Polyethylene-Glycol Formulations

Subjects: Pharmacology & Pharmacy
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Background: Soft tissue regeneration and remodeling is fundamental in periodontal surgery, thus we investigated the angiogenic response elicited in the subcutaneous tissue of rats by a proprietary, polyethylene glycol hydrogel formulation (PEG) alone or conjugated with specific amelogenins (EMD) or nanobioglass particles (NBG).

Methods: Discs with three different formulations (PEG, PEG-EMD, and PEG-NBG) were inserted into four unconnected subcutaneous pouches, produced on the back of Sprague-Dawley rats (*n*=56, divided into three groups), and used for blood flow evaluation by Laser Doppler analysis at 1, 2, 4, 8, and 16 weeks or for histological and immunohistochemical analysis at 1, 2, 4, 8, and 16 weeks.

Results: All formulations showed tissue integration, absence of inflammatory reaction (as revealed by myeloperoxidase staining), and increased vascularization (by counting microvascular density following CD31 staining). Laser Doppler analysis revealed a statistically significant increase in blood flow after 1 week for PEG-EMD and after 2 weeks for PEG-NBG. The angiogenic response was significantly increased at 1, 2, and 8 weeks for PEG-EMD, but only at 4 weeks for PEG-NBG.

Conclusions: The studied biomaterials revealed equal biocompatibility and tissue integration properties. PEG-EMD showed the most pronounced and consistent angiogenic response in the early phases of wound healing, while the PEG-NBG formulation provided a slower and delayed, but relevant, response.

Keywords: polyethylene glycol ; angiogenesis ; hydrogel ; Formulations Show ; periodontal ; Subcutaneous ; angiogenic ; Glycol Formulations ; Different Soft ; EMD

1. Introduction

Guided bone regeneration (GBR) in the oral cavity is a procedure that attempts to restore bone tissue in a specific area, by the use of a cell occlusive membrane. To reach this goal, the cellular proliferation originating from the adjacent bone onto a scaffold support must have a higher proliferative rate than fibrogenesis coming from surrounding connective and soft tissues.[1] Soft tissue regeneration and remodeling is fundamental, especially in the case of exposed roots and gingival recession, and to increase peri-implant mucosal tissues. The PEG membrane fulfils the main criteria for a resorbable GBR membrane having a cell-occlusive function,[2] slow reabsorption time,[3] and the ability to allow bone regeneration.[4] PEG membrane thus lead to similar amounts of bone regeneration as a polytetrafluoroethylene (ePTFE) membrane.[5] PEG hydrogel shows unique biochemical properties that make it well suited for use as a carrier for other bioactive materials, as for RGD,[3] enamel matrix derivative (EMD), and nanobioglasses (NBG). RGD is a tripeptide (Arg-Gly-Asp) that by binding with integrins (transmembrane receptors that bind cells to the extracellular matrix) enhances bone regeneration.[6] EMD is an extract of enamel matrix and contains amelogenins of various molecular weights, involved in the formation of enamel and periodontal formation during tooth development. EMD stimulates the regeneration of the periodontium, reduces crevicular depth, and enhances the formation of a new epithelial attachment in cases of periodontal bone defects.[7] Recent studies[8] have shown that EMD, and more specifically amelogenin (very likely the 28.9-kDa protein), stimulates angiogenesis. Angiogenesis is a fine-tuned mechanism intimately linked to a wide variety of both normal and pathological processes. Inadequate or inappropriate bone vascularity is associated with decreased bone formation and repair.[9] PEG hydrogel can also be conjugated with NBGs, a group of surface reactive glass–ceramic biomaterials able to stimulate bone regeneration and form a bond with the bone itself. Moreover, NBG display an antimicrobial effect due to the release of alkaline species.[10] Considering these observations, this study was designed to investigate the angiogenic response elicited by PEG hydrogel alone or preconditioned with EMD or NBG in skin wounds produced in experimental animals.

