of EMD(2 and 4 vs. 1 and 3) $p < 0.05$
SCT (%)	91.7 ± 45.6	100.8 ± 17.0	87.6 ± 30.8	70.5 ± 17.1	NS
NB (%)	16.8 ± 9.6	13.6 ± 7.6	18.6 ± 19.7	12.6 ± 16.9	NS
NBA (μm^2)	33.3 ± 25.0	47.6 ± 39.7	89.6 ± 112.4	38.9 ± 60.7	NS

GR, gingival recession; JE, length of junctional epithelium; NB, length of new bone; NBA, area of newly formed bone; NS, not statistically significant; SCT, length of supracrestal soft connective tissue; SD, sulcus depth.

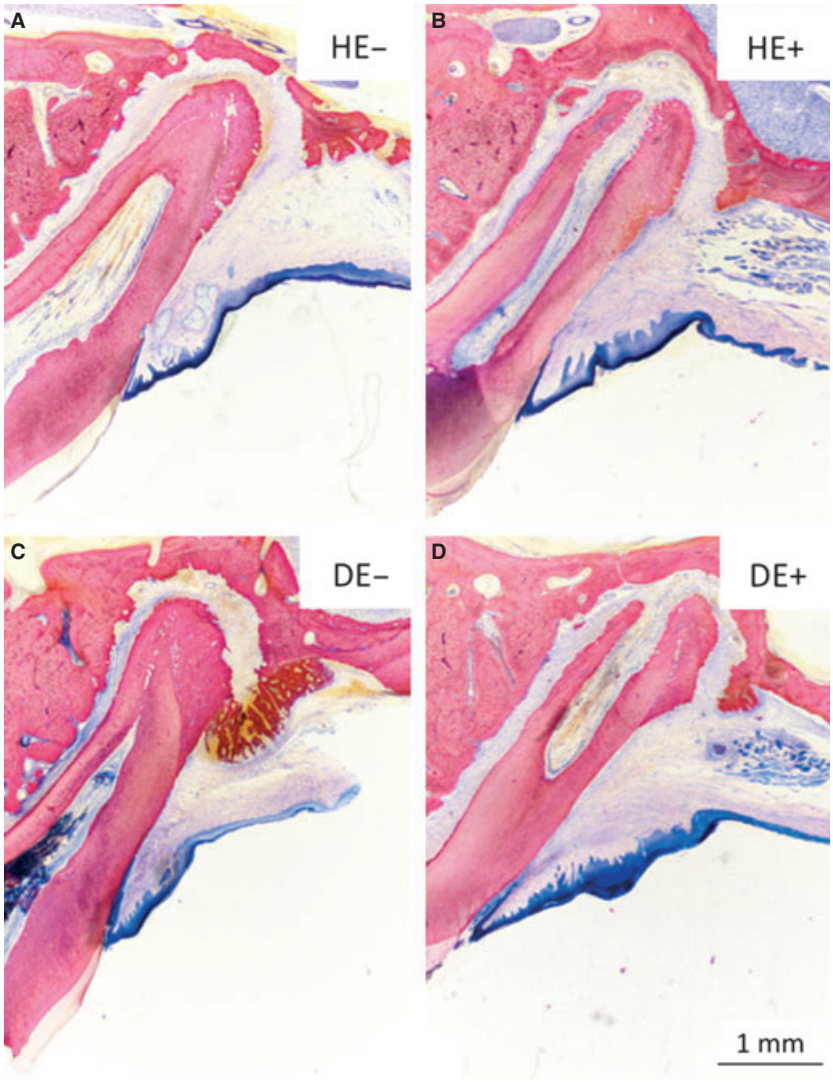

Fig. 2. Overview micrographs showing: (A) non-EMD-treated defect in a healthy rat (HE−); (B) EMD-treated defect in a healthy rat (HE+); (C) non-EMD-treated defect in a diabetic rat (DE−); and (D) EMD-treated defect in a diabetic rat (DE+).

in all groups (data not shown). Perpendicular and oblique collagen fibers were seen at some distance from the denuded root surface in the HE− and HE+ groups. However, cell density appeared higher and structural soft tissue organization/maturation lower in the HE+ group than in the HE– group (Fig. 3). In the diabetic animals, tissue formation and maturation adjacent to the denuded root surface appeared less advanced compared to the non-diabetic animals (Fig. 3). In the DE− group, collagen fibers and cells were often oriented parallel to the denuded root surface, whereas less new tissue formation and a higher cell density was observed adjacent to the denuded root surface in the DE+ group. In addition, there were more round and small cells observed in the DE+ group compared to the DE– group.

