Bone Morphogenetic Protein 7 Expression in Alveolar Bone Addition With Autologous Blood, Lyophilized Bone and Atelocollagen

BOGDAN-CĂTĂLIN ALEXANDRU1, MONICA POPA1, LIVIU OANA2, COSMINA IOANA BONDOR3, CARMEN GEORGIU4, CAMELIA-SIDONIA LAZĂR4, ALINA SIMONA ȘOVREA5, ANNE-MARIE CONSTANTIN5 and GABRIELA DOGARU6

1Discipline of Medical Hygiene, “Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca, Romania; 2Department of Surgical Pathology, University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania; 3Department of Medical Informatics and Biostatistics, “Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca, Romania; 4Discipline of Pathology, Department of Morphological Sciences, “Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca, Romania; 5Discipline of Histology, Department of Morphological Sciences, “Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca, Romania; 6Discipline of Medical Rehabilitation, “Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca, Romania

Abstract. Background/Aim: The biomaterials used in guided bone regeneration have undergone significant diversification in recent years. This study aimed to evaluate alveolar bone addition and bone morphogenetic protein 7 (BMP7) expression using an improved autologous and xenogeneic biomaterial. Materials and Methods: Chronic marginal periodontitis was induced in sheep; the intervention group received bone addition as periodontal therapy, using a composite system with lyophilized bovine bone enriched with atelocollagen type 1, platelet-rich plasma and advanced platelet-rich fibrin (A-PRF). Six weeks after the intervention, the dentoalveolar structures were evaluated using hematoxylin-eosin and immunohistochemical staining, to evaluate bone addition and BMP7 expression. Results: The untreated sheep showed inflammation, periodontal ligament destruction, remnants of calculus and bacterial plaque as well as foreign bodies in the desmodontal space, without signs of repair. In the treated sheep, fibroblasts/fibrosis, cartilage and/or new bone, cellular cementum and desmodontium, along with remnants of biomaterial with various degrees of cellularity were observed. In the untreated group, the presence of BMP7 was found in osteoblasts and osteocytes while in the treated group, it was mainly found in the biomaterial remnants, while immunohistochemical staining was less intense in the newly formed osteo-periodontal tissues. Quantitative analysis using the Mann-Whitney U-test showed highly statistically significant differences between the two groups, demonstrating the efficiency of this composite system. Conclusion: The current composite system meets all the necessary conditions for promising guided alveolar bone regeneration.

Over the past years, special attention has been given to biomaterials used for guided alveolar bone regeneration (GTR); the best results in GTR have been shown by bone autografts and biomaterials containing recombinant growth factors. However, bovine xenografts have also been among the biomaterials of choice, and platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) autologous blood derivatives have also been frequently mentioned as sources of growth factors. One of these growth factors, bone morphogenetic protein BMP-7, which belongs to the transforming growth factor beta (TGF-β) family and is involved in osteoblast differentiation with a main role in the transformation of mesenchymal stem cells into bone and cartilage, is predominantly found in autologous blood derivatives (1). In this regard, the...
improvement of these biomaterials, especially by certain combinations, might have a beneficial effect on GTR. For this purpose, xenografts of bovine origin with a macroporous structure, enriched with atelocollagen type 1, responsible for osteoconduction, might be used. Atelocollagen type 1 stimulates and accelerates new bone formation, by initiating platelet aggregation and releasing growth peptides like TGF-β, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and insulin growth factor (IGF) (2, 3). Regarding PRP, different studies have demonstrated its role in bone regeneration (4-12). In order to obtain PRP, test tubes with heparin addition can be used for the optimization of growth factor release (13). Besides its anticoagulant effect, heparin presents growth factor binding properties, allowing the biomimetic sequential release of these growth factors (2, 13). The advanced platelet-rich fibrin (A-PRF), unlike PRF, has an increased content of platelets, erythrocytes, neutrophils/granulocytes (leukocyte subfamilies) and an increased amount of stem cells (1). Moreover, the A-PRF membrane presents high amounts of growth factors, stimulating the cell proliferation and differentiation (14, 15). A-PRF can thus be used as an augmentation mass for hard tissues, both alone or in combination with other bone addition materials (1).

