The Impact of Different Augmentative Methods on the Expression of Inflammatory Factors

Marcus Stoetzer, DDS[1]
Vasilios Alevizakos, DDS[2]*
Björn Rahlf, DDS[1]
Nils-Claudius Gellrich, MD, DDS[1]
Andreas Kampmann, Dr rer nat[1]
Constantin von See, DDS[2]

Many animal studies show that an intact periosteum plays an important role in osseous regeneration. The potential effect of an in vivo periosteal barrier membrane on the expression of specific proteins has not been examined sufficiently. The aim of the present study is to investigate the influence of the flap preparation method and collagen membrane on the emission of inflammatory factors. This study examines 20 patients with dental implants who had previously undergone an augmentation. A soft tissue sample was taken during augmentation and 3 months later from the same location. Samples were always taken from the margins of a previously prepared mucoperiosteal flap. The flap was raised with a conventional periosteal elevator in the control group and with a piezoelectric device in the test group. In both groups, we covered half of the augmented bone with a native collagen membrane (NCM; Geistlich Bio-Gide). This allowed us to examine the same incision area with and without a membrane. An immunohistochemical analysis was performed for collagen IV, fibronectin, and inflammatory factors such as cluster of differentiation 31 (CD31), cyclooxygenase-2 (COX-2), and interleukin 6 (IL-6). There was a clear difference in the expression of specific proteins after the piezoelectric device and the periosteal elevator were used. The expression of fibronectin, IL-6, and COX-2 was higher after preparation with the periosteal elevator than after piezoelectric periosteum dissection. The expression of collagen IV was higher after the piezoelectric procedure. No difference was observed for CD31. The membrane had no effect on the expression of collagen IV, fibronectin, IL-6, and COX-2. The type of periosteal preparation influences the expression of specific proteins. With regard to the factors examined here, NCM did not appear to influence the wound healing cascade.

Key Words: piezoelectric, collagen membrane, bioactivity, preparation of the periosteum, tissue factors, Bio-Gide

INTRODUCTION

In medicine, and especially in dentoalveolar surgery, membranes are regularly placed beneath the periosteum in order to cover native or augmented bone. To display the field of interest the mucosa on the top of the alveolar crest is cut and then a mucoperiosteal flap prepared. The flap preparation can be performed either by pushing strictly a conventional raspatory between bone and periosteum along the bone surface in order to raise the periosteum from the bone or by using a piezoelectric-driven raspatory instead. The primary purpose for using membranes is to prevent the ingrowth of soft tissue into the bone defect, which is caused by the rapid proliferation of epithelial cells and connective tissue.[1] Membranes are also used to close wounds especially in surgery involving the maxillary sinus and to cover bone augmentations under the gums. In the latter case they act as a mechanical buffer to the maxillary sinus and/or oral cavity. Membranes can thus serve as placeholders for osteoblasts and osteoclasts, which proliferate slowly. Membranes are used in augmentation procedures to protect the ossification and integration of added bone.[2] Another advantage of collagen-based membranes is the positive haemostatic effect, as platelet aggregation is induced by exposed collagen.[3]

Guided bone regeneration (GBR) and guided tissue regeneration (GTR) are currently standard therapeutic procedures in dental surgery. These procedures are based on the isolation of regenerative cell types, such as periodontal fibroblasts and osteoblasts, from rapidly proliferating epithelial and connective tissue cells.[1] Over the years, absorbable membranes have become more commonly used since a second intervention for the removal of the membrane is no longer necessary[4,5] In most cases, absorbable membranes are xenogenic collagen membranes.

One of the most commonly used membranes in dentoalveolar surgery is the Bio-Gide membrane (Geistlich, Baden-Baden, Germany). This porcine-based membrane consists of noncross-linked type I and III collagens. The membrane can still be detected in the tissue after 3 to 4 months. It later disappears, more likely as a result of integration into the new tissue than absorption. Porcine type I and III collagen membranes are some of the most widely used membranes.[6] They are absorbed by endogenous collagenases and proteases with little or no inflammation.[5] Absorption starts with the activity of the