2. Materials and Methods

2.1. Production of PEG discs

All PEG discs were prepared and supplied by Straumann (Basel, Switzerland), a sterilized microscopy glass slide and a 1-mm spacer were used to produce the discs. The PEG two-chamber applicator was prepared according to the manufacturer's instructions and the liquid polyethylene glycol mixture (MembraGel™, Straumann®) was evenly distributed on one glass slide within the spacer area. A second glass slide was put on top and polymerization was allowed for 60 s. After removal of the top glass slide, a 5-mm tissue sample obtained by mucosa punch was used to produce the discs. All discs were 1.5 mm thick and kept at 4°C until surgery, which was performed within 48 h after preparation of the discs. For addition of EMD (Straumann Emdogain), the discs were immersed on both sides in a droplet of EMD using tweezers and excessive liquid was removed using a spatula. For addition of NBG (Bioactive Glass, 30–60 nm, Smartodont Lic., Zurich, Switzerland), the discs were immersed on both sides in a droplet of a sonicated suspension of 70% ethanol and 20% NBG (w/w). The ethanol was allowed to evaporate for 60 s in a laminar flow, which produced an even layer of NBG adherent to the disc surface.

2.2. Animals and surgical procedures

For this study, 56 Sprague-Dawley rats (aged 3 months, weighing 225–250 g) were obtained from Charles River (Calco, Italy). Animals were maintained according to procedures approved by the local Ethics Committee and in accordance with the ARRIVE Guidelines.[11] Experiments were performed in accordance with the Guidelines of the U.S. National Institute of Health (NIH) regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations.

Rats were microbiologically clean, allocated in single cages, and fed with standard chow and water *ad libitum*. Temperature and humidity, as well as the day/night light cycle were kept stable and constantly monitored.

After 1 week of stabilization, animals were randomly divided into groups as specified below and underwent surgery. Animals were anesthetized with pentothal sodium (50 mg/kg/ip in saline solution), shaved on the back, and four pouches were produced with a scalpel at equal distance from the spine following the cardinal points. The different biomaterials were inserted and wound edges were closed with a 2-0 silk suture. Rats were allowed to recover from anesthesia under a heating lamp to maintain body temperature and housed in separate cages for the duration of the experiment.

2.3. Experimental groups for blood flow evaluation

Surgery was conducted for 7 working days and rats were randomly divided into different groups, as follows:

- 1- PEG (n=7); four pouches were created, two pockets were filled with the biomaterial and two left empty. The animals were monitored with Laser Doppler at 1, 2, 4, 8, and 16 weeks for blood flow evaluation.
- 2- PEG+EMD (n=7); four pouches were created, two pockets were filled with the biomaterial and two left empty. The animals were monitored with Laser Doppler at 1, 2, 4, 8, and 16 weeks for blood flow evaluation.
- 3- PEG+NBG (n=7); four pouches were created, two pockets were filled with the biomaterial and two left empty. The animals were monitored with Laser Doppler at 1, 2, 4, 8, and 16 weeks for blood flow evaluation.

2.4. Experimental groups for histological analysis

The remaining 35 rats were treated as follows:

Four pouches were created, three were filled with the different biomaterials and one left empty as an internal negative control (or sham-control pouch). Seven animals were then randomly sacrificed at 1, 2, 4, 8, and 16 weeks for histological evaluations. Sacrifice was done by maximal i.p. injection of the anesthetic.

2.5. Blood flow evaluation

For blood flow evaluation, Laser Doppler tests were performed at baseline, 1, 2, 4, 8, and 16 weeks using a perfusion imager (PIM 3.0 Perimed/Periscan, Sweden). Based on the Doppler principle, the instrument scans the tissue with a laser beam and collects back-scattered light without tissue contact. Color-coded images are generated showing the spatial distribution of the tissue perfusion (red=high perfusion, yellow=moderate, green=low, blue=very low perfusion).

The generated images together with the graph representing flow variation were used for the subsequent analysis. A region of interest (ROI) was selected around the same pocket at all time points in all time points (basal, 1-2-4-8-16 weeks) and the perfusion of the empty pocket was set as reference. To select the ROI, the area underneath the suture thread (for the first time points) or the scar (for the last three time points) was used in the recorded photos (Fig. 1A).

