Efficacy of Platelet-Rich-Plasma (PRP) and Highly Purified Bovine Xenograft (Laddec®) Combination in Bone Regeneration after Cyst Enucleation: Radiological and Histological Evaluation

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ABSTRACT

Objectives: The purpose of the present study was to evaluate the efficacy of adding platelet-rich plasma (PRP) to a new highly purified bovine allograft (Laddec®) in the bone regeneration of cystic bony defects augmented following cystectomy.
Material and Methods: Study sample included 20 patients undergoing cystectomy in which the bone defect was filled with PRP and Laddec®. All patients were examined with periapical radiographs before operation and at follow-up. After 3 months, at re-entry surgery for implant placement, bone core was taken for histological and histomorphometric analysis.
Results: The postoperative successive radiographs showed a good regeneration of bone in the height of bony defects with application of PRP to bone graft. By the first postoperative month, about 48% of the defect was filled, which gradually increased in each month and showed about 90% of defect-fill by 6 months. Histological and histomorphometric analysis, showed a significant presence of bone tissue and vessels, with newly formed bone in contact with anorganic bone particles. The mean volume of vital bone was 68 ± 1.6% and the mean percentage of vital bone was 48 ± 2.4%. The mean percentage of inorganic particles in tissues was 20 ± 1.2% of the total volume. All the samples analyzed did not evidence the presence of inflammatory cells.
Conclusions: The results of this study showed how the use of Laddec® in association with platelet-rich plasma allows bone regeneration and has a potential for routine clinical use for regeneration of cystic bony defects.

Keywords: platelet-rich plasma; sintered bovine bone true bone ceramics; cystectomy; histology; xenograft.

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INTRODUCTION

Bone-replacement graft materials have played an important role in regenerative dentistry for many years. Several types of filling biomaterials have been evaluated for bone regeneration, and the choice of the biomaterial mostly depends on its features and application site [1]. The grafts could be classified according to their origin as autologous, homologous, heterologous and alloplastic materials [2,3], and according to their mechanism as osteogenic, osteoconductive, and osteoinductive materials [4]. Osteogenic materials directly stimulate bone cells to synthesize bone tissue; osteoconductive materials facilitate cell proliferation, migration and new bone apposition; osteoinductive materials induce differentiation of mesenchymal cells into osteoblasts [5].

Since 1978, autologous material has been used for bone regeneration and presently it is considered the gold standard in bone grafts since it has osteogenetic, osteoconductive and osteoinductive features [6,7]. The advantage of autogenous bone is that it maintains bone structures such as minerals and collagen, as well as viable osteoblasts and bone morphogenetic proteins (BMP); furthermore, there is no immunological response to autologous grafts [7]. Its main disadvantages are increased surgical time and patient morbidity [8].

Homologous grafts, are composed by non-vital osseous tissue taken from one individual, stocked in bone banks, and transferred to another individual of the same species [9]. There are three forms of homologous bone or allograft: fresh frozen, Freeze-Dried Bone Allograft (FDBA) and Demineralized Freeze-Dried Bone Allograft (DFDBA). Homologous graft is thought to be osteoinductive and osteoconductive [10], but the amount of BMPs in any single allograft has shown dramatic variability [11,12], and contradictory opinions about its biological properties are still present in literature [13-17].

Heterologous grafts consist of deproteinized cancellous skeletal bone tissue that is harvested from one species and transferred to the recipient site of another species; bovine bone being the most common source [9]. Heterologous materials have been used in several types of bone defects with satisfactory results; the advantage is the maintenance of the physical dimension, and the disadvantage is that they are only osteoconductive [18].