[1] Hanover Medical School, Hanover, Germany.
[2] Danube Private University, Center for Digital Technologies in Dentistry and CAD/CAM, Krems an der Donau, Austria.
* *Corresponding author,* e-mail: vasileios.alevizakos@googlemail.com
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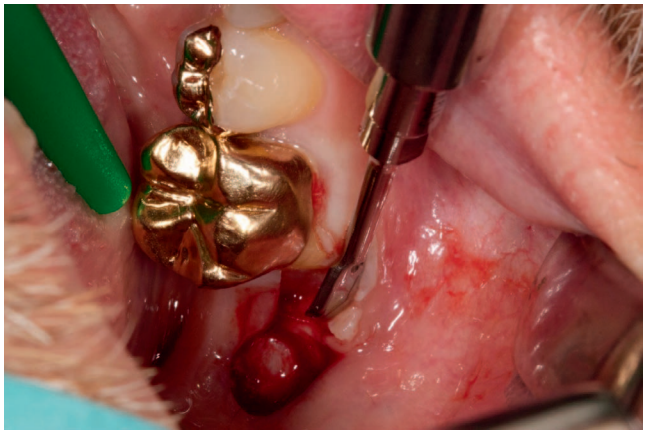


Figure 1. Piezoelectric periosteal raise.

collagenases, which cleaves the collagen at a specific location in the molecule. As a result of this cleavage, the larger fragments become more sensitive to thermal exposure and denature at 37°C. Gelatinases and proteinases then cause a further breakdown into oligopeptides and natural amino acids. In addition, collagen membranes are tissue friendly and support the attachment of different cell types like fibroblasts and osteoblasts.[7] After 10 to 14 days, capillary sprouts start to develop on a collagen membrane.[8] Another important feature of these membranes is their hydrophilic properties, which promote their adaptation to the surrounding tissue. Newly formed blood vessels penetrate into the membrane so that it eventually becomes vascularised. With a thickness of 0.25 to 0.35 mm, these membranes are highly tear resistant and easy to use in surgery. The fibrous collagen microstructure of the cross-linked membrane is hydrophilic and thus maintains its shape even if fluid is added. Absorption is completed within 4 to 8 weeks.

Protein expression in the periosteum

Various proteins are involved in the healing process, each at a specific time, for a specific duration, and with a specific task and target. In case of a disturbance of the healing process these specific characteristics can change; for example, proteins are still present in a large amount although they should not be. Type IV collagen is a major component of the lamina densa of the basement membrane where it forms a network and thus the basic structure of the basal lamina. Since type IV collagen is also found in the periosteum, it is used as a marker to indicate periosteal damage.[9,10] Fibronectin is a glycoprotein of the extracellular matrix and plays an important role in tissue repair, embryogenesis, coagulation, and cell migration/adhesion. Fibroblasts as well as chondrocytes, endothelial cells and macrophages produce insoluble fibronectin, which they deposit in the extracellular matrix. Fibronectin forms between collagen fibrils and other extracellular matrix molecules and is an adhesion molecule for various migrating cells.[11] The platelet endothelial cell adhesion molecule (PECAM-1) also known as cluster of differentiation 31 (CD31) is found on the surface of different cells such as endothelial cells or monocytes. Its key role is to remove aged neutrophils. Among other things, it controls the transmigration of leucocytes. In immunohistochemistry CD31 demonstrates the presence of endothelial cells and is consulted in evaluating the degree of angiogenesis.[12,13] Cyclooxygenase-2 (COX-2) is an enzyme that metabolises arachidonic acid to the eicosanoid prostaglandin H2 in two steps. COX-2 synthesis is induced by cytokines and mitogens after injury, inflammation, or cell growth. COX-2 is transcribed at higher levels during inflammatory processes.[14] Interleukin 6 (IL-6) is a signal of the immune system and belongs to the group of pro-inflammatory cytokine/interleukins. It has a key role in nonspecific innate immune response.[15]

Study objective

The objective of this study was to clarify the extent to which the periosteum is compromised by surgical procedures and usage of membranes by analyzing the amount of expression of specific proteins and inflammatory factors in the augmented tissue.

Materials and Methods

Study design

The following study was approved in advance by the ethics committee of the Hannover Medical School under the number 2372-2014.

This study included patients (n = 20) with dental implants (all implants position 036; Straumann BL 4.1 RC10 mm, SLActive, Institute Straumann AG, Basel, Switzerland) who had previously required an augmentation. The patients were 31 years old (±4 years). We used the random sampling method for this study.

A 4 mm tissue probe consisting of mucosa and periosteum was punched during the augmentation procedure and, at the same location, 3 months later when the implant was inserted. The samples were always taken at the margin of a mucoperiosteal flap, which had been previously prepared. In one group, the flap was raised with a conventional periosteal elevator. In the other group, the flap was prepared with a new piezoelectric device (PR1, Mectron, Carasco, Italy; Figure 1). In both groups, we covered half of the augmented area (autologous bone from retromolar region) with Bio-Gide membranes (Geistlich, Baden-Baden, Germany) and particulate bone chips. The preparation device to be used was predetermined. Immunohistochemical and histopathological preparations, analyses, and evaluations were then carried out on the tissue samples. Two samples from each mucoperiosteal flap were taken.