Until now, sheep have been used in many bone regeneration studies, including periodontal alveolar bone regeneration (16-19). The current experimental study was performed on sheep with chronic marginal periodontitis, a disease very similar in sheep to that found in humans. The aim of the current experimental study was to evaluate the alveolar bone regeneration on sheep, after a treatment using a combination of different biomaterials, a composite system containing bovine xenografts enriched with atelocollagen type 1, combined with PRP and covered with membranes made of A-PRF. Another aim of this study, was to evaluate the presence of bone morphogenetic protein 7 (BMP7) in the bone addition areas. All parameters were evaluated 6 weeks after the intervention.

Materials and Methods

Animal groups
Experimental group. The study was conducted based on the Ethics Committee’s approval no 23 of 23.01.2015, issued by “Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca. The study included five sheep (Ovis aries, Romanian Turcana breed), aged five years, non-pregnant, multiparous females, with a weight ranging between 40-60 kg, without any cardiac diseases or other organic disorders, but with chronic marginal periodontitis. Three of these sheep received periodontal treatments, including bone addition, representing the treated group. In order to avoid animal suffering, general anesthesia was used before any examination or intervention on the dento-periodontal structures (20, 21). After the diagnosis of chronic marginal periodontitis, prophylactic treatments and closed field periodontal curettage, followed by alveolar bone addition using a composite system containing Hypro-Oss preparation (Bioimplon GmbH, Gielen, Germany) were performed. The Hypro-Oss preparation consists of lyophilized original bovine bone enriched with atelocollagen type 1, with granule sizes ranging between 0.5 mm and 1 mm. PRP was obtained from autologous blood by centrifugation of glass test tubes containing heparin. The test tubes also contained a thixotropic gel that separated blood into the two fractions. The A-PRF membranes were obtained following centrifugation of autologous blood in simple vacuum test tubes. After centrifugation, the platelet clot was extracted and pressed, obtaining the A-PRF membranes. The membranes were cut and placed over the Hypro-Oss combined with PRP, covering the entire grafts, then placed on the vestibular part of the surgically treated periodontal structures. Subsequently, the mucoperiosteal flap was reapplied, also covering the biomaterial, and adequately sutured coronally, at cervical level. Antibiotic therapy was performed, using 1 ml Amoxicillin Retard 20% per 10 kg live body weight; the suspension was administered after stirring the vial in the neck muscle (22, 23), for a period of five days, starting 12 hours before initiation of the periodontal treatments.

Control group. In order to compare and quantify the results, the two other multiparous female sheep, left with untreated chronic marginal periodontitis were also studied, representing the control or untreated group (Figure 1A). All five animals were euthanized and dentoalveolar samples were collected after the same period of six weeks after the initial diagnosis, with or without treatment (Figure 1B).
Staining techniques. In both animal groups, histological and immunohistochemical examinations were performed in order to evaluate the process of repair, meaning the appearance of fibrosis or bone neoformation (if present) and the presence of BMP7 in the addition mass and/or in the newly formed periodontal tissue. All the dento-osseous samples were fixed in formalin 10% for 48 hours, decalcified in trichloroacetic acid/hydrochloric acid for 1 week and longitudinally sectioned, perpendicularly to the bone addition area (Figures 1C).

HE staining. From the treated group three pieces were collected, from which 21 dentoalveolar samples were sectioned. From the control untreated group, two samples were taken, from which 14 dentoalveolar samples were obtained. Then they were embedded in paraffin and cut at four microns, being subsequently stained with hematoxylin-eosin (HE).