Alloplastic materials are synthetic materials that have been developed to replace human bone and that are available in different sizes, forms and textures. They are biocompatible and are the most common type of graft materials utilized [4]. The varying nature of commercially available synthetic graft materials, such as porosity, geometries, different solubility, and densities, determines their biological features and their resorption times. There are several types of alloplastic substances in clinical use nowdays: calcium phosphate-based (CaPs), other ceramics (e.g. Hydroxyapatite - HA), Biphasic Calcium Phosphate (BCP), Tricalcium Phosphate (TCP), Calcium Sulfate, and Biocompatible Composite Polymers. Calcium phosphate cement composites (CPCs) are osteoconductive materials rapidly integrate into the bone structure and are transformed into new bone by the action of bone cells responsible for the local bone remodelling [19]. However, in spite of these good properties, synthetic materials have limitations due to their poor mechanical properties and slow biodegradation *in vivo* [20]. In view of the biological limitations associated individually with graft materials, surgeons have attempted to augment the activity and physical properties with composite grafts combining molecular, cellular, and genetic tissue engineering technologies [21-23]. The molecular approach using BMPs has received the most attention over the past decade. BMPs are differentiation factors that are part of the transforming growth factor superfamily [24], but many other factors also contribute, such as transformating growth factor (TGF-βs), insuline-like growth factor (IGFs), fibroblast growth factor (FGFs), plateled-derived growth factor (PDGFs) [5]. The commercial availability of these growth factors (GF) has given oral and maxillofacial surgeons an additional option for the reconstruction of bony defects, but despite their potential usefulness, GF are still not available for routine use in practice. Another GF approach is to use the patient's own blood, separating out the platelet-rich plasma (PRP) and adding this concentrated group of autologous GFs to the grafting material [25]. PRP is considered to be a rich source of autologous GFs, and the contribution of PRP formulations to the bone healing process is thought to be based on the GFs contained [26]. The addition of PRP to autogenous grafts showed a more rapid and dense bone formation compared to autogenous grafts used alone for bone augmentation [25]. PRP has been also used in conjunction with allografts as a source of autologous GFs [27], but an improvement in bone formation when PRP is added to these graft materials has not been demonstrated clearly [28].

Recently has been proposed to surgeons an highly purified bovine allograft characterized by preservation of the type I collagen matrix associated with spindle-shaped hydroxypatite crystals (Laddec®, BioHorizons, Birmingham, USA) [29,30]. The results of preliminary studies suggest that presence of type I collagen fibbers in the matrix of a bone biomaterial could be of major interest to determine cell attachment, spreading

and orientation of osteoblasts and that type I collagen can bind osteoblasts via specific cell surface receptors, the integrins [31,32]. Hence, the present study was undertaken to assess the role of platelet rich plasma with this highly purified bovine allograft in regeneration of osseous defects of jaws caused by cysts enucleation.

MATERIAL AND METHODS

The present study included 20 consecutively treated patients, between 34 and 68 years of age, who needed cystectomy for some kind of pathology in the oral cavity (Figures 1 - 4). In all the patients, after cystectomy, PRP mixed with Laddec® was used for bone regeneration. Subjects with systemic diseases, renal disorders, regional malignancies, and respiratory problems were excluded from the study. All patients were examined with panoramic radiographs (Promax, Planmeca. Helsinky, Finland) converted into digital images using the computed system Regius (Konica, Minolta, Tokyo, Japan). Periapical radiographs (Ultra-speedA, Eastman Kodak Co, Rochester NY, USA), by means of 65 kV dental X-ray unit equipped with a longcone (Oralix 65 S, Gendex Dental System S.r.l., Milano, Italy) were used before surgery for preoperative evaluation of size lesion, and at 1 month, 2 months, 4 months, and 6 months postsurgery, respectively, to assess the rate of bone regeneration. A silicone index material was fixated to the adjacent teeth, and a radiograph holder was constructed for each patient. This technique ensures that the same position of the radiograph could be reproduced at each visit. All these radiographs were compared with preoperative radiographs to check the height of bone regenerated (defect bone fill) from the base of the defect to cemento-enamel junction (CEJ) of the adjacent teeth. The nature of the study was explained to the patients and informed consent was obtained. The study was conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki, The protocol and consent form were approved by the institutional ethics committee of University of Catania (Italy).

