Histological Effects of Enamel Matrix Derivative Proteins (Emdogain®) on the Healing of Rats Wounds

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Introduction

The regeneration of tooth supporting tissues lost to periodontal disease has been an important goal of periodontal treatment [1]. Several approaches have been classically proposed to achieve this goal including the use of grafting materials and barrier membranes [1]. More recently, biologic mediators, such as Enamel Matrix Derivative (EMD), have also been applied for periodontal regeneration [2-4]. EMD was originally described as a protein extract capable of regenerating root cementum and consequently promoting the formation of a new periodontal ligament and alveolar bone [2,3,5,6]. In fact, a number of animal and clinical studies have demonstrated that EMD can successfully regenerate lost periodontal tissues in intrabony defects, class II furcation defects, and dehiscence defects [1,3,7,8]. Furthermore, EMD has been combined with coronally advanced flap for root coverage procedures, and recent evidence has demonstrated that this combined approach yields predictable long-term results [9,10].

In addition to the regenerative effects of EMD on the periodontium, it has been described that EMD application has a positive effect on post-operative symptoms and soft tissue healing [11,12]. When compared to other surgical regenerative procedures, EMD application results in less pain and post-operative swelling [11,13,14], which could be attributed to the anti-inflammatory effects of EMD [15], since EMD down-regulates the expression of genes involved in the early inflammatory phases of wound healing [16]. Likewise, the anti-inflammatory and anti-microbial effects of EMD could explain the improved healing of gingival tissue and skin wounds [17,18]. Another possible mechanism behind the healing effects of EMD is that it stimulates angiogenesis by inducing secretion of VEGF, and other molecules involved in the healing process such as PDGF, IL-6 and matrix metalloproteinase-2 [12,19,20]. Apparently, these beneficial effects of EMD in wound healing induce a faster healing as demonstrated in an animal skin wound healing model, when EMD application resulted in wound closure twice as fast as control wounds [20]. EMD has also been used in the healing of venous leg ulcers and hard-to-heal wounds in humans’ subjects promoting a significant reduction in ulcer size and pain, compared to control wounds [21-23]. Mirastchijski et al. 2004 [20] suggested that EMD acts as a temporary scaffold for cellular adherence, which in turn stimulates cell functions related to wound healing process such as migration, proliferation, and synthesis of mediators that regulate the wound healing processes.

Although a few studies have addressed the beneficial effects of EMD on skin wound healing, a histological time-course analysis of the healing process has not been conducted. Furthermore, the assessment of the overall quality of the healed tissue is lacking. Thus, in the present study, we applied a rat skin wound healing model to investigate morphological changes throughout the healing process, to conduct morphometrical evaluation of the healed tissue, and to assess the quality of the newly formed tissue.

Materials and Methods

Surgical procedure

The experiments were conducted under a protocol approved by the Institutional Animal Research and Care Ethics Committee at the Juiz de Fora Federal University (MG, Brazil), SUPREMA and Centro de Pesquisas São Leopoldo Mandic. Male Wistar rats (n = 50), weighing 250-300g were used in this study. A combination of ketamine and xylazine (ket-a-xyl®, Agrovetsmarket, Brazil) was intramuscularly injected to anesthetize the rats in doses of 40 and 15 mg/kg, respectively. A rectangular area of 5 cm x 5 cm in the dorsal central region of the back of each rat was cleared by hand pulling the rat’s fur. We have noticed that this method avoids unnecessary cuts and nicks of the skin that would create some in-
flammation, which could interfere with the wound-healing assay. The surgical area was disinfected with 70% alcohol and two 4.0 cm-long incisions extending to the muscular layer, bilateral and parallel to the spinal cord, were made on each animal, with #15c surgical blades. The lengths and the thicknesses of the incisions were standardized and measured with digital calipers (Mitutoyo, UK). The wounds were randomly assigned for the experimental group (EMD group) or control group (saline solution group). In the experimental group, Emdogain (0.7 ml, Institute Straumann, AG, Basel, Switzerland) was delivered inside the wound edges, whereas in the control group saline solution was used. The incisions were then closed using simple interrupted sutures (Nylon 6-0). Immediately after suture, Emdogain and saline solution were placed over their respective incisions for 5 minutes. Care was taken to avoid cross contamination during this procedure.