Immunohistochemical staining. For logistical reasons, only 12 slides from the treated group and four slides from the untreated group were immunohistochemically stained and evaluated. Immunohistochemical staining for BMP7 expression was performed on 4 micron sections displayed on silanized slides. BMP7 antigens were unmasked using sodium citrate buffer, pH 6.0. The primary antibody used was rabbit polyclonal Anti-BMP7 antibody predicted to work with sheep (ab56023, Abcam, Cambridge, UK), diluted at 1:200. The detection kit “Super Sensitive TM Polymer-HRP IHC Detection System” (BioGenex, Hague, Netherlands) was used according to the protocol, with secondary HRP-conjugated anti-rabbit antibodies diluted in the blocking solution for an hour, subsequently revealed with the DAB chromogen (3,3’-diaminobenzidine). This caused a brown staining of antigen positive sites, against a blue background, obtained with hematoxylin counterstaining. On the negative control slides, primary antibodies were excluded.

Statistical analysis. A statistical study was performed by evaluating and comparing the two groups (treated and control). For this purpose, several parameters were quantified as markers of periodontal complex regeneration: the biomaterial and the cellular biomaterial (exclusively...
in the treated group), the fibroblasts/fibrosis, the newly formed cartilage and bone, osteocytes, osteoblasts, desmodontal ligaments, cellular cementum, cementum and mucosa (in both groups). These parameters were quantitatively evaluated in the HE stained histological preparations. The quantitative and qualitative BMP7 expression was also assessed. The bone addition score was determined by adding the grades of 12 parameters. To assess bone addition as well as biomaterial differentiation for each evaluated parameter, values from 0 to 3 (0=absent, 1=5%-25%, 2=25%-60%, 3=>60%) were used; these represented the amount of tissue found on the histological slides by direct visual assessment by two different examiners (any differences being next mediated by discussion). The total score represented the sum of the evaluated parameters. The highest possible value of the bone addition score was 36 points. The categories were established depending on the presence and extensions of: biomaterial, cellular biomaterial, cartilage and biomaterial, cartilage, new bone and biomaterial, new bone, osteocytes, osteoblasts, cellular cementum, cementum, desmodontal ligaments, mucosa. The analyzed elements depended on the characteristics of the differentiating periodontal tissues, similarly to previous studies (24).

The statistical study was performed by evaluating and comparing the samples collected from the treated and untreated/control groups. First, the obtained data were presented as descriptive statistical parameters: median (25th, 75th percentile). The statistical test used for comparison was the Mann-Whitney U-test. The relationship between the analyzed parameters was assessed by Pearson correlation in the case of data without outliers and with Spearman correlation in the case of data with outliers. A p-value less than 0.05 was considered statistically significant. Statistical analysis was performed using IBM Corp SPSS Version 25.0 and Microsoft Excel (2018) (Microsoft Corporation Armonk, New York).

Results

Regarding the control group, histological HE examination at 6 weeks after diagnosis revealed the persistence of chronic inflammatory infiltrate, periodontal desmodontal ligament and alveolar bone destruction, as well as presence of calculus deposits, bacterial plaque and foreign bodies in the desmodontal region, without reparatory signs (Figure 2A-D). Given the presence of chronic marginal periodontitis, BMP7 was evidenced by immunohistochemistry in the osteoblasts and osteocytes of the remaining initial bone (Figure 3A) and slightly in the desmodontium (Figure 3B), signaling an attempt to stimulate regeneration in response to the inflammatory bone destruction, while it was negative in all other sites.

In the treated group, at 6 weeks after the biomaterial mass addition, the histological evaluation on HE staining revealed important bone addition areas, represented by well-formed but thin new bone trabeculae growing on the vestibular part of the original alveolar bone, sometimes with islands of cartilage (cartilaginous ossification) (Figure 4D-J), adjacent to fibroblasts and desmodontal like fibrotic tissue (Figure 4C) and remnants of biomaterial with various degrees of cellularity (Figure 4A, B). In some areas we also observed new cellular cementum (Figure 4K, L). In this group, immunohistochemistry revealed the presence of BMP7 in all these areas, the highest expression (highest staining intensity) being observed in the biomaterial remnants (Figure 5A, B). However, the biomaterial showed a reduction of staining intensity with increasing cellularity, being less intense in the cellular biomaterial, containing undifferentiated mesenchymal cells (Figures 5A, B). The intensity of BMP7 expression was also gradually reduced in more differentiated areas, like those containing fibroblasts or newly formed cartilage and bone trabeculae (Figure 5C-F), however persisting in the periodontal desmodontium, lateral and also apical (Figure 5G, H).
Figure 4. Continued.
Statistically, the parameters investigated on the untreated and treated groups were new bone formation, the presence of cartilage, fibroblasts and desmodontal like fibrotic tissue, the cellular and acellular biomaterial remnants, isolated or mixed with bone or cartilage, the new cellular cementum, the original destroyed cementum, the density of osteoblasts and osteocytes (for the control group from the original bone and for the treated group from the new bone) and separately, the BMP7 expression in each site.