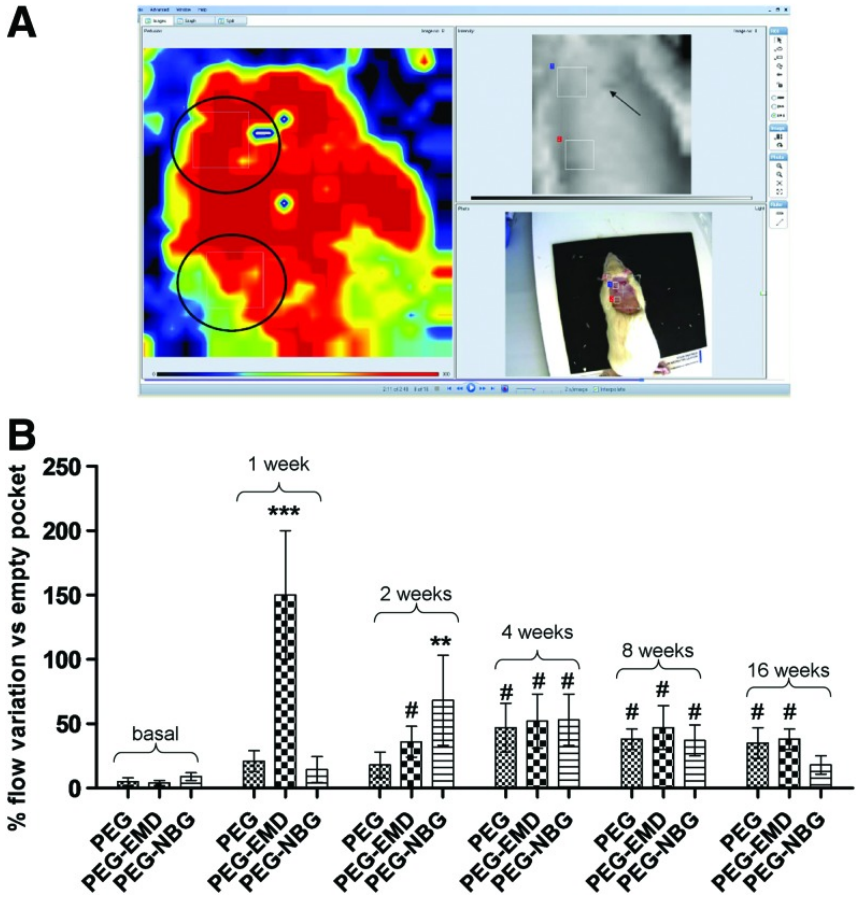

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FIG. 1.

(A) Representative Laser Doppler software appearance during the evaluations. **(B)** Results from Laser Doppler analysis at 1, 2, 4, 8, and 16 weeks following biomaterial insertion. *Bars* in the graph represent the mean of the results obtained from seven animals. ***p<0.0001 versus other PEG preparations, **p<0.001 versus PEG, #p<0.005 versus empty pouch. Color images available online at www.liebertpub.com/tea

Data generated by the PIM 3 software were then plotted in the GraphPad Prism 5 software and analyzed.

2.6. Histological examination and immunostaining

At the scheduled time point, animals were sacrificed and the skin around the pocket, including the remaining material, was excised and left in 10% buffered formalin for at least 24 h before being embedded in paraffin. Slices of 5 µm were used for the subsequent hematoxylin and eosin staining and observed with a Leica microscope at magnifications from×4 to×40. The samples were coded and the operator was blinded during the analysis. The ROI was selected in the subepidermal layer, between the epidermis and hypodermis, where the discs were inserted.

An immunohistochemical study was performed to evaluate the presence or absence of an inflammatory reaction. All specimens obtained from week 1 to 16 were tested with myeloperoxidase (MPO, Thermo scientific, Freemont, CA) as an indicator of neutrophil accumulation in the skin. Slices of 5 µm were rehydrated in graded alcohol and antigen retrieval was performed with a pH 6.0 buffer citrate and endogenous peroxidase blocking with 1% H_2O_2 in PBS. The primary antibody was incubated overnight at 4°C in a moisturized chamber and the reaction visualized with DAB (Sigma-Aldrich, Milan, Italy) the day after the secondary antibody was added. Negative control slices were tested using PBS instead of a primary antibody. To avoid any loss of positivity, no counterstaining was performed.

