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Enamel matrix derivative *versus* guided tissue regeneration in the presence of nicotine: a histomorphometric study in dogs

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Abstract

Aim: The goal of this histometric study was to compare the healing process of dehiscence-type defects treated by enamel matrix derivative (EMD) or guided tissue regeneration (GTR) under the effect of nicotine in the dog model.

Materials and Methods: Eight mongrel dogs were used. Buccal osseous dehiscences were surgically created on the mesial roots of the mandibular third and fourth premolars. The defects were exposed to plaque accumulation for 3 months. After this period, the defects were randomly assigned to one of the treatments: open flap debridement (OFD), EMD or GTR with a resorbable membrane. During 4 months, the dogs received subcutaneous administration of nicotine (2 mg/kg twice a day with a 12 h interval between the applications). After this period, the animals were killed and the blocks were processed. The histometric parameters evaluated included gingival recession, epithelial length, connective tissue adaptation, new cementum and new bone.

Results: A superior length of new cementum was observed in the sites treated by EMD in comparison with OFD ($p \leqslant 0.05$). No statistically significant differences were observed between GTR and the other groups.

Conclusions: In the presence of nicotine, EMD may promote more new cementum formation than OFD while GTR failed to provide a significant difference.

Key words: enamel matrix derivative; guided tissue regeneration; nicotine; periodontal regeneration

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The goal of periodontal therapy includes not only the arrest of progressive periodontal disease but also the restitution of those parts of the supporting apparatus that have been destroyed by the disease. Guided tissue regeneration (GTR) involves the placement of a barrier covering the periodontal defect in such a way that the gingival tissues (epithelium and connective tissue) are prevented from contacting the root surface during healing, thus allowing periodontal ligament and bone cells to repopulate the isolated space selectively (Karring et al. 1980, 1985, 1993, Nyman et al. 1980, 1982, Gottlow et al. 1984, 1986). Using this surgical approach, the periodontal defects can be successfully treated (Becker & Becker 1993, Cortellini et al. 1993, 1995). It has also been shown that resorbable and non-resorbable membranes can produce similar histological and clinical results (Caffesse et al. 1994, Bouchard et al. 1997, Pereira et al. 2000).

An alternative regenerative approach that has been studied in the last few years is the use of enamel matrix derivatives (EMD). A heterogeneous mixture of proteins containing amelogenins as a major component has been shown to induce the formation of an acellular cementum adjacent to the dentin, together with collagenous fibres that developed over a newly formed bone (Hammarstrom et al. 1997). The application of EMD mimics the events that take place during the development of the dental root. It is accepted that Hertwing's root sheath cells secrete enamel matrix protein during root formation and that these proteins are involved in the formation of an acellular cementum during nascent tooth development (Slavkin & Boyde 1975, Slavkin 1976, Lindskog 1982a, b, Hammarstrom 1997)

After the clinical application of EMD, new cementum, connective tissue and bone could be observed on the root

surface (Heijl 1997, Mellonig 1999). The use of EMD in the treatment of intra-bony defects resulted in a superior clinical response when compared with the control (modified Widman flap with placebo application) on a long-term basis (Heijl et al. 1997).

Cigarette smoking has been shown to be a strong risk marker, and possibly, a true risk factor for periodontal disease (Bergstrom 1989, Bergstrom et al. 2000). Smokers showed a significantly less favourable response compared with non-smokers after scaling and root planing (Grossi et al. 1996), adjunctive antimicrobial therapy (Kinane & Radvar 1997), modified Widman flap surgery (Preber & Bergstrom 1990), root coverage procedures (Martins et al. 2004), regenerative procedures (Tonetti et al. 1995, Trombelli & Scabbia 1997, Trombelli et al. 1997) and during periodontal maintenance (Kaldahl et al. 1996). The precise mechanisms by which tobacco smoke interferes with healing are not completely understood, due to the fact that there are thousands of toxins in tobacco smoke, many of which have not been identified. Most of these negative effects have been attributed to nicotine, which is the major component of the particulate phase of tobacco smoke and its most citotoxic and vasoactive substance (Benowitz 1988). Nicotine and its metabolite cotinine have been found in the saliva and gingival crevicular fluid of smokers with periodontal disease (McGuire et al. 1989). The presence of nicotine on the root surfaces of periodontally diseased teeth in smokers has also been reported (Cuff et al. 1989). In vitro studies have shown that nicotine inhibits the growth of gingival fibroblasts and increases collagenase activity (Triton & Dabbous 1995). Inhibition of the neutrophil and monocyte antimicrobial function was reported after exposure to nicotine (Pabst et al. 1995).