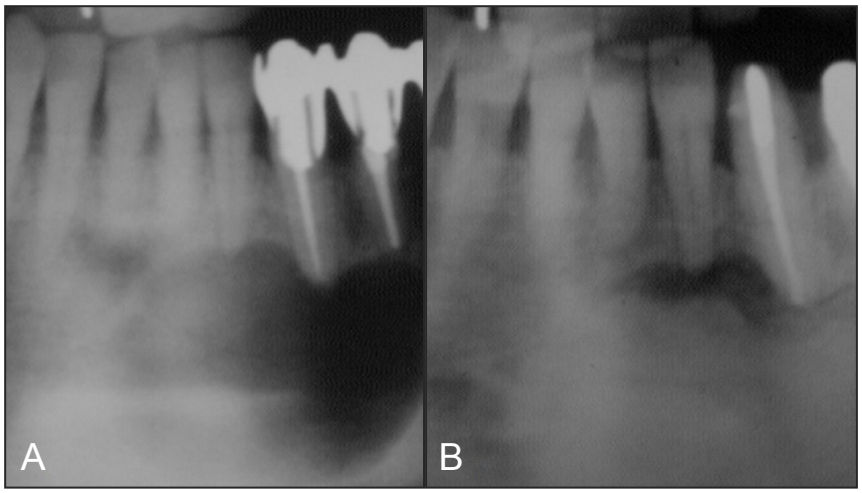

Figure 1. Example of radiographic cystic cavity shrinkage: A = preoperative, B = 6 months postoperative.

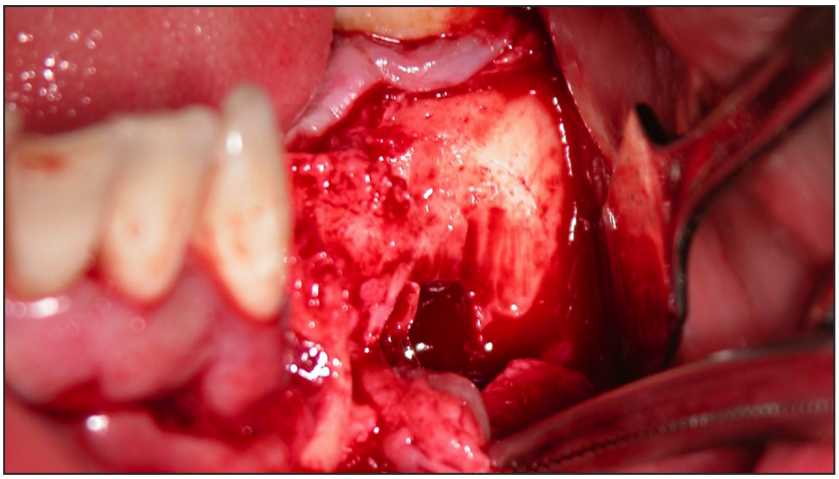

Figure 2. Intraoprative view: enucleation of the cyst.

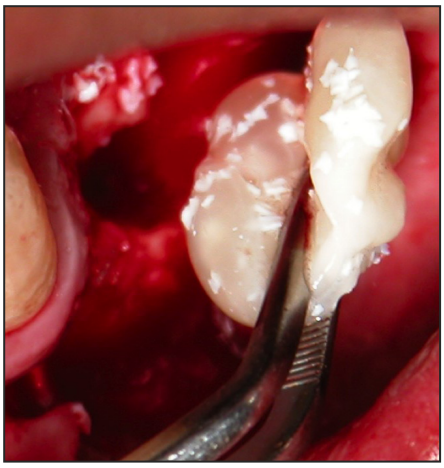

Figure 3. Intraoprative view: positioning of the PRP.

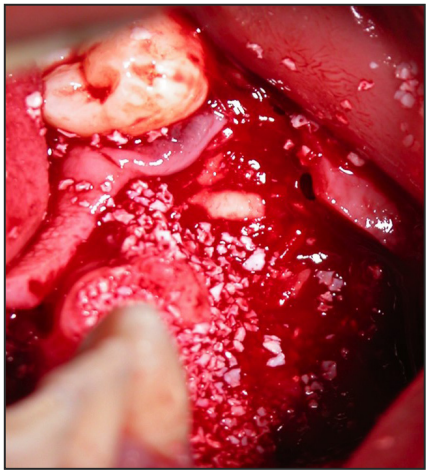

Figure 4. IIntraoprative view: positioning of PRP and Laddec®.