To support the evaluation of perfusion by Laser Doppler CD-31, immunostaining was performed to evaluate microvascular density (MVD). All specimens obtained from week 1 to 16 were tested with CD-31 (Abcam, Cambridge, United Kingdom), as described above. Counterstaining with hematoxylin was performed to enhance discrimination of the subcutaneous

layers. MVD was obtained by counting the positively stained vessels in at least three different microscopic fields.

2.7. Statistical analysis

To ensure reproducibility and statistical significance of the results, seven animals were used in each group. Values were reported as mean±standard deviation and data were compared using ANOVA. For perfusion analysis, all results were expressed as a percent of blood variation versus the empty pocket. The Student–Newman–Keuls *post hoc* test was used when ANOVAs demonstrated significance. Differences between groups were considered significant if $p \leq 0.05$.

3. Results

3.1. Perfusion results

At baseline, all the biomaterials showed the same impact on tissue perfusion and there was no significant difference between the filled versus the empty pouches (Fig. 1B).

After 1 week, we observed a remarkable increase in the blood flow in the pockets containing PEG+EMD discs compared to the other materials. At the same time, the pockets with PEG alone or PEG+NBG showed a minimal, but not significant, increase in tissue perfusion compared with the empty pocket. After 2 weeks, tissue perfusion showed a statistically significant increase in the pockets containing PEG+NBG, while PEG alone and PEG+EMD did not enhance tissue perfusion. After the fourth week and until the end of the experiment, the level of tissue perfusion remained homogeneous among all biomaterials and was increased compared to the empty pocket.

3.2. Histology results

At 16 weeks, PEG+EMD appeared to be the biomaterial, which was most integrated in the surrounding tissues, in agreement with the highest blood flow observed by Laser Doppler. Between the first and the last week, no further differences were observed in the analyzed samples (Fig. 2). No inflammatory reaction was observed in all the evaluated samples at any time point, as evidenced by the negative MPO staining (Fig. 3).

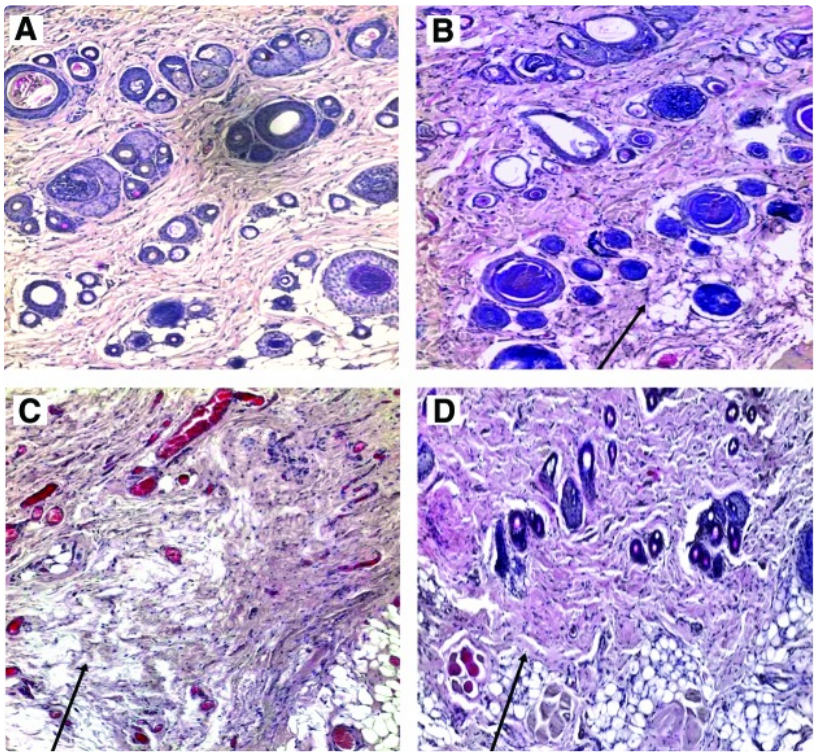

FIG. 2.