There are clinical evidences to support the negative impact of smoking on the healing response after regenerative procedures (Tonetti et al. 1995, Zucchelli et al. 2002, Stavropoulos et al. 2004). It has been shown by our laboratory that nicotine administration might affect bone density but may not prevent bone healing in defects treated by guided bone regeneration in dogs (Saldanha et al. 2004). In addition, we demonstrated that cigarette smoke reduces the self-healing capacity of periodontal tissues in fenestration defects in the rat model (Benatti et al. 2005). However, there is a paucity of information about the histological outcome after different regenerative procedures under the effect of nicotine. Therefore, the goal of the present study is to evaluate, histometrically, the healing pattern after the treatment of dehiscence-type defect with EMD or GTR in the presence of systemic administration of nicotine in dogs.

Material and Methods

Eight mongrel dogs in good general health were used (approximately 15 kg body weight). The animals were kept in individual cages with access to food and water ad libitum. Before the surgical procedures, all animals were allowed to acclimatize to the facility environment for a period of 7 days. The surgical procedures were performed under general anaesthesia with an intravenous injection of a 3% sodium pentobarbital solution (0.5 ml/kg). The protocol was approved by the University of Campinas Institutional Animal Care and Use Committee.

Surgical creation of the defects

After raising mucoperiosteal flaps, buccal osseous dehiscences were created by removing, with hand instruments, 4×6 mm of bone covering the mesial roots of the mandibular third and fourth pre-molars. In each defect, the cementum was curetted and a strip of Toffelmire matrix band was secured to the crown of the tooth with cyanoacrylate glue, extending subgingivally into the defect area. The flaps were replaced to the original position and sutured with 4-0 vicryl inter-proximal sutures. The sutures were removed 1 week later. Areas were permitted to heal without any special oral hygiene care.

Preparation period

After 3 months of plaque accumulation, the band matrices were removed and a supragingival plaque control regimen was instituted consisting of daily brushing and topical application of 0.12% chlorhexidine digluconate for 15 days before the surgical procedures.

Surgical treatment of the defects

Full-thickness flaps were raised and apical notches were placed at the base of the obtained defects (bone level), and coronal notches were placed at the cemento-enamel junction.

The randomized block design was used in the study, with each dog receiving all the treatments. First, the side of the mandible receiving EMD was randomly determined. After that, the other side received the other two treatments. Therefore, the defects were randomly assigned to one of the following treatment groups:

1. OFD: open flap debridement.
2. GTR: root instrumentation and placement of a resorbable membrane (Resolut® bioresorbable matrix barrier; W.L.Gore & Associate Inc., Flagstaff, AZ, USA) covering the defect and extending to the surrounding bone (2–3 mm). It was stabilized with absorbable sling sutures.
3. EMD: root instrumentation and etching with ethylene diamine tetraacetic acid (EDTA) 24% (Prefgel, Biora AB, Malmo, Sweden) (2 min.). After irrigation with saline solution, EMD (Emdogain, Biora AB) was applied on the root surface.

In all sites, the flaps were coronally positioned (approximately 1–2 mm) and ePTFE-interrupted sutures were applied.

Post-operative care

The sutures were removed after 2 weeks. After the surgical procedures, the animals were given a single intra-muscular injection of 1.5 ml of penicillin (150,000 IU).

The post-operative plaque control was performed by daily application with 0.12% chlorhexidine digluconate solution.

Systemic nicotine administration

Subcutaneous administration of nicotine (Sigma, St. Louis, MO, USA) was initiated 1 day after the regenerative procedures and continued during the entire experimental period. A total dose of 4 mg/kg/day was administered (2 mg/kg twice a day with a 12 h interval between the applications).