PRP preparation

The PRP was performed at the Department of Hematology Hospital "Canizzaro" Catania. Prior to the start of the surgery, 300 ml of blood was drawn intravenously from patients and collected in sterile plastic vacuum tube coated with anti-coagulant citric acid and dextrose. Automated centrifugation machine was used for obtaining PRP with a speed of 1300 rpm for 10 min. After centrifugation, 3 layers were obtained: 1) an upper straw coloured fluid - PPP (Platelet Poor Plasma); 2) a middle buffy coat rich in platelets; 3) a lower layer rich in red blood cell (RBC). The straw coloured plasma was collected along with buffy coat and 1 ml of the RBC layer. This was centrifuged at 2000 rpm for 10 min. The PRP was obtained in the form of a red button at the bottom of the test tube. This was collected with the help of a pasture pipette and transferred into a sterile tube. The PPP was discarded. PRP obtained after second centrifugation was placed in a sterile tube. For activation, 6 ml of calcium chloride and thrombin were added to PRP. A first layer of PRP gel was introduced into the residual bone cavity after cystectomy to stimulate the capillary regeneration in wound healing [33], and a second layer was mixed with Laddec® in a volume preparation of 1:1.

Histological and histomorphometric evaluation

3 months after surgery a bone biopsy was performed in all patients. Specimens were taken through the use of a milling cutter of 2.5 mm diameter trephine (Figure 5). The cylinder marrow was used for histological and histomorphometric analysis. The biopsies were immediately fixed in 4% formaldehyde in a buffered solution of 0.1 M phosphate (pH 7.3) at 4 °C for 24 hours to their dispatch to the laboratory. Samples were hydrated gradually with ethanol and soaked in Epon 812 (Shell Chemical Co., New York, NY, USA). Decalcified sections of a thickness of 30 ± 10 µm were obtained by cutting, by means of Buehler Isomet (Buehler, An ITW Co, Lake Bluff, Il, USA) along the vertical axis of the cylinder marrow. The bone sections were stained with toluidine blue and were used for qualitative histological analysis and for quantitative histomorphometric analysis, carried out with FOMI III (Carl Zeiss, Ovberkochen, Germany) equipped with a microscope with image resolution (DC 280 Leica, Wetzlar, Germany).

Statistical analysis

The height of regenerated bone (defect bone fill) in mm at four follow-up periods was compared with that from the preoperative periapical radiographs. The data set was analysed with the aid of the SPSS 13.0 package (SPSS, Chigaco, IL, USA) and a Student *t*-test was used for comparision between values of different time periods. Results were considered statistically significant at $P < 0.05$.

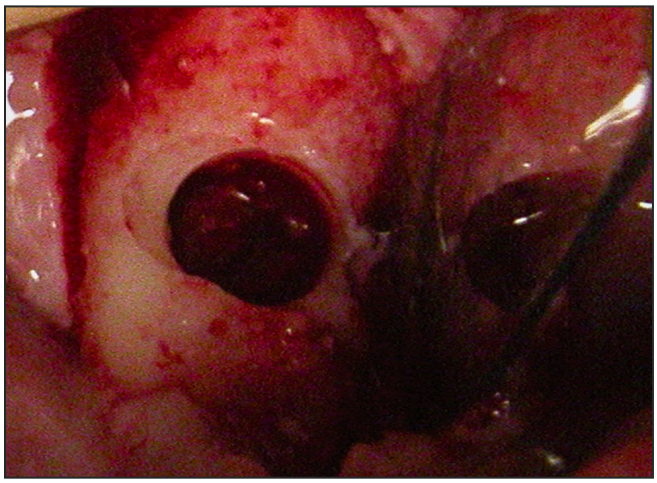

Figure 5. Re-entry surgery after 3 months.