Animals were caged individually after surgery to avoid damage to the wounds. Five rats were assigned to each of the following subgroups: E1: sacrifice at 1 day post-op; E3: sacrifice at 3 days post-op; E7: sacrifice at 7 days post-op; E14: sacrifice at 14 days post-op; E21: sacrifice at 21 days post-op; C1: sacrifice at 1 day post-op; C3: sacrifice at 3 days post-op; C7: sacrifice at 7 days post-op; C14: sacrifice at 14 days post-op; C21: sacrifice at 21 days post-op.

At the time of sacrifice, animals were killed by deepening anesthesia with halothane.

Macroscopic Evaluation

Just after the rats were killed, digital photographs of each wound were taken for macroscopic evaluation. A blind investigator, not related to the study, evaluated the wounds. Deposition of granulation tissue, characterized by the typical red appearance, was scored as the percentage coverage of the wound on a scale from 0 to 5 [0, none (0%); 1, minimal (1-20%); 2, slight (21-40%); 3, moderate (41-60%); 4, marked (61-80%); 5, extensive (81-100%)]. The mean of the scores of each wound in each group was calculated to determine the nonepithelialized wound area, and wound tracings were scanned (ScanJet 5300C, Hewlett-Packard, Palo Alto, CA) together with a millimeter ruler for calibration in cm2. The day of wound closure, defined as complete granulation tissue, characterized by the typical red appearance, was harvested for histopathological examination. Each specimen was cut in two parts, and of the halves was processed according to standard histological protocols, sectioned, stained with Haematoxylin-Eosin (HE), and observed under a light microscope (binocular microscope, WILD, Germany). The following histological structures were investigated: the epidermis (re-epithelialization and keratinization), the dermis (creation of fibrin network, presence of polymorphonuclear leukocytes - PMNL, tissue macrophages, migration, proliferation and orientation of fibroblasts, creation of new Extracellular Matrix [ECM] - especially new collagen fibers, neoangiogenesis, and the muscle layer alone). The histological structures and processes were semi-quantitatively evaluated in coded slides. Photomicrographs were taken using the system NIKON MICROPHOT (Japan).

Immunohistochemistry

An analysis of the levels of TNF-beta e iNOS was performed to evaluate the possible influence of the EMD on the cellular composition of the animals. Cells were obtained by grinding the other half of the tissue specimens with a homogenizer in an incomplete RPMI medium. The cells were re-suspended in not supplemented RPMI with 5% fetal bovine solution, non-essential amino acids, antibiotics and L-glutamine and the supernatants were gathered for cytokine measurement.

Measurement of cytokines by ELISA (TNF-beta e iNOS)

ELISA plates were sensitized with the capture antibody, diluted in a carbonate-bicarbonate buffer, incubated for two hours at room temperature and blocked with PBS-Tween 20 (PBST) + 10% FCS (fetal calf serum) for thirty minutes. After this period, the plates were washed four times in PBST and then added to the capture antibodies. After incubation for two hours at room temperature, the recombinant cytokine standards were serially diluted. The culture supernatant samples were distributed. The plates were then incubated for 18 hours at 4°C. The plates were washed four more times, and, after placement of the enzyme conjugate, they were incubated for another hour. After this period, the reaction was detected by the addition of a substrate containing 0.1 M citric acid, 0.2 M sodium phosphate, distilled water, ABTS chromogen and hydrogen peroxide 30%. The reaction was blocked with 0.2 M citric acid, and the reading was made in an ELISA reader (SPECTRAMAX 190, Molecular Devices) at 410 nm. The quantities of cytokines were calculated based on standard curves obtained by different concentrations of recombinant cytokines.

Statistical Analysis

Results regarding the study and comparison of variables in-
volved in the assays were expressed in whole numbers or mean ± standard error for in vitro experiments or mean ± standard error (SE) for in vivo assays. When these numbers were distributed as independent samples for in vitro assays, double entry variance analysis (ANOVA) was used. A significance level of 0.05 (p less than 0.05) was accepted; therefore the limit of 5% probability of error was established. To perform the calculations, the computer program Primer of Biostatistics software was used [24].

**Results**

**Macroscopic evaluation**

The macroscopic aspect of the experimental and control wounds was distinct, since the experimental wounds healed faster than the control wounds and this difference was clear at days 14 and 21 (Figure 1). At earlier stages (days 1 and 3) this difference was not noticed, however, the experimental wound appeared slightly better healed than the control wound at day 7. Table 1 depicts the overall distribution of the healing scores. In the both groups score 2, meaning complete closure of the wounds, appeared in day 14, however with higher figures in the experimental group.