Representative histological skin appearance after 16 weeks from biomaterial insertion. **(A)** empty, **(B)** PEG, **(C)** PEG-EMD, and **(D)** PEG-NBG. The *arrows* indicate nonhomogeneous areas where likely the biomaterial was integrated into the tissue. Color images available online at www.liebertpub.com/tea

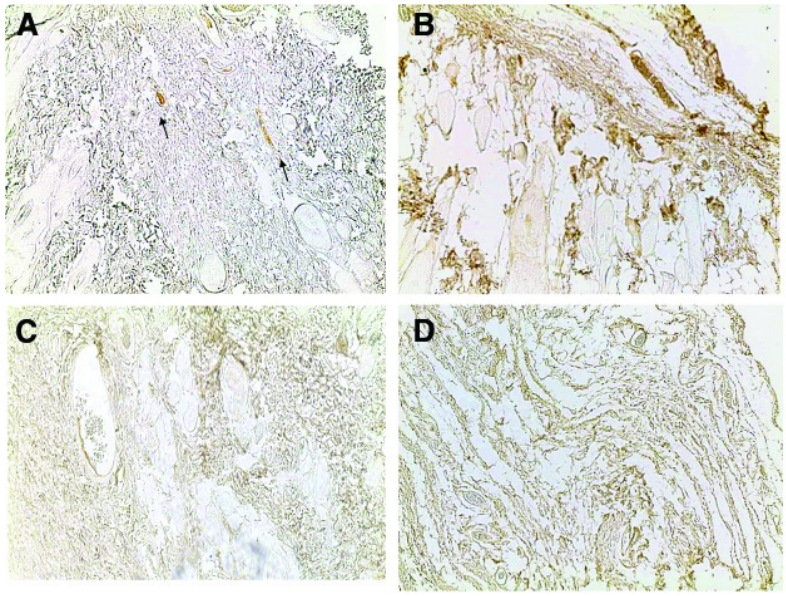

FIG. 3. Representative MPO staining in the skin after 1 week from biomaterial insertion. **(A)** empty, **(B)** PEG, **(C)** PEG-EMD, **(D)** PEG-NBG. The *arrows* in **(A)** indicates slight positive reaction around the vessel wall. Color images available online at www.liebertpub.com/tea

The staining with CD-31 (Fig. 4A–D) showed the presence of few and slightly marked new vessels from week 2 up to week 8 when angiogenesis and remodeling occurred following surgery in the empty pocket. PEG alone was able to improve the number of small-caliber vessels (representing active angiogenesis), especially at week 4. Preconditioning of PEG with EMD showed the most impressive results with the largest increase in positive staining as early as week 1 and persistence throughout week 8. PEG combined with NBG also showed a significant increase in angiogenesis starting from week 4 to 8 (Fig. 4E).

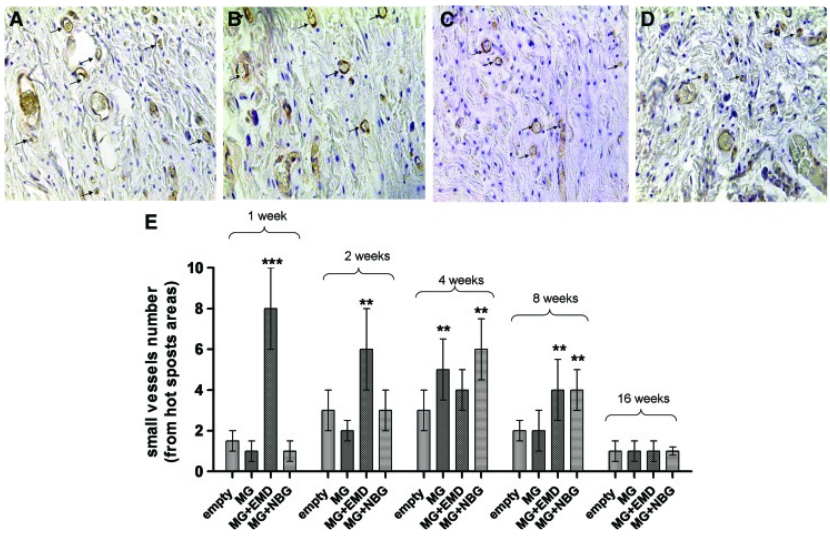

FIG. 4.