In all samples, the boundary between old and new bone was distinguishable. New bone formation was mainly localized in the apical (infrabony) portion (Fig. 4). The vertical extension and area of new bone varied considerably within each group and between groups, but the differences were statistically not significant due to large standard deviations (Table 1). There were signs of bone resorption and fibers inserting in the bone surface (Fig. 4). Ankylosis was only observed in diabetic animals. In the DE− group, ankylosis occurred in two of the nine defects (Figs. 2C and 4C), whereas in the DE+ group, ankylosis was observed in one of the nine defects. In addition, the ankylotic union was smaller in the DE+ group than in the DE− group. Three teeth in the DE– group and two teeth in the DE+ group showed periodontal infection. In some specimens, the inflammation was massive and the epithelium migrated down to the root apex (Fig. 5). Separation of the epithelium from the root surface and deposition of organic material resembling a bacterial biofilm were indicative of pocket formation. Intensely blue stained cementocyte lacunae were indicative of bacterial penetration into the cellular cementum. Numerous polymorphonuclear neutrophils, macrophages and proliferating granulation tissue were observed.

Discussion

In the present study, STZ-induced hyperglycemic rats were used as an animal model for type 1 DM for the following reasons. It is known that rats are cost-effective, easy to handle (41) and the general metabolism of STZ-induced diabetic rats is similar to that in human DM (42,43). In all cases, animal and experimental models have to be regarded cautiously and placed in perspective relative to the study hypothesis (44).

In the HE– group, only two teeth were excluded from the histomorphometric analysis because of histological complications, whereas massive inflammation accompanied with extensive apical migration of the epithelium, bacterial invasion and bone and root fracture were observed in 10 teeth of the diabetic animals regardless of the use of EMD. The severe periodontal infection in the diabetic animals may be accounted for by hyperglycemia, as the primary factor, which leads to impaired wound healing due to a defective immune response with impaired leukocyte chemotaxis, decreased polymorphonuclear microbicid-