Nicotine and cotinine serum level

Blood samples were taken hourly from 15 min. to 8 h after the first injection of the day, on the first and last days of nicotine administration. Serum samples were assayed for concentrations of nicotine and cotinine by high-pressure

liquid chromatography, as described previously (Saldanha et al. 2004).

Histometric measurements

After 4 months, the animals were killed with an overdose of sodium pentobarbital 3%. The jaws were dissected and the blocks containing the experimental specimens were obtained. The blocks were fixed in a 10% neutral formalin solution for 1 week. They were decalcified in a solution of equal parts of 50% formic acid and 20% sodium citrate for 3 months. The decalcified specimens were washed in water, dehydrated and embedded in paraffin. Buccolingual sections of 7 μm were stained with Haematoxylin and Eosin and with Masson's Trichrome.

The image analysis system used for the histological/histometric evaluation comprised a Zeiss Axioskop 2 microscope (Carl Zeiss Instruments, Gottingen, Germany) equipped with the following objectives: ×1.25, ×2.5, ×5, ×10 and ×40. The microscope was associated with a video camera (DXC-107 A/107 AP–Sony electronics Inc., Shinagawa-Ku, Japan)/computer/software (Image Pro Plus v 3.0, Media Cybernetics, Silver Spring, MD, USA). In order to describe the healing pattern and to establish clearly the limits of each tissue for the histometry, all five magnifications were used, according to the dimension of the structure to be evaluated. After that, the measurements were performed using the ×2.5 objectives.

The mean for each histometric parameter in each dog was obtained after measuring three sections per site (selected from the middle portion of the defect). The following parameters were measured by one of the authors:

1. *Gingival recession:* from the gingival margin to the coronal notch, measured in the sites where the gingival margin was located apically to the coronal notch. Negative values were assigned. If the gingival margin was located at the level of the coronal notch or coronal to this notch, a ''0'' value was applied.
2. *Epithelium*: from the coronal notch to the apical border of the junctional epithelium, measured when the gingival margin was coronal to the coronal notch. If gingival recession was present, the epithelium was measured from the gingival margin to the apical border of the junctional epithelium.
3. *Connective tissue (without cementum)*: from the apical border of the junctional epithelium to the coronal end of new cementum.
4. *New cementum*: from the apical notch to the most coronal part of new cementum.
5. *New bone*: from the apical notch to the most coronal part of the bone.
6. *Defect extension*: from the apical notch to the coronal notch.

Statistics

The mean for each parameter in the treatment group was calculated using the values of the eight dogs. The data were statistically analysed by repeated measures ANOVA and the Student–Newman–Keuls' test for multiple comparisons. Statistically significant results were corroborated applying non-parametric statistics using the Kruskal–Wallis test.

Results

Serum levels of nicotine and cotinine

Over time, follow-up of nicotine and cotinine serum levels demonstrated a similar pattern during the first and last days of nicotine administration. The highest serum level of nicotine was noted 15 min. after its administration (first day: 1799.8 ng/ml and last day: 1552 ng/ml), and a time-dependent decrease was observed. A significant decrease in the serum level of nicotine was observed 1 h after its administration (first day: 634.7 ng/ml and last day: 690.2 ng/ml), with a tendency for stabilization after the third hour (first day: 209 ng/ml and last day: 107.9 ng/ml). Regarding the cotinine serum levels, the highest value was observed 1 h after nicotine administration (first day: 334.2 ng/ml and last day: 490.9 ng/ml), and a tendency to stabilize was then noted after the fourth hour (first day: 143.2 ng/ml and last day: 60.7 ng/ml). On the first day of nicotine administration, nicotine/cotinine serum levels were slightly higher than on the last day of administration. However, for both periods, similar values were obtained.

Clinical observations

Healing progressed uneventfully in the three groups. No suppuration or abscess formation was observed. Clinical coverage of the defect was obtained in 20 out of the 24 treated sites. A small gingival recession was observed in two sites of OFD and two sites of GTR (ranging from 0.5 to 1 mm in both groups). The membrane exposures associated with the recession in these sites were treated with 1% chlorhexidine gel (daily) until the degradation of the exposed portion of the barrier and the healing progressed with no complication other than a slight inflammation.