A-D Representative CD31 staining in the skin after different weeks from biomaterial insertion. **(A)** PEG-EMD at 1 week, **(B)** PEG-EMD at 2 weeks, **(C)** PEG-NBG at 4 weeks, and **(D)** PEG-NBG at 8 weeks. The *arrows* indicate positive small-caliber vessels. **(E)** Microvascular density evaluated at each time point in several groups. ***$p<0.0001$ versus empty and other PEG preparations, **$p<0.05$ versus empty pouch. Color images available online at www.liebertpub.com/tea

4. Discussion

In recent years, several biocompatible materials belonging to different categories have been successfully used for GBR procedures, e.g. nonresorbable (ePTFE, titanium mesh), and resorbable materials (dura mater, polylactic acid, polyglycolic acid, and polyurethane). To be used in GBR procedures, each material must meet specific criteria such as biocompatibility, tissue integration, cell occlusivity, nutrient permeability, degradation kinetics, and ease of use during surgery.[3] Nonresorbable membranes require a second surgery to be retrieved and, in case of premature exposure, there is a higher chance of bacterial colonization.

Consequently, current investigations are focused on resorbable devices, especially on type I and type III porcine or bovine collagen membranes.[12] Collagen meets certain important requirements of a barrier for GBR procedures, including early wound stabilization, chemotactic properties to attract fibroblast, and semipermeability, but its degradation could be accelerated by polymorphonuclear leucocytes, macrophages, and periodontal bacterial pathogens resulting in early membrane collapse. The heterologous nature of collagen by-products can be related to adverse reactions, thus compromising tissue integration. Moreover, all prefabricated devices must be adapted to the individual defect, therefore compromising the handling in sites that are difficult to reach.

To overcome these drawbacks, recent experimental studies, conducted on both animal and human models, have investigated the efficacy of a newly developed synthetic hydrogel made of polyethylene glycol (PEG) to be used in bone regeneration therapy.

The purpose of this study was to analyze the role of a proprietary PEG gel formulation as a synthetic barrier, able to stimulate the angiogenic response in skin wounds, in an experimental animal model. In this study, the hydrogel was studied alone or preconditioned by adsorption of specific amelogenins or nanobioglass particles, to evaluate whether these components might modify the ability of the hydrogel to increase angiogenesis. The animal model and the surgical procedure were conducted in accordance with the previous studies proposed by Herten *et al.*[3]

To the best of our knowledge, this is the first published report of the use of Laser Doppler analysis to study perfusion of superficial skin layers following the insertion of biomaterials commonly used for oral tissue regeneration. This technique allowed creation of a perfusion map of blood flow variations at the surgical site following different times after the surgical procedure. The PEG gel formulation was shown to be a valid resorbable barrier membrane with evidence of hydrogel remnants at 16 weeks, but no evidence of inflammatory reaction or tissue necrosis.

Laser Doppler analysis highlighted statistically relevant modifications at 1 week for EMD and 2 weeks for nanobioglass. Histological analysis confirmed the angiogenetic response to both EMD and nanobioglass. EMD, whose angiogenetic potential was already reported,[8] is able to raise a statistically significant response after 1, 2, and 8 weeks, but not after 4 weeks. One possible explanation for this pattern may be the decreasing progression of angiogenesis, which reaches a plateau at the fourth week and does not further progress until the eighth week.

In contrast, the nanobioglass reaches the highest point of vascular formation after 4 weeks, with a slower response. One possible explanation is that EMD, as an active biomolecule, is able to cause an angiogenetic burst in the early phases of wound healing, while the nanobioglass, as a scaffold, shows a slower, less intense but more steady action. The MPO assay suggests that the EMD angiogenetic stimulus is a direct action and not a consequence of an inflammatory reaction.[3]

Angiogenesis plays a fundamental role in the wound healing process, so wherever the vascular conditions are lacking at the surgical site, consistent neoangiogenetic stimuli could enhance the healing of the wound.[13][14][15]The results described here suggest that PEG is able to interact with the host tissue without any inflammatory reaction, regardless of the previous preconditioning of the material with other substances. In addition, we have demonstrated an increase in blood flow and angiogenesis in the area where PEG was inserted in combination with EMD, which was able to further improve PEG integration in the host tissues as well as the angiogenic response for up to 8 weeks.