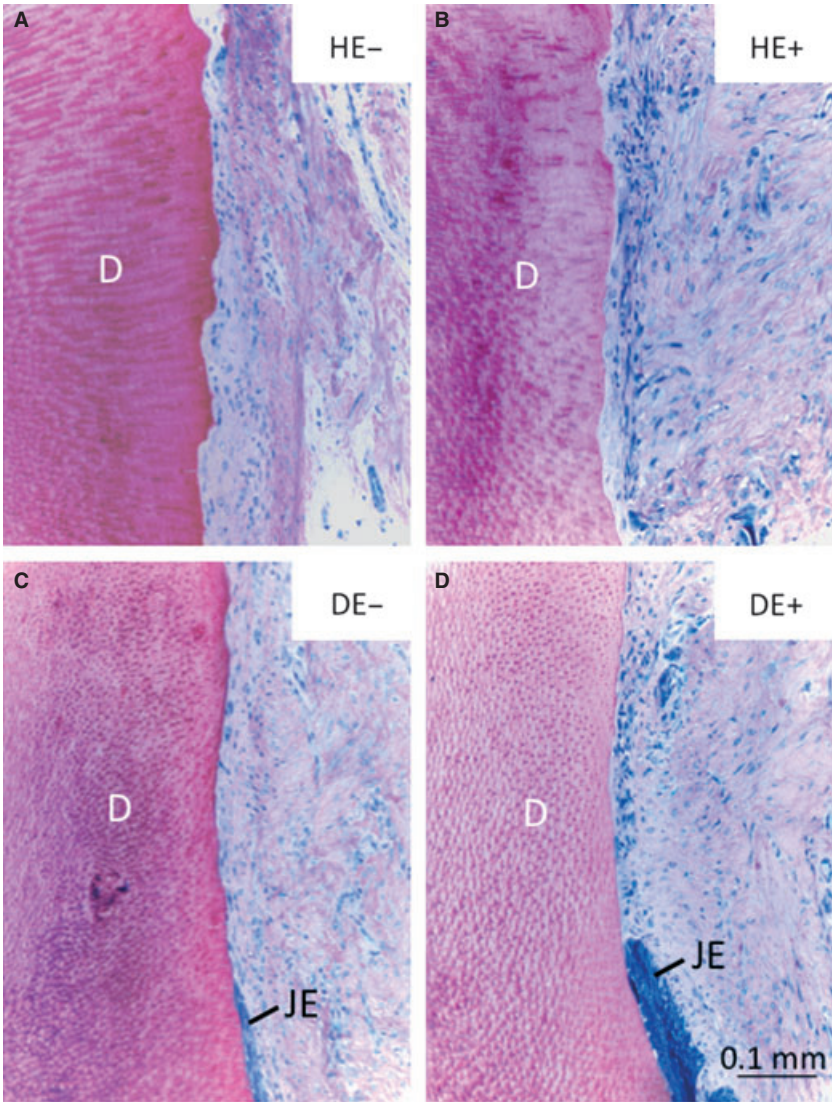

Fig. 3. Micrographs illustrating the root surface in: (A) non-EMD-treated defect in a healthy rat (HE−); (B) EMD-treated defect in a healthy rat (HE+); (C) non-EMD-treated defect in a diabetic rat (DE−); and (D) EMD-treated defect in a diabetic rat (DE+). Note that there are no signs of new cementum formation and that there is a high cell density on the root surface in EMD-treated animals. D, dentin; JE, junctional epithelium.

al activity, faulty delivery of the humoral and cellular immune system components (22,45). Our results are consistent with those from a previous study where aggressive bone loss and distortion of collagen fiber attachment were observed in diabetic animals (46).

Furthermore, it has been reported that the level of monocyte chemoattractant protein-1, which is considered to be a major signal for chemotaxis of mononuclear leukocytes, was similarly increased in gingival tissue of diabetic rats without periodontitis when compared to non-diabetic rats with periodontitis (47). These results indicate that DM may have an independent influence on periodontal tissue destruction irrespective of the presence or absence of periodontal disease (46,47).

In the present study, statistically significant gingival recession was only observed in the two diabetic groups. Silva *et al*. (45) have reported that diabetic animals presented a reduced height of the connective tissue papillae and concluded that this may be due to a direct response to inflammation or an adaptive remodeling of the weakened connective tissue. Furthermore, several studies have demonstrated decreased collagen synthesis, increased intracellular degradation and solubility in the gingiva of diabetes-induced rats (25,46,48). These findings may explain the gingival recessions observed in the present study.

The length of the junctional epithelium was significantly shorter in EMD-applied sites. This finding is in agreement with previous reports showing shorter epithelium after EMD treatment than after surgical debridement (33,40) and supported by *in vitro* studies showing that EMD inhibits the proliferation of oral squamous cell carcinoma-derived (SCC25) (49) as well as normal human gingival epithelial cells (50). New bone formation was observed mainly in the infrabony region in all groups but there was no statistically significant difference among the groups.

New bone formation is considered to be the result of the early phase of spontaneous healing and repair after surgical intervention with the host bone (51). The histological findings on bone formation are in agreement with our previous study that demonstrated no significant influence of EMD on bone healing (40). The combined supra/infrabony defects in this animal model mostly consist of suprabony periodontal defects, and the infrabony region was relatively limited and shallow. Actually, the defects may be regarded as being very similar to horizontal type periodontal defects. It has also been reported that new bone was significantly stimulated in narrower intrabony defects by the application of EMD (51,52). Thus, the defect configuration in the present study appears to be too challenging for an EMD application. Ankylosis between bone and tooth was detected in the two diabetic groups only, although the total number was small. These results indicate that bone metabolism is extensively altered in rats with type 1 diabetes. Our observation of a higher incidence of ankylosis in the DE− group compared to the DE+ group is in line with a previous study