![Figure 1: The macroscopic aspect of the experimental and control wounds: control group (A), first postoperative day. (B) third postoperative day. (C) seventh postoperative day. (D) fourteenth postoperative day. (E) twenty-first.](image)

**Histological evaluation**

The amount of newly formed blood vessels was similar in experimental and control groups at initial stages of wound healing (GRAPHIC 1, p>0.05) (Figure 2 groups test and group control respectively). However, at days 7 and 14, new blood vessel formation was significantly greater in the experimental group than in the control group (GRAPHIC 1, p<0.001)

![Figure 2: Histological cut of skin sample of Wistar rats. (A) Control group, third postoperative day. An inflammatory infiltrate is observed with predominance of neutrophils (blue arrows), intense angiogenesis (asterisk) and fibrinous formation (arrowhead). (B) Control group, seventh postoperative day, we observed angiogenesis (X) and collagen fibers (arrows). (C) Treated group, third postoperative day. An inflammatory infiltrate with predominance of lymphocytes (arrows), angiogenesis (asterisk) and fibrinous formation (arrowhead) is noted. (D) Treated group, seventh postoperative day More mature neoformed vessels are observed in relation to untreated (X) and denser collagen fibers (arrows). Staining by hematoxylin and eosin. Original magnification of 400x.](image)

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GRAPHIC 1: Blood vessel section values. Anova shows statistical significant differences between groups at days 7 and 14 (p<0.001); and at the intragroup group comparison no statistical difference was found through time, for both experimental and control groups (p>0.05).

The healing process of the experimental wounds was better at all time points with the exception of day 1 that demonstrated a similar healing aspect of both groups. At day 3, it was observed greater superficial clot coverage of the wounded area and the presence of a more pronounced inflammatory infiltrate in the experimental group (Figure 3A). Likewise, the subcutaneous tissue in the experimental group seemed to be more stable despite the greater edema, as compared with the control group (Figure 3B). Furthermore, on both groups, it was observed fibrin deposition, which is compatible with that healing period (day 3). However, the inflammatory content of the control group is predominantly composed of polymorphonuclear leucocytes (neutrophils), whereas mononuclear leucocytes (lymphocytes) were more prevalent in the experimental group (GRAPHIC 2 and 3).

Leucocyte and lymphocytes counting revealed significant differences between the experimental and control groups at days 7 and 14 (GRAPHIC 2 and 3, p<0.001). The newly formed vessels in the test group seemed to be more mature (Figure 3C). At day 7, the epithelial borders and dermal layers of the experimental group were stable, whereas in the control group the wounds were still filled with a more immature healing tissue. The granulation tissue in the papilar and reticular dermis is denser and more organized in the experimental group, and less edema was present in this group at this time-point (day 7). No differences were observed in the degree of re-epithelialization of the healing area, although the angiogenesis was more evident in the experimental wounds (Figure 3B). At day 14, the subcutaneous tissue in the control group depicted greater edema and more disorganization of the muscular layer. Both groups presented re-epithelialization with keratin formation, however, the experimental group showed slightly better cellular/architectural organization and an evident greater maturation degree of the sub-epithelial regions (Figure 3C, G). At day 21, newly formed epithelial tissue with superficial keratin formation can be observed in both groups. In the experimental group, the initial formation of epithelial attachments can be seen, whereas that cannot be observed in the control group. The muscular fibers in the experimental group are more evident and organized as compared to the test group (Figure 3H, D, test and control respectively).

Figure 3 (A-H): Histological cut of the skin sample of Wistar rats. (A) Lower surface clot cover is observed. Group control. Third postoperative day (B) Reepithelialization is observed. Group control. Seventh postoperative day. (C) A lower wound closure is noted. Group control. Seventh postoperative day (D) Muscle layer formation is observed. Group control. Twenty-first postoperative day. (E) Greater surface clot cover is noted. Treated group. Third postoperative day. (F) Epithelial borders and more stable dermis layers are observed. Treated group. Seventh postoperative day. (G) Greater closure of the wound is noted. Treated group. Fourteenth postoperative day. (H) The formation of epidermal attachments in the cicatricial area and the more organized and mature muscular layer is remarkable. Treated group. Twenty-first postoperative day. Staining by hematoxylin and eosin. Original increase of 100x.
Immunohistochemistry