In the treated group, multiple statistical correlations between the evaluated parameters were found, with the Spearman or Pearson \( r \) correlation coefficient being statistically significant and positive, sometimes tending towards the maximum +1, meaning the growth of one parameter accompanied closely the growth of the other one. Thus, the biomaterial was significantly correlated with: cellular biomaterial \( (r=0.91, p<0.001) \), cartilage \( (r=0.52, p=0.005) \), cartilage+biomaterial \( (r=0.68, p=0.008) \). Consequently, the amount of biomaterial led to a statistically significant increase in the amount of cellular biomaterial, and cartilage. The newly formed bone was significantly correlated with: new bone and biomaterial \( (r=0.86, p<0.001) \), cellular cementum \( (r=0.71, p=0.003) \), cementum \( (r=0.46, p=0.014) \) and osteocytes \( (r=0.38, p=0.042) \). In addition, fibroblasts were significantly correlated with: newly formed bone \( (r=0.70, p=0.001) \), new bone \( (r=0.79, p<0.001) \), cementum \( (r=0.76, p<0.001) \) and cellular cementum \( (r=0.78, p<0.001) \).

Regarding BMP7, the amount of immunohistochemically positive biomaterial and desmodontal like fibrotic tissue was correlated with: new bone volume \( (r=0.5, p=0.019) \), new bone and biomaterial \( (r=0.75, p=0.072) \), cementum \( (r=0.25, p=0.910) \) and cellular cementum \( (r=0.75, p=0.315) \), providing a statistically significant correlation.
Figure 5. BMP7 expression in different areas of the periodontal complex from the treated teeth: (a) Positive biomaterial (40×); (b) Highly positive granular biomaterial and moderately positive cellular biomaterial (100×); (c) Transforming biomaterial, at the junction between the root and the alveolar old bone and desmodontal remnants; decreasing intensity from the cellular part to the fibrously transformed biomaterial (100×); (d) Moderately positive cellular biomaterial and less intense fibroblastic biomaterial (100×); (e) Delicate trabeculae of new bone, presenting slightly positive osteoblasts/osteocytes, and negative old bone (100×); (f) Positive osteoblasts and some osteocytes in new bone trabeculae, at very high magnification (1000×); (g) Moderately positive lateral periodontal desmodontium and negative cellular cementum, dentin and old alveolar bone (100×); (h) Moderately positive apical periodontal space and negative root (200×). Immunohistochemical staining; positivity is indicated by the brown staining of the cytoplasm and/or nuclei on a blue background.
As expected, there were significant differences regarding the reparatory signs between the two groups (treated and untreated). The development of the bone addition mass, i.e., the new periodontal tissue, was very significant in the treated group; the differences between the two groups showed high statistical significance for each parameter as well as for the total bone addition score ($p < 0.001$) (Table I).

### Discussion

Bovine xenografts were introduced into clinical practice in 1954 (25) and since then processing technologies have been constantly improving. At present, commercially available xenografts are much safer.

The Hypro-Oss preparation was introduced into clinical practice in the early 2000’s, obtained by the lyophilization technology, which allows preserving the natural, crystalline structure of the components. It contains hydroxyapatite (70% of osteoconductive bovine hydroxyapatite) and atelocollagen (30% collagen type I without telopeptide, which maintains the triple helicoidal structure of the matrix and contains the naturally osteoinductive elements TGF-β, BMPs and IGF). Hypro-Oss is taken from the bone structure of young animals, given that growth factors and BMPs are quantitatively reduced in old bovines (26).