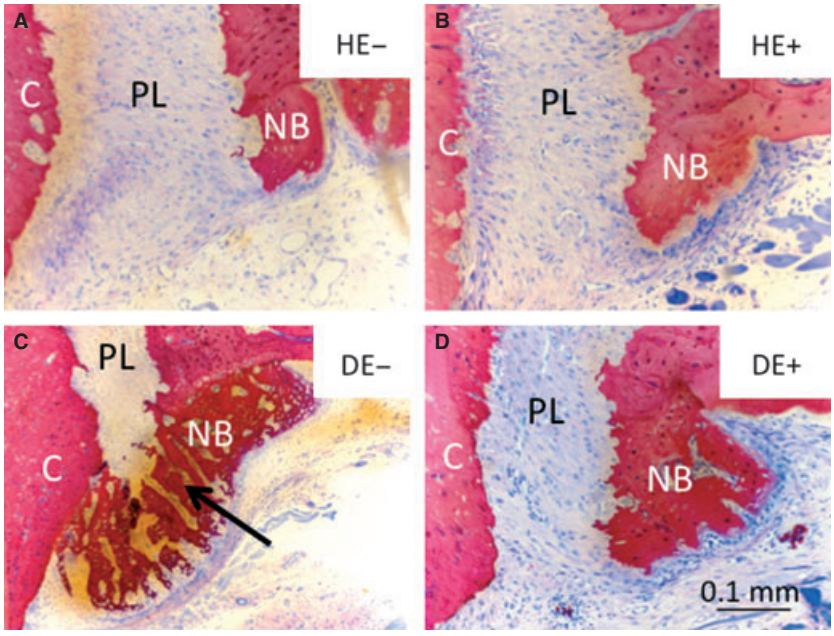

Fig. 4. Micrographs illustrating the region of the alveolar crest in: (A) non-EMD-treated defect in a healthy rat (HE−); (B) EMD-treated defect in a healthy rat (HE+); (C) non-EMD-treated defect in a diabetic rat (DE−); and (D) EMD-treated defect in a diabetic rat (DE+). New bone (NB) is present in all groups. Note the ankylosis (arrow) in the defect from the DE− group. C, cementum; PL, periodontal ligament.

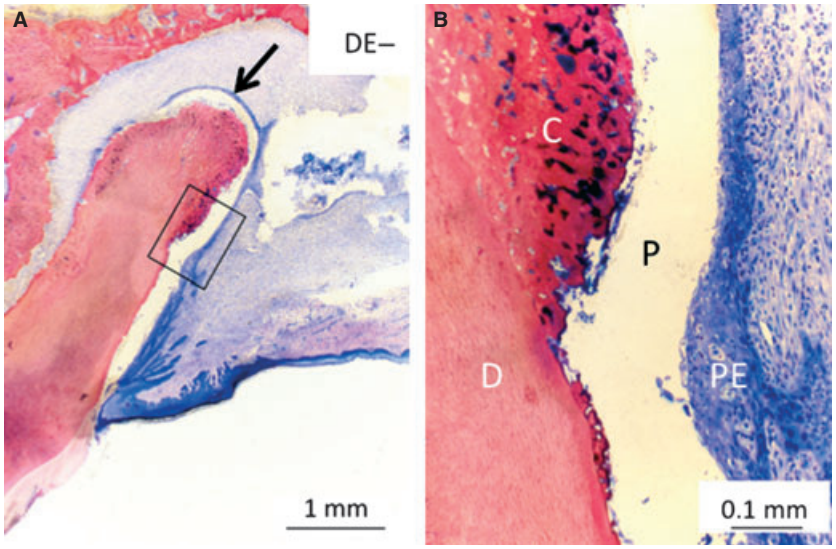

Fig. 5. (A) Histologic overview and (B) detail micrograph from a non-EMD-treated defect in a diabetic rat (DE−). The rectangle in (A) is enlarged in (B). Note the highly inflamed periodontal tissues, apical migration of the epithelium down to the root apex (arrow), and periodontal pocket (P) formation. C, cellular cementum; D, dentin; PE, pocket epithelium.

showing a six times greater extension of ankylosis in non-EMD-treated compared to EMD-treated teeth in rats (40) and dogs (53).

In contrast to previous *in vivo* (34,54,55) and *in vitro* (56,57) findings, the results of the present study cannot confirm that EMD enhances wound healing and connective tissue regeneration. A measurable layer of new cementum was not detected in any of the teeth in the present study. In a few tissue samples, however, an extremely thin matrix layer was observed on the denuded and EDTA-decalcified surface. This matrix layer was considered to be either a superficially modified dentin matrix (58) or the first deposited layer of precementum. Nevertheless, our results suggest that in this defect model EMD had no observable positive effect on cementum formation in both diabetic and non-diabetic animals. Thus, our results are in line with findings from another study in rats (59) but in contrast to many other histological studies in various species, including the human (36) and the rat (40). Discrepancies between studies may be related to species differences, defect models and/or healing periods. For instance, the healing period in our previous study using the same rat periodontal defect model was 12 wk (40), whereas the healing period in the present study was 3 wk only. We have chosen this short-term observation period to lower detrimental systemic influences of uncontrolled diabetes and to alleviate the suffering of the animals. Our analysis showed that 3 wk of healing appeared to be too short to see an effect of Emdogain® in this model.

The large standard deviations seen for most of the parameters measured may be due to the fact that many teeth, particularly in diabetic animals, had to be excluded from the histomorphometric analyses. As the resulting lower number of defects per group may have lowered the statistical power, a larger number of animals may be considered in future studies of this kind. Nevertheless, within the limits of the present study, it can be concluded that periodontal healing was clearly impaired in the STZ-induced diabetic rats. Furthermore, EMD had no measurable beneficial effects on new bone and cementum formation in this model and could not ameliorate the observed adverse effects in the systemically compromised animals.

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