Histological preparation

Formalin-fixed specimens were embedded in paraffin for light microscopy. Thin sections (5 μm) were stained with haematoxylin and eosin (Merck KGaA, Darmstadt, Germany) according to standard procedures and examined by light microscopy (Leica DM4000 B, Leica Microsystems, Wetzlar, Germany).

In order to detect relevant proteins, thin sections (5 μm) were immunohistochemically stained using specific antibodies. We used an Alexa Fluor 594-conjugated goat anti-rabbit antibody (Dianova, Hamburg, Germany) as a secondary

antibody. We mounted the specimens with the help of a DAPI containing mounting medium (Roti-Mount FluorCare DAPI, Carl Roth GmbH, Karlsruhe, Germany) and examined them with fluorescent microscopy (Leica DM4000 B, Leica Microsystems) and the program ANAlysis 3.1 (Soft Imaging System GmbH, Münster, Germany). First, we searched the interface of gingiva to bone. Second the area of interest was marked with a square. The program measured the area in the square. By omitting the primary antibody step, negative controls were performed, none of which showed detectable staining.

Statistical analysis

All data were documented and stored with Microsoft Excel (Microsoft, Redmond, Wash). The standard deviation and the mean were calculated using the two-sample *t* test. The *P* value, by convention, is commonly set to .05. The statistical analysis was conducted using SPSS (SPSS Statistics 23, SPSS Inc, Chicago, Ill).

Results

We obtained the following results for CD31 (Figure 2) with the periosteal elevator and the piezoelectric device and with and without a collagen membrane (Bio-Gide) before (R1/R1m and P1/P1m) and after augmentation (R2/R2m und P2/P2m). This diagram shows the differences between the two devices at the two time points. There was no significant difference between the two devices at the different time points although the values of the piezoelectric dissection group tended to be higher. The results obtained for IL-6 are shown in Figure 3. We observed a significant difference between the periosteal elevator and the piezoelectric device as well as a decrease in IL-6 at the second time point. The expression of collagen type IV was considerably higher for periosteum dissection with a piezoelectric device than with a periosteal elevator. A significant difference between the two devices is shown in Figure 4. When it came to fibronectin, we observed a highly significant difference between the devices at the second time point (R2/R2m compared with P2/P2m). We did not observe a significant difference at the first time point (Figure 5).

For COX-2, there was a significant difference between the piezoelectric device and the periosteal elevator (R1/R1m compared with P1/P1m and R2/R2m compared with P2/P2m), as is shown in Figure 6.

Discussion

The study was carried out on patients requiring an augmentation prior to the insertion of implants. The periosteum was dissected with a periosteal elevator in one group and a piezoelectric device in the other. In both groups, the bone was covered with a membrane after the augmentation procedure. Tissue samples were taken from all patients during and after the augmentation. Since the augmented sites were only partly covered with a membrane, two areas were able to be examined under the same conditions.

An immunohistochemical analysis was performed for collagen IV, fibronectin, and inflammatory factors such as CD31, COX-2, and IL-6.