GRAPHIC 1 and 2 reveals the immunohistochemical analysis for cells positively-stained for iNOS and TGFβ1, respectively. There was a significantly higher number of iNOS positively-stained inflammatory cells between groups at all time points (p<0.001), with exception on day 1. In the intragroup comparison, there was a significant increase in i-NOS positively-stained through time (p<0.001). Quantification of positive cells showed that, at day 7, 20% of the inflammatory cells in the control group were positive for iNOS, whereas, 70% of those cells were positive in the experimental group (GRAPHIC 1). Similarly, at day 14, 20% of the inflammatory cells were positive for iNOS in the control group, and 80% of those cells were positive in the experimental group (GRAPHIC 4).

GRAPHIC 3: polymorphonuclear values. Anova shows statistical significant differences between groups at days 7 and 14 (p<0.001); and at the intragroup group comparison no statistical difference was found through time, for both experimental and control groups (p>0.05).

GRAPHIC 4: iNOS values. Anova shows statistical differences between groups for all time points, with exception of day 1; and intragroup through time, for both experimental and control groups (p<0.001).

There were no statistical differences between groups at any time point in the number of TGFβ1 positive-stained cells (p>0.05), however the intragroup comparison showed a statistically significant increase in those positive-stained cells for TGFβ1 trough time (p<0.001). At day 7, 60% of the inflammatory cells were positive for TGFβ1 in the control group, and 80% of those cells were positive in the experimental group, whereas at day 14, the percentage of positive cells was 80% and 90% for control and experimental groups, respectively (GRAPHIC 5).

Discussion

Several reports have demonstrated that EMD is capable of markedly improving soft tissue healing [20,25]. In the present study we further explored the soft-tissue healing capacity of EMD.
and performed a time-course analysis of the healing process of different soft tissue layers in an animal model. Our results confirm previous findings that EMD-treated soft tissue wounds heal much faster with minimal post-operative inflammatory response [20]. We observed that EMD treatment increased the amount of granulation tissue and accelerated the time to complete epithelialization by day 14 of treatment. Mirastschijski et al. (2004) [20] showed that the healing time of circular full-thickness 2-cm skin wounds in rabbits was twice as fast when treated with EMD, associated with more granulation tissue formation, which also appeared more resistant. They also observed that in the EMD-treated group, it was observed less scab formation, earlier epithelial coverage, more mature scar tissue architecture reflected by regression of blood vessels, meaning an advanced healing. Histopathological examination was only performed 28 days after therapy, whereas in our study the histological response was monitored throughout the entire experiment and since we observed an improved healing of the wounds much earlier than 28 days, no analysis at that time-point was performed. This difference in the speed of healing may be explained by the type of skin wound created; i.e., in our study we made an incision, whereas Mirastschijski and co-workers removed a circular portion of the skin including the epithelium. Another interesting aspect regarding the incision wound used in our study is that EMD was applied underneath the wound edges. Although we do not know for how long EMD-related proteins remained inside the wound, it is likely that the approximation of the incision edges by suturing served as a protective factor for EMD to act longer in deeper layers of the tissues. Nevertheless, it is noteworthy that, regardless the wound type, EMD is capable of accelerating the healing process in both incisional and excisional wounds.

Topical application of EMD in soft tissue wounds has been shown to be beneficial of healing. It has been reported in a number of studies that EMD accelerates wound healing with reduced pain and exudates in different wound types [11,12], including in large wounds such as hard-to-heal ulcers in human subjects [21-23]. With that in mind, besides applying EMD into the wounded area, we also applied EMD topically onto the sutures. However, the fact that EMD induces proliferation of fibroblastic cells, enhances mesenchymal cell recruitment, and at the same time is cytotstatic for epithelial cells is puzzling [26,27]. Obviously, the faster wound closure cannot be explained by the direct inhibitory effects of EMD on epithelial cells, but possibly by an indirect effect. It is known that EMD enhances several processes associated with wound healing such as angiogenesis, granulation tissue formation and proliferation of fibroblasts, in part by stimulation of VEGF, PDGF, IL-6, and MPM-2 [12,19,20]. Therefore, EMD promotes a faster formation of the foundation for the spread and proliferation of epithelial cells, which in turn results in a faster wound closure. In fact, in the present study we could observe especially from day 14 a much higher number of completely closed wounds as compared with the control group, and that is in line with other studies that also observed faster wound closure after EMD therapy [20,25]. By analyzing different time-points throughout the healing process, we could observe that, in the EMD-treated wounds, all the skin layers besides the epithelium presented an organized and mature structure much sooner than the control wounds. According to the hypothesis presented above, this faster tissue maturation is conducive for an improved and faster wound closure.