Histological observations

The predominant healing pattern for OFD was the development of a long junctional epithelium on the coronal half of the defect (Fig. 1). A continuous layer of new cementum had formed in the specimens of all groups extending coronally to a varying degree (Figs 1–3). However, in GTR (Fig. 2) and EMD (Fig. 3), the coronal extension of new cementum seemed to be more evident than in OFD. The new periodontal ligament, which was confined between the new cementum and new alveolar bone contained fibroblasts, functionally oriented collagen fibres and a relatively large num-

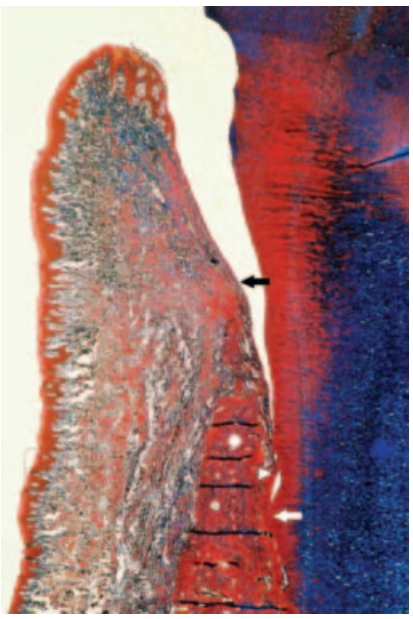

Fig. 1. Open flap debridement. The long junctional epithelium is the predominant healing pattern in the coronal part of the defect. The apical limit of the junctional epithelium is indicated by the black arrow. New bone (white asterisk) and new cementum (white arrowhead) are observed in the apical part of the defect. The white arrow indicates the apical limit of the defect. Original magnification × 25. Masson's Trichrome.

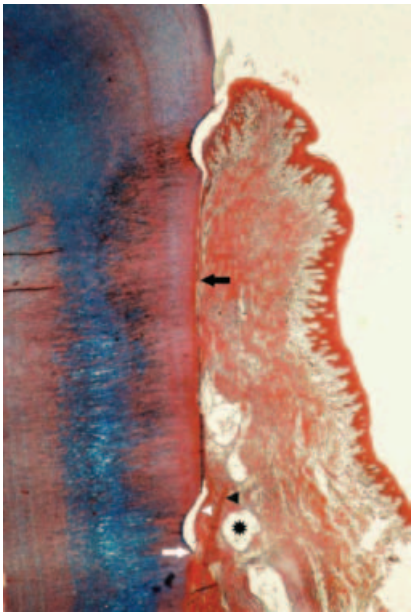

Fig. 2. Guided tissue regeneration. The epithelium is located at the coronal part of the defect. The apical limit of the junctional epithelium is indicated by the black arrow. New connective tissue attachment comprised by a newly formed cementum (white arrowhead) extends coronally, covering most of the defect. The new alveolar bone (black arrowhead) is located in the apical notch area. The remnants of the membrane (black asterisk) could be observed adjacent to the external surface of the bone and root surface surrounded by non-infiltrated connective tissue. The white arrow indicates the apical limit of the defect Original magnification ×25. Masson's Trichrome.

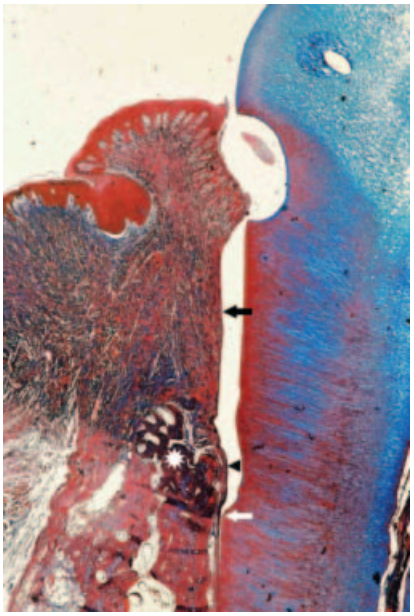

Fig. 3. Enamel matrix derivative. The epithelium is located at the coronal portion of the defect. The apical limit of the junctional epithelium is indicated by the black arrow. New connective tissue attachment with new cementum (black arrowhead) extends up to a region near the coronal notch. New bone (white asterisk) is observed coronally to the apical limit of the defect (white arrow). Original magnification ×25. Masson's trichrome.