This study shows how PEG, besides being a valid barrier membrane, can act as a carrier for different bioactive molecules, likely mediating their release. The substances used in our experiments, especially the EMD, appeared to improve neoangiogenesis, possibly enhancing the healing course in all places that a strong vascular support is needed, as in the GBR.

The use of biomaterials has dramatically improved tissue regeneration procedures and patient outcomes, however, several conditions may alter this process. In particular, altered blood flow supply, reduced production, and release of growth factors and their related molecules could negatively affect the healing process, as it occurs in diabetes.[16] These results provide new insights on the regenerative efficacy of biomaterials, exploring the angiogenic and the tissue remodeling response, making these biomaterials useful for a possible immediate use in the clinical setting. If confirmed, these data could be extremely important to improve the poor outcome, due to other preexisting conditions (i.e., smoking habit or diabetes), in regenerative procedures.

Future investigations could be directed toward a combination of these three materials compounded in a single formulation, thus combining all advantages and verifying the interactions between the components.

References

1. polyethylene glycol;angiogenesis;hydrogel;Formulations Show;periodontal;Subcutaneous;angiogenic;Glycol Formulations;Different Soft;EMD
2. Format correct
3. Herten M., Jung R.E., Ferrari D., et al. Biodegradation of different synthetic hydrogels made of polyethylene glycol hydrogel/RGD-peptide modifications: an immunohistochemical study in rats. Clin Oral Implants Res 20,116, 2009
4. Thoma D.S., Dard M.M., Hälg G.A., Ramel C.F., Hämmerle C.H., and Jung R.E. Evaluation of a biodegradable synthetic hydrogel used as a guided bone regeneration membrane: an experimental study in dogs. Clin Oral Implants Res 23,160, 2012
5. Jung R.E., Zwahlen R., Weber F.E., Molenberg A., van Lenthe G.H., and Hammerle C.H. Evaluation of an in situ formed synthetic hydrogel as a biodegradable membrane for guided bone regeneration. Clin Oral Implants Res 17,426, 2006
6. Jung R.E., Hämmerle C.H., Kokovic V., and Weber F.E. Bone regeneration using a synthetic matrix containing a parathyroid hormone peptide combined with a grafting material. Int J Oral Maxillofac Implants 22,258, 2007
7. Sculean A., Schwarz F., Becker J., and Brecx M. The application of an enamel matrix protein derivative (Emdogain) in regenerative periodontal therapy: a review. Med Princ Pract 16,167, 2007
8. Kauvar A.S., Thoma D.S., Carnes D.L., and Cochran D.L. In vivo angiogenic activity of enamel matrix derivative. J Periodontol 81,1196, 2010
9. Hu X., Zhang P., Xu Z., Chen H., and Xie X. GPNMB enhances bone regeneration by promoting angiogenesis and osteogenesis: potential role for tissue engineering bone. J Cell Biochem 114,2729, 2013
10. Waltimo T., Brunner T.J., Vollenweider M., Stark W.J., and Zehnder M. Antimicrobial effect of nanometric bioactive glass 45S5. J Dent Res 86,754, 2007
11. Kilkenny C., Browne W.J., Cuthill I.C., Emerson M., and Altman D.G. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol 8,e1000412, 2010 [PMC free article]
12. Bunyaratavej P., and Wang H.L. Collagen membranes: a review. J Periodontol 72,215, 2001
13. Bitto A., Polito F., Altavilla D., Minutoli L., Migliorato A., and Squadrito F. Polydeoxyribonucleotide (PDRN) restores blood flow in an experimental model of peripheral artery occlusive disease. J Vasc Surg 48,1292, 2008
14. Galeano M., Bitto A., Altavilla D., et al. Polydeoxyribonucleotide stimulates angiogenesis and wound healing in the genetically diabetic mouse. Wound Repair Regen 16,208, 2008
15. Polito F., Bitto A., Galeano M., et al. Polydeoxyribonucleotide restores blood flow in an experimental model of ischemic skin flaps. J Vasc Surg 55,479, 2012
16. Shirakata Y., Eliezer M., Nemcovsky C.E., et al. Periodontal healing after application of enamel matrix derivative in surgical supra/infrabony periodontal defects in rats with streptozotocin-induced diabetes. J Periodontal Res 49,93, 2014