The term PRP was introduced in the 1970s to describe autologous preparations and platelet enrichment in the plasma concentrate (27). PRP was introduced in the 1990s by top scientists such as Whitman and Marx (28). The main objective of PRP was the isolation of the greatest amount of platelet growth factors associated with their collection and reuse. The technique involved $\text{CaCl}_2$ or bovine thrombin addition, also requiring a long preparation time (1). In the early 2000’s, injectable PRP was developed, which was obtained by a single centrifugation (29).

A-PRF was developed by Choukroun in 2001 in France (30). A-PRF is a platelet concentrate with a high immunological value, because it incorporates all the elements involved in the immune response and the healing of soft and hard tissues in the form of a fibrin membrane, obtained from a 10 ml blood sample (1).

Our study demonstrated the capacity of the composite graft to initiate, promote and maintain osteogenesis and odontogenesis in sheep. The graft, with a complex structure, contained lyophilized bovine bone enriched with atelocollagen type 1, combined with PRP (enriched with heparin) and A-PRF (with a membrane role). Histological examination showed its gradual replacement. The correlations of tissue differentiation were also statistically quantified. Graft biodynamics was characterized according to previous similar studies; the ideal addition mass was represented by cell differentiation and differentiated regeneration of tissues that progressively replace the graft (31), which also demonstrates the combined release of growth factors and BMPs (13). The presence of a multitude of BMPs in bone grafts is known and supported; for autologous blood derivatives, we refer to BMP2 in the first place (1). This experimental study supports the literature hypothesis according to which BMP2 and BMP7 induce osteogenic and odontogenic tissue differentiation without affecting each other (32), while being classified as strong osteoinductors (33). The presence of BMP7, which also plays the role of enhancing ossification and bone regeneration modeling as well as collagen accumulation (34) was demonstrated here by immunohistochemistry.

### Table I. Comparison of the evaluated parameters (each graded from a minimum of 0 to a maximum of 3) and the bone addition total score between the control and treated groups.

| Parameters                          | Control group (n=14) | Treated group (n=26) | $p$-Value |
|-------------------------------------|----------------------|----------------------|-----------|
|                                     | Median value         | Median value         |           |
|                                     | (minimum; maximum value) | (minimum; maximum value) |           |
| Biomaterial                         | 0 (0; 0)             | 2.5 (1; 3)           | $<0.001$  |
| Cellular biomaterial                | 0 (0; 0)             | 3 (2; 3)             | $<0.001$  |
| Fibroblasts/Desmodontal like        | 0 (0; 0)             | 0 (0; 3)             | 0.023     |
| Cartilage                           | 0 (0; 0)             | 1 (0; 1)             | 0.014     |
| Cartilage and biomaterial           | 0 (0; 0)             | 1 (0; 1.5)           | 0.009     |
| New bone                            | 0 (0; 0)             | 1.5 (0; 2.5)         | 0.019     |
| New bone and biomaterial            | 0 (0; 0)             | 0 (0; 2)             | 0.072     |
| Cellular cementum                   | 0 (0; 0)             | 0 (0; 1)             | 0.315     |
| Cementum                            | 0 (0; 0.5)           | 0 (0; 1.5)           | 0.921     |
| Osteocytes                          | 2 (1.5; 3)           | 3 (2.5; 3)           | 0.166     |
| Osteoblasts                         | 1.5 (1; 3)           | 2.5 (2; 3)           | 0.145     |
| Mucosa                              | 0 (0; 0)             | 0 (0; 0)             | 0.443     |
| **Total score**                     | **5 (3.5; 6)**       | **14 (12; 18)**      | **<0.001**|

Bold values indicate statistical significance.
BMP7 is generally expressed after two weeks during the healing process following trauma or interventions on bone structures (2). PRP rapidly releases proteins in high amounts (35), thus being considered an alternative to the use of rhBMP7 (36). A-PRF releases more growth factors compared to PRP in a period of up to ten days (35). In the case of the graft used, BMP7 was seen even after a period of six weeks due to sequential BMP release, favored by heparin and collagen, which facilitates cell proliferation and differentiation. Collagen, found in A-PRF and in the bovine xenograft, is considered a protein carrier (2), being at the same time an indicator of osteoblast differentiation (34). The test tubes utilized for the production of PRP contain heparin; heparin has the capacity to bind various growth factors (including TGF-β) (2) and at the same time, BMPs. The latter (being hydrophilic when released) are limited, having low bioavailability due to their poor stability and short biological half time in the circulatory system (31). The binding effect provided by heparin and collagen, respectively, was demonstrated in our study.