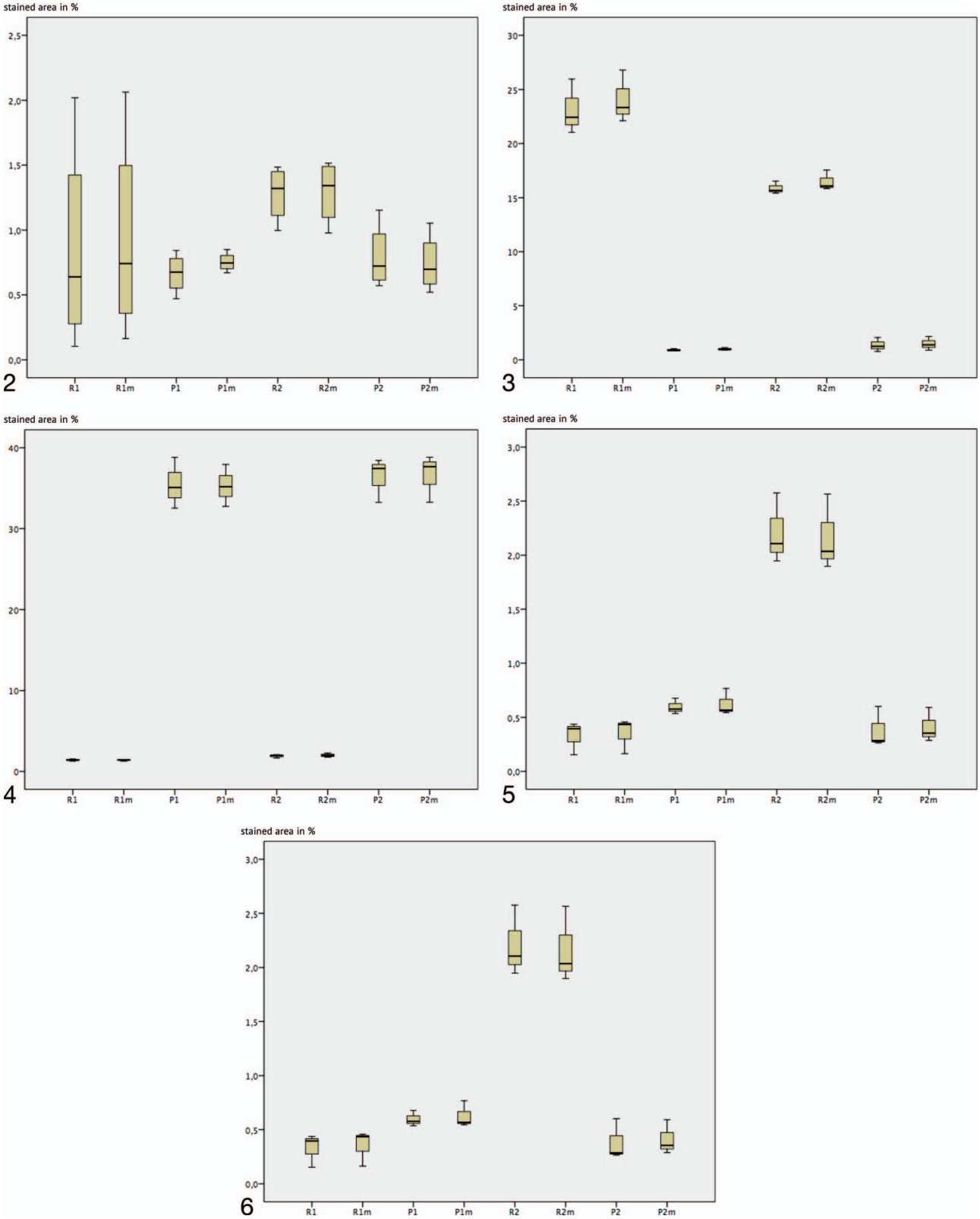
There was a clear difference in the expression of specific proteins after the piezoelectric device and the periosteal elevator were used. The expression of fibronectin, IL-6, and COX-2 was higher after preparation with the periosteal elevator than after piezoelectric periosteum dissection. The expression of collagen IV was higher after the piezoelectric procedure. No difference was observed for CD31. The membrane had no effect on the expression of collagen IV, fibronectin, IL-6, and COX-2.

For utilizable samples the tissue punches have to be clearly removed from the bone avoiding any laceration and they may not layer. Layering of the tissue punch and unclear removal have a negative impact on the immunohistochemistry investigation, which leads to incorrect results and conclusions. Furthermore, in order to avoid errors in the staining of the samples during immunohistochemistry analysis, the samples should be stained fresh if possible and should not be stored for too long. Storage should ideally be at 4°C. In principle, the correct dilution must be observed when preparing the various solutions for fixing and buffering, and all solutions must be fresh, otherwise insufficient coloring will occur. The measurement results are strongly affected by this. Incorrect preparation of tissue punches and immunohistochemical analysis represent potential biases. Furthermore, the small number of patients could weaken the meaningfulness of the findings.