RESULTS

In the periapical radiographs, it was observed that the mean preoperative defect size was 22.5 mm with standard deviation of ± 4.5 when calculated from the base of the defect to the CEJ of the adjacent tooth. In the first month, the defect size reduced to 9.4 ± 1.1 mm, the difference from the preoperative radiograph was 13.1 ± 4.2 mm, and the size of the defect was filled by 56%. In the second month the defect size reduced to 8.4 ± 0.6 mm, the difference from the preoperative radiograph was 14.1 ± 4 mm, and the size of the defect was filled by 62%. In the fourth month, the defect size reduced to 4.7 ± 1.5 mm, the difference from the preoperative radiograph was 17.8 ± 4.4 mm, and the size of the defect was filled by 74%. In the sixth month, the defect size reduced to 1.1 ± 2 mm, the difference from the preoperative radiograph was 21 ± 4.5 mm, and the size of the defect was filled by 92%. The difference was significant between the postoperative 4 months and 6 months results (Table 1).

The postoperative successive periapical radiographs showed adequate consolidation (regeneration) of the bone, as manifested by homogeneous radiopacity. On observation for comparison of height of regenerated bone, it was noticed that, by first postoperative month about 56% of the defect was filled; this gradually increased in each month and showed about 92% of defect fill at 6 months.

Table 1. Observation for the height of regenerated bone (defect bone fill) with platelet rich plasma + Laddec® application seen on periapical radiograph

Examination periods	Bony defect (mm) Mean ± SD	Difference operative defect size (mm) Mean ± SD	Defect bone fill (%)
Preoperative	22.5 ± 4.5		
1st months postoperative	9.4 ± 1.1	13.1 ± 4.2	56
2nd months postoperative	8.4 ± 0.6	14.1 ± 4	62
4th months postoperative	4.7 ± 1.5	17.8 ± 4.4	74
6th months postoperative	1.1 ± 2	21.4 ± 4.5	92
$P < 0.05^a$			

SD = standard deviation.
[a]The difference was significant between the post-operative 4 months and 6 months results.

In all the specimens, histological analysis showed a significant presence of bone tissue and vessels. Both, histological and histomorphometric analysis, showed newly formed bone in contact with anorganic bone particles. The mean volume of vital bone was 68 ± 1.6% and the mean percentage of vital bone was 48 ± 2.4%. The mean percentage of inorganic particles in tissues was 20 ± 1.2% of the total volume. All the samples analyzed did not evidence the presence of inflammatory cells (Figures 6 - 9).

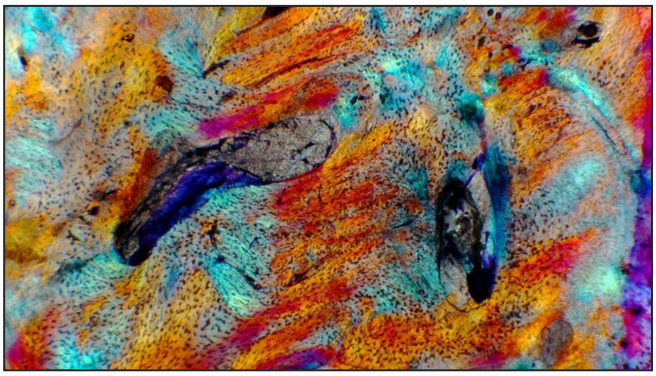

Figure 6. Histological view of new bone and vascular proliferation. Toluidine blue stain, original magnification x40 (Courtesy of Dr. Roberto Crespi).

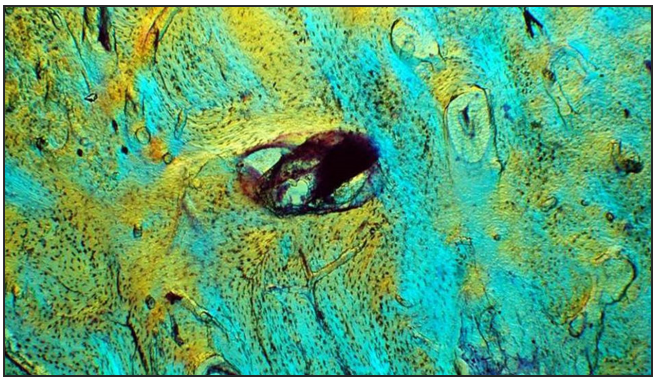

Figure 8. Increased cellular activity with lines of newly formed bone tissue. Toluidine blue stain, original magnification x40 (Courtesy of Dr. Roberto Crespi).