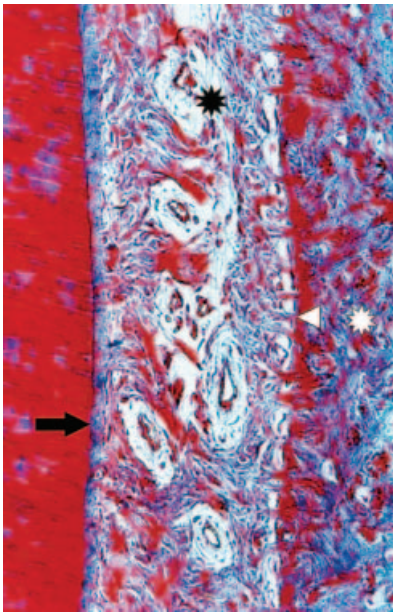

Fig. 4. Enamel matrix derivative. Higher magnification showing new cellular cementum (black arrow) adjacent to the periodontal ligament (black asterisk) and new bone (white asterisk) with inserting collagen fibres (white arrowhead). Original magnification ×100. Masson's Trichrome.

ber of blood vessels in all groups (Fig. 4). In the group treated with EMD, areas of acellular and cellular cementum were observed. Areas of connective tissue adjacent to the root surface without cementum formation were infrequent in all groups. The apical termination of the junctional epithelium coincided with the coronal border of the newly formed fibrous attachment in most of the sites, regardless of the treatment. Bone formation was observed in the apical part of the defects in all groups (Figs 1–3). In GTR, the remnants of the resorbable membrane could be observed in some sections adjacent to the external surface of the bone and were surrounded by a non-infiltrated connective tissue (Fig. 2).

Histometric measurements

The histometric data are shown in Table 1. A small mean gingival recession was observed in all groups, with no significant differences among them. The mean epithelium length observed in EMD (0.63 ± 0.35 mm) tended to be smaller than the one observed in OFD (1.71 ± 0.61 mm) and GTR (1.23 ± 0.99 mm). However, no statistically significant differences were observed among the groups. In addition, no statistically significant differences were observed in the new connective tissue adaptation, bone formation and defect extension. Nevertheless, the magnitude of the values indicated a superior amount of new bone for EMD. A statistically significant difference in the new cementum formation was observed between the sites treated with EMD (5.32 ± 0.73 mm) when compared with OFD (3.6 ± 1.14 mm). GTR showed no significant differences in new cementum (4.05 ± 1.80 mm) when compared with the other groups.

Discussion

The present study was designed to evaluate, histometrically, the healing process of dehiscence-type defects treated by GTR or enamel matrix proteins in the presence of systemic administration of nicotine. It was based on the hypothesis that there may be a differential susceptibility of EMD and membranes to the negative effects of smoking (due to nicotine). It is well accepted that the periodontal ligament is an important source of cells for periodontal regeneration (Melcher 1976, Nyman et al. 1982). Therefore, any factor that might jeopardize the function of such cells could also impair tissue repair and regeneration. It has been shown that tobacco products (nicotine and cotinine) may inhibit attachment, proliferation and chemotaxis of human periodontal ligament fibroblasts in vitro (Giannopoulou et al. 1999, James et al. 1999). The results derived from clinical studies showed the negative influence of smoking on the outcome of regenerative procedures (Tonetti et al. 1995, Trombelli & Scabbia 1997, Trombelli et al. 1997, Zucchelli et al. 2002, Stavropoulos et al. 2004). However, at present, there is a lack of histological data about the outcome of different regenerative procedures under the effect of nicotine (isolated from other compounds of cigarette smoke). In the present study, an animal model was used where identical contra-lateral lesions were created to serve as control