There is scientific evidence of resemblances of periodontitis development between sheep and humans (37, 38). Thus, BMP7 plays an important role in desmodontal development in both species, triggering the development of periodontal ligament (PDL) and cell differentiation into osteoblastic and cementoblastic phenotypes and, at the same time, playing a key role in the transformation of mesenchymal cells into bone and cartilage (39). In our study, BMP-7 was observed extensively in the experimental group in the desmodontal regeneration areas, and also in the control untreated group in the chronic marginal periodontitis areas. In the treated experimental group BMP-7 was locally synthesized, mediating the differentiation of cell lines such as cementoblasts and osteoblasts (37, 40) and also playing an anabolic and anticatabolic role (41).

Compared to other addition masses, e.g. rhBMP7 (released in the form of microspheres) (31), which starts to replace the biomaterial with bone structure only during the eighth week, our composite graft presents newly formed bone as early as the sixth week (confirmed by the presence of BMP7). In addition, this composite graft model does not produce catabolic effects (13, 41) or complications such as cell apoptosis, extensive inflammatory reactions (sometimes with vital risk), fibroblast infiltration, abnormal stimulation of bone resorption, ectopic ossification or increased oncogenic risk, as demonstrated in the case of rhBMP7. Furthermore, compared to bone autografts, additional surgeries with bone sacrifices were avoided.

Statistically, the comparison made between the two groups led to promising results. Histologically, the differences between the two groups (untreated and treated) were clear; however, due to the lack of newly formed cartilaginous elements and new bone in the untreated group and to the small number of samples, statistically significant differences were found only in the "new bone" and "biomaterial" categories. Compared to the untreated group, the treated group showed the highest significant statistical differences for the cell differentiation and biomaterial (p<0.001).

Conclusion

The results of this study are compatible with those regarding the differentiation of osteo-periodontal tissues of the biomaterial studied in vivo. The current composite graft meets all the optimal conditions required for alveolar bone regeneration, due to the inclusion of the xenograft components (osteonenic cells, osteoconductive factors, porous osteoconductive matrix), as well as through the additional sequential release of growth factors and BMPs found in the autologous blood derivatives (including BMP7). Regarding autologous platelet plasma, the use of test tubes with heparin addition proves to be effective, due to the ability of heparin to bind growth factors and BMP7.

Although more extensive studies on much larger animal samples evaluated after a longer time period are required, given the clinico-histological aspect of the graft transformation and the lack of complications, the evolution of such bone addition appears to be favorable and more useful compared to other commercially available materials.

Conflicts of Interest

Bogdan-Cătălin Alexandru, Monica Popa, Liviu Oana, Cosmina Ioana Bondor, Carmen Georgiu, Camelia-Sidonia Lazăr, Alina Simona Șovrea, Anne-Marie Constantin, and Gabriela Dogaru declare that they have no conflicts of interest regarding this study.

Authors’ Contributions

Bogdan-Cătălin Alexandru: Conceptualization, Methodology, Investigation, Writing of original draft, Visualization. Monica Popa: Conceptualization, Methodology, Visualization, Supervision. Liviu Oana: Conceptualization, Visualization. Cosmina Ioana Bondor: Conceptualization, Formal and Statistical analysis. Carmen Georgiu: Investigation, Writing-original draft. Camelia-Sidonia Lazăr: Investigation, Writing-review & editing. Alina Simona Șovrea: Visualization, Supervision. Anne-Marie Constantin: Methodology, Investigation, Writing-review & editing, Visualization. Gabriela Dogaru: Writing-review & editing, Visualization.

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