DISCUSSION

Cystectomy includes the removal of all inflamed soft tissues and sometimes application of different biomaterials to enhance new bone formation in the defect site [34,35]. Various bone grafts and barrier membranes can be used to achieve optimal healing and regeneration of the cystic cavity. All these approaches are known as regenerative therapies. Recently it has been developed a procedure for bony defects regeneration utilizes PRP

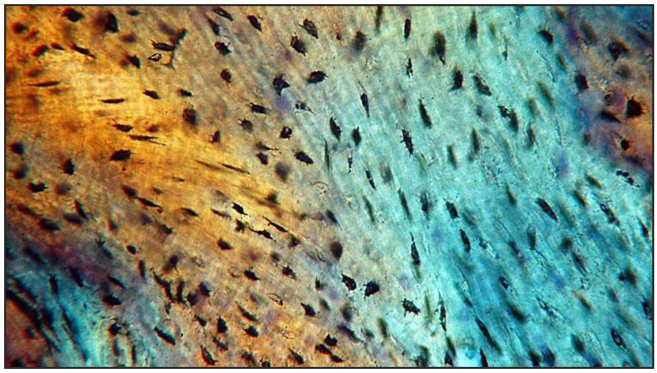

Figure 7. Histological view of lines of osteocytes. Toluidine blue stain, original magnification x120 (Courtesy of Dr. Roberto Crespi).

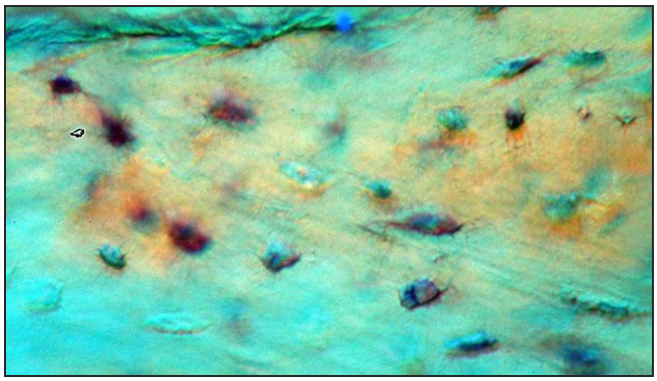

Figure 9. At high magnification it is possible to observe osteoblasts surrounding particles of graft material. Toluidine blue stain, original magnification x160 (Courtesy of Dr. Roberto Crespi).

in addition to bone grafts, and several studies have showed that a combination of PRP with bone grafts promote bone regeneration [36-42]. However, other authors still argue the lack of scientific evidence for defending the use of PRP associated with bone grafts in bone regeneration and recommend the surgeon to maintain a critical mind regarding its efficacy [43,44]. In our opinion, the controversy found in the literature regarding the use of this technique could be probably related to a lack of standardization in the different PRP formulations, and in the protocols, experimental models and surgical techniques employed.

Cystic cavity regeneration is a complex process involving both tissue repair and regeneration. The cellular events responsible for healing are controlled and regulated by specific signalling molecules, growth factors, and cytokines. TGF-b1, Bone morphogenetic protein-2 (BMP-2), and PDGF-A are secreted by cells recruited to the healing wound which are released in response to wounding stimuli detected at the cell surface [45,46]. The local availability of these growth factors is enhanced by about threefold or greater in concentration by addition of autologous PRP. It has been also reported that during the early stages of wound healing PRP has a strong stimulant effect on capillary regeneration [33]. Particularly, the publications, which point out positive features of PRP over the last years, have stressed:

1. Importance of controlled release systems of growth and differentiation factors using biomaterials in combination with PRP [47];
2. Enhancement of osteogenesis and angiogenesis [48];
3. Inhibition of osteoclast activation [49];
4. The enhancement of bone density adding PRP to a suboptimal dose of recombinant human BMP-2 (rhBMP-2) [30];
5. A significant increase of early bone marrow stromal cells (BMSCs) proliferation and differentiation using the combination of rhBMP-2 and bFGB (one of the signalling molecules of PRP) [50];
6. Relevance of PDGF and transforming growth factors (TGF-al and TGF32) for bone regeneration [51].