To evaluate the healing process by immunohistochemistry 5 specific proteins were chosen. These proteins play a major role during the healing process, indicating with their presence and absence if the healing process is disturbed or not.[12,16,17]

Type IV collagen was used as a marker to identify potential damage to the tissue.[17–19] The results show that more collagen IV was expressed when the periosteal elevator was used, which indicates greater damage. Comparable results were obtained in the studies of Şurlin et al.[20]

IL-6 is also released when tissue is damaged and inflamed.[16,21–24] As was the case with type IV collagen, a higher level of interleukin 6 was observed when the periosteal elevator was used and a lower expression when surgery was performed with the piezoelectric device. IL-6 has already been sufficiently examined as an inflammation marker in connection with periodontitis.[25] As a marker, CD31 unfortunately had limited validity and did not provide adequate results. However, it has to be taken into account that CD31 is a good marker for tissue vessels.[12,13,26,27] There were likely not enough vessels in the present samples.[28] Fibronectin plays a vital role in tissue repair.[29–31] Since the fibronectin level was significantly higher in the periosteal elevator group at the second time point, it may be concluded that this device destroyed considerably more tissue during the second surgical intervention. This may be connected to reduced vascular density and thus a decreased regenerative capacity. Immunohistochemistry was used to identify the parameters (IL-6, COX-2, collagen IV, fibronectin, and CD31). As other studies have shown, this is a valid procedure for identifying these parameters.[32] There are only a few studies available on the behavior of tissue factors in connection with absorbable membranes. This study might be the first to provide data on this topic. We were able to show that membranes might play only a minor role in the expression



Figures 2–6. Figure 2. Expression of CD31. **Figure 3.** Expression of IL-6. **Figure 4.** Expression of collagen type IV. **Figure 5.** Expression of fibronectin. **Figure 6**. Expression of COX-2. R1 indicates periosteal elevator, day 0, without membrane; R1m, periosteal elevator, day 0, with membrane; P1, piezoelectric device, day 0, without membrane; P1m, piezoelectric device, day 0, with membrane; R2, periosteal elevator, after augmentation, without membrane; R2m, periosteal elevator, after augmentation, with membrane; P2, piezoelectric device, after augmentation, without membrane; P2m, piezoelectric device, after augmentation, with membrane.

of tissue factors. However, the method of periosteum preparation might be decisive. The periosteal elevator has a considerable effect on tissue factor expression. Nevertheless, with this device the same results were obtained whether a membrane was used or not.

No significant differences in CD31 were observed between the groups. This is not surprising since this factor is typically used to detect tumor angiogenesis. COX-2 is expressed, among others, in macrophages following the activation of the CD14 receptor in response to lipopolysaccharides. Macrophages then produce prostaglandin E2, which modulates the inflammatory response.[33] The results of this study demonstrate a higher COX-2 level after the periosteum is detached from the bone with a periosteal elevator. Since no difference was found between the presence and absence of the membrane in any group, it may be concluded that membranes neither cause nor prevent an inflammatory response. The same applies to IL-6. The obtained data are comparable to those for COX-2. The basement membrane is a special layered extracellular matrix. The main component of the basement membrane is collagen IV. In this study, considerably more collagen IV was observed after the piezoelectric device was used. Since collagen IV is already present in the tissue, it may be concluded that tissue damage was less severe than it was with the periosteal elevator. Only a higher fibronectin level was measured in tissue prepared with a periosteal elevator after the augmentation procedure. It can be assumed that the first preparation with the periosteal elevator caused a scar, which provoked the higher fibronectin level measured after the second procedure. Further studies are required in order to investigate the exact regeneration mechanisms, healing, and postoperative scar formation in the periosteum.

Conclusions

Regarding the findings of the present study it might be assumed that the use of absorbable membranes does not have an effect on tissue factor expression; however, type of periosteum preparation does play a role. Within the limitations and possible biases of the present study it could be concluded that the piezoelectric device allowed to detach the periosteum from the bone with fewer adverse biological consequences than the manual approach. Membranes can be used as internal patches and prevent premature angiogenic sprouting into the augmentation area without adverse effects on the tissue and do not prevent tissue regeneration. Further studies with more participants, and more accurate and less sensitive analysis procedures containing fewer potential biases are needed to support the findings of the present study.

Abbreviations

CD14: cluster of differentiation 14
CD31: cluster of differentiation 31
COX-2: cyclooxygenase-2
DAPI: 4′,6-diamidino-2-phenylindole
GBR: guided bone regeneration
GTR: guided tissue regeneration
IL-6: interleukin 6
NCM: native collagen membrane
P1: piezoelectric device, day 0, without membrane
P1m: piezoelectric device, day 0, with membrane
P2: piezoelectric device, after augmentation
P2m: piezoelectric device, after augmentation, with membrane
PR1: piezoelectric raspatory 1
R1: periosteal elevator, day 0, without membrane
R1m: periosteal elevator, day 0, with membrane
R2: periosteal elevator, after augmentation
R2m: periosteal elevator, after augmentation, with membrane

Note

We declare that there are no conflicts of interest.

Acknowledgments

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