A proposed mechanism for the healing effects of EMD is that it has an important anti-inflammatory effect since it down-regulates inflammatory genes, whereas genes related to repair and growth molecules are up-regulated [16]. In the present study we observed that EMD may also significantly modulate the levels of TGFβ around the wound sites and thereby promoting healing and tissue regeneration. Although an absolute explanation for the effects of EMD on soft tissue wound healing has yet to be established, all the findings of previous studies and ours put together, may partially explain the wound healing effects of EMD on soft tissues. EMD is suggested to trigger mesenchymal cells to express important factors behind healing, growth and regeneration processes [28,29]. That would probably lead to angiogenesis stimuli and production of VEGF, PDGF and matrix metalloproteinase-2. EMD is also helpful for healing due to its inherent anti-bacterial capacity [30-33].

Angiogenesis is one essential component of a normal wound healing and repair and assists the granulation tissue formation, which is essential for wound closure. VEGF is related to that neoangiogenesis and also modulates the recruitment and activity of osteoblasts and osteoclasts and stimulates osteoblasts by up-regulating other osteoinductive factors, such as TGF-β1, IGF-1, and FGF-2 [34]. Immunohistochemically observed that VEGF expression and micro vessel density was statistically enhanced in periodontal pockets treated with scaling and root planing following 48 hours of EMD application. Our results histologically support their findings since we also observed that sites treated with EMD also increased neoangiogenesis. However, in our study that effect was only evident at days 7 and 14. Differences in the models (human vs rats) and evaluation methods (immunohistochemistry vs histology) might, at least partially, explain that discrepancy.

Our immunohistochemical results also showed that inflammatory cells were positive for TGFβ1 with higher numbers, although not statistically significant, for the experimental sites. Transforming growth factor β (TGF-β1) is a pleiotropic cytokine, which has been demonstrated to regulate a wide array of biological processes. It plays a major role in the regulation of vascular function, hemostasis, facilitates migration of wound keratinocytes and, thereby, successful reepithelialization, stimulates fibroblasts to deposit new extracellular matrix proteins, which supports cell and vascular in-growth neoangiogenesis [35]. It has been show a positive correlation with EMD and increased expression of TGF-β1 [19,36]. Our results are in line with this possible positive cor-
relation with the increase TGF-β1 and neo blood vessel formation, also partially explaining the faster healing of the EMD-treated sites.

It has been suggested that one of the factors related to skin and muscle healing is the expression of inducible Nitric Oxide Synthase (iNOS), which seems to have important role on all aspects of tissue repair (angiogenesis, cell proliferation, matrix deposition, and remodeling) [37,38]. Histologically evaluated the “Wound gap” on incisions in the back of guinea pigs treated or not with EMD. In all time points (5, 20 and 35 days) it was observed a statistically smaller gap in the treated sites, and also, through time they noted that in the test group the formation and organization of the connective tissue and muscle layer was better. At the end of the experimental time, over 50% of sites of the test group were completely closed. They hypothesized that EMD might modulate the level of NOS around wounded area promoting healing. The present study, as far as we are concerned, is the first study to show immunohistochemically an increase in the iNOS modulation on EMD-treated skin wounds, increasing the body of evidences suggesting that EMD foster and improves soft tissue healing. Our study is also in line with the previous study [25] in regards muscle formation. At day 14 of healing, the muscular layer was better organized in the EMD-treated group than in the control group. Furthermore, at day 21 a striking difference was observed between those two groups in regards to muscle formation, maturation and organization. Although we did not investigate any possible explanation for these findings, it is conceivable that EMD induces the synthesis of factors involved in muscle formation, including iNOS.

In summary, this time-course study showed that healing evolution observed macroscopically was positively sustained and marked by well defined histological and immunohistochemical events, suggesting that EMD modulates the natural healing response and might be associated in changes of iNOS and TGF-β1. Some statistical discrepancies found in this study seem to be related to random event and not necessarily directly related to the intervention itself. New inflammatory markers should also be measured and investigated to further explore the EMD mechanism of action in the healing process.

Conclusion

EMD-therapy improves and accelerates the healing of skin wounds.

References