Our study used these principles for enhancing the osteoconductive property of a new highly purified bovine allograft (Laddec®) by addition of autologous PRP in regeneration of osseous defects of jaws caused by cystectomy. Radiographic assessments of present study indicated that this association induced a fast new bone growth in the cystic cavities. It was observed that the defect was filled by 56% at the first month, and after a time interval of 6 months postoperatively the defect was filled by 92%, showing a significant increase in vertical height on radiographs. The clinical efficacy of the association PRP and Laddec® reported from our study is also supported by histological data that we have documented, since the mean volume of vital bone at 6 months was found to be 68 ± 1.6% and the mean percentage of vital bone 48 ± 2.4%. These data are in accordance with and in support of what has been previously suggested by others studies that have shown that adding PRP to graft material significantly accelerates the rate of bone formation and improves trabecular bone density as compared to sites treated with only graft material [25,52]. Contrary to this, there are also reports that suggest that the use of PRP in combination with anorganic bovine bone mineral does not benefit bone regeneration [53-60], highlighted that osteoblasts have difficulties in adhering to allograft smooth surfaces. Since some allograft has a smooth surface [61,62], most attempts of creating new bone using bovine allografts may not be able to provide close contact between bone and the bovine material under reproducible conditions. Baslè and co-workers [31] suggested that treatments applied to bovine allografts to prevent immunological, inflammatory, bacteriological or virological adverse responses, may also interact with type I collagen, to which can bind osteoblasts via specific cell surface receptors, the integrins. The authors have compared *in vitro* two different bovine allografts that displayed similar architectural organization with connected plates and rods and similar surface topography and roughness. They differed by the presence or not of collagen type I. The first one was characterized by preservation of the type I collagen matrix associated with spindle-shaped hydroxypatite crystals and the second was solely composed by heat-modified apatite crystals. Osteoblast-like cells (Saos-2) were cultured on both biomaterials and examined in scanning and transmission electron microscopy after 7 and 14 days. Both biomaterials were cytocompatible as demonstrated by good ultrastructural cell preservation. At the surface of the collagen containing biomaterial, cells were elongated in shape and oriented according to the trabecular architecture and to the superficial collagen network. After 14 days of culture, cells were confluent and the biomaterial surface was hidden by the cell sheet. The beta 1 integrin subunit was detected by immunogold in transmission electron microscopy in close relationship with the superficial collagen fibbers of the biomaterial and with the outer cell surface. When cultures were carried out in presence of anti beta 1 integrin subunit, cells were packed and piled up with lack of specific orientation. At the surface of the deproteinized biomaterial, cells were globular without specific disposition and often partially attached to the surface. After 14 days of culture, large areas of the biomaterial surface remained uncovered. Anti beta 1 subunits conjugated with gold particles

were detected around the cells but with no specific association with the deproteinized biomaterial. These results strongly suggest that the chemical nature of the surface of bovine allografts directly influences adhesion process, shape, and spatial organization of cultured osteoblastic cells. Furthermore, the presence of type I collagen fibbers in the matrix seems to be of major interest to determine cell attachment, spreading and orientation via interaction between type I collagen and beta 1 integrin subunit of osteoblasts. In contrast, at the surface of the single mineral matrix, cells were round shaped with random disposition. This data has been also confirmed by another research [33] in which has been documented that the beta 1-integrin subunit was localized at the outer surface of cells, in close association with collagen and at the contact points between cells and Laddec® allograft. These *in vitro* results are still limited and must be confirmed by other studies, but they could support favourable outcomes concerning newly grown bone achieved in our present clinical study performed using a combination of PRP and Laddec®. We have to add that it is not possible to compare the published clinical studies and animal trials concerning the association between PRP and heterologous grafts, due to the varying methodologies applied and due to the varying nature and biological features of commercially available bovine graft materials. The results of our clinical and histological study seems however to confirm that preservation of the type I collagen matrix associated with spindle-shaped hydroxaypatite