Table 1. Mean* standard deviation and median for the parameters evaluated after the use of open flap debridement (OFD), resorbable membrane (GTR) and enamel matrix derivative (EMD)

	Mean ± SD median			*p*-value
	OFD ($n = 8$)	GTR ($n = 8$)	EMD ($n = 8$)	
Recession	− 0.36 ± 0.48 (− 0.3)	− 0.25 ± 0.34 (0)	− 0.02 ± 0.07 (0)	0.31
Epithelium	1.71 ± 0.61 (1.65)	1.23 ± 0.99 (0.85)	0.63 ± 0.35 (0.55)	0.07
Connective tissue	0.15 ± 0.22 0	0.15 ± 0.21 0.05	0.36 ± 0.s27 0.35	0.41
New cementum	3.60 ± 1.14 B (3.65)	4.05 ± 1.08 AB (4.2)	5.32 ± 0.73A (5.3)	0.01†
New bone	1.55 ± 0.93 (1.55)	1.56 ± 0.96 (1.25)	2.52 ± 1.34 (2.45)	0.09
Defect	5.85 ± 1.05 (5.75)	5.66 ± 1.13 (5.35)	6.36 ± 0.71 (6.25)	0.07

*Mean values in millimetres.

†Means followed by different letters are statistically different ($p \leqslant 0.05$) – ANOVA and Student–Newman–Keuls for multiple comparisons. The Kruskal–Wallis test was used to corroborate the significant results obtained with ANOVA.

(Sallum et al. 1998, 2004, Pereira et al. 2000) and all animals received nicotine administration under the same regimen (4 mg/kg/day). It was previously demonstrated that this form of administration was efficient to provide nicotine serum levels within the range reported for smokers (Saldanha et al. 2004). It is important to note that the aim of the present study was not to compare groups with or without nicotine, as the negative effects of smoking on regenerative outcomes are well documented. It was focused on the comparison of the two regenerative procedures under the effect of nicotine.

The reduced gingival recession observed in the study could be attributed to the careful management and stabilization of soft tissues during the surgical procedures and the rigidity of the plaque control regimen during the healing period. Membrane exposures were treated with 1% chlorhexidine gel (daily) until the degradation of the exposed portion of the barrier with no complication other than a slight inflammation. The complete defect coverage achieved in the majority of the sites was important to assure the full potential of the tested therapies in terms of healing response. This is particularly important for the sites treated with GTR where excessive membrane exposure could have been an interfering negative factor (Simion et al. 1994). It is interesting to note that, clinically; no recession could be detected in the EMD-treated sites. Histometrically, the mean gingival recessions were 0.36, 0.25 and 0.02 mm for OFD, GTR and EMD, respectively, with no significant difference among the groups.

The length of the epithelium did not reach a statistically significant difference among the groups. This is in accordance with previous studies evaluating regenerative therapies in the same experimental model (Pereira et al. 2000, Sallum et al. 2004). Nevertheless, the magnitude of the values indicated that the group treated with EMD tended to show a shorter epithelium. The extension of the epithelium in EMD (0.63 mm) was half of that observed in GTR (1.23 mm) and one-third of that observed in OFD (1.71 mm), suggesting that the epithelium was consistently maintained in a more coronal position with EMD.

The amount of new cementum formation observed with EMD (5.32 mm) was significantly different from the one observed with OFD (3.6 mm). This is in accordance with previous studies showing the possibility of using EMD to promote the formation of new connective tissue attachment, i.e. new cementum with inserting collagen fibres (Hammarstrom et al. 1997, Heijl 1997, Sallum et al. 2004). GTR assumed an intermediate position and tended to be slightly superior to OFD and inferior to EMD, but was not statistically different from the other groups. In spite of the extreme care during the surgical procedures and post-operative period to avoid problems with the soft tissues and the consequent contamination of the barriers, membrane exposure could not be avoided in all GTR-treated sites of the present study. However, it would be important to recognize that not only should the risk of exposure be considered when using membranes (Simion et al. 1994) but also the presence of the barrier itself, interfering physically with the local blood perfusion (Zanetta-Barbosa et al. 1993). A previous investigation showed that e-PTFE membranes may interfere with the re-vascularization during the early phase of healing while bioabsorbable membranes allow earlier anastomosis of the vasculature of the flap and regenerated tissues (Vergara et al. 1997). For this reason and to eliminate the need for a second surgery, a bioresorbable membrane was preferred for the GTR procedure in the present study. Morphologic alterations in the microvasculature of the rat oral mucosa following systemic nicotine administration were characterized by an inferior length of the capillary fragments in the nicotine-treated groups (Johnson et al. 1989). The interference of smoking and nicotine on gingival blood flow was also reported (Clarke et al. 1981, Clarke & Shephard 1984, Johnson et al. 1991, Palmer et al. 1999, Meekin et al. 2000, Mavropoulos et al. 2003, Morozumi et al. 2004, Nakamura et al. 2005). Some articles showed that nicotine is able to reduce the marginal gingival circulation (Clarke et al. 1981, Clarke & Shephard 1984, Nakamura et al. 2005), while others showed that it may increase gingival blood flow (Johnson et al. 1991, Mavropoulos et al. 2003). One of the studies evaluated the relative blood flow of healthy gingiva and forehead skin in smokers and non-smokers showing no significant differences in the relative gingival blood flow between the groups; however, a significant increase in the relative blood flow to the forehead skin of light smokers was observed when compared with heavy smokers or with non-smokers, 2 min. following the smoking experience (Meekin et al. 2000). Therefore, their results do not support the theory that there is a localized vasoconstriction within the gingival tissues (Palmer et al. 1999, Meekin et al. 2000). Considering the amount of new cementum achieved with EMD in the present study, coupled with the possibility of a simultaneous interference of membrane and nicotine in the local microvasculature, it would be reasonable to assume that EMD might be preferred to GTR, especially in cases where the soft tissue coverage can hardly be performed, or the membrane adaptation and fixation is difficult to achieve.

Regarding the quality of the newly formed cementum, the present study showed that a mixed cellular and acellular cementum was formed in the group treated with EMD, rather than a predominantly cellular type of cementum observed in the GTR-treated sites. This observation does not corroborate the results from previous studies showing that the treatment with EMD results exclusively in the formation of acellular cementum (Hammarstrom 1997, Hammarstrom et al. 1997) and it is in agreement with previous studies showing areas of mixed cellular and acellular cementum after EMD application (Sculean et al. 2000, Sallum et al. 2004). The significance of these differences in the type of cementum formed after different regenerative procedures remains to be determined.

Considering new bone formation, no statistically significant differences were observed among groups. However, the magnitude of the values indicated that EMD might positively influence bone healing, even in the presence of nicotine. This is in accordance with previous studies showing that bone tissue might be influenced by EMD at the histological (Hammarstrom et al. 1997, Heijl 1997, Casati et al. 2002), clinical (Mellonig 1999) and cellular levels (Gestrelius et al. 1997, Boyan et al. 2000, Hoang et al. 2000). GTR and OFD showed similar values for bone formation. In this respect, some studies showed more bone formation after GTR when compared with controls without membranes (Magnusson et al. 1988, Pitaru et al. 1988, Pereira et al. 2000), while others showed no difference (Caffesse et al. 1988, Sallum et al. 1998, 2004). Additional comparative studies are necessary to clarify the potential for bone re-growth after the use of different materials and techniques.

Within the limits of this study, it can be concluded that a significant new cementum formation can be achieved with EMD when compared with OFD, even under systemic nicotine exposure. GTR may not provide a superior amount of new cementum formation than OFD under the same circumstances.

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Clinical Relevance

Scientific rationale for the study: Clinical studies have suggested that there may be a differential susceptibility of EMD and membranes to the negative effects of smoking (due to nicotine). However, no histological/histometric information is available about the performance of different regenerative procedures under the effect of nicotine. Therefore, the present study aims to compare, histometrically, the healing pattern of defects treated with EMD or GTR in the presence of systemic administration of nicotine in dogs.

Principal findings: In the presence of nicotine, EMD may promote more new cementum formation than OFD, while GTR does not provide a significant difference.

Practical implications: It would be reasonable to assume that EMD might be preferred to GTR under the effect of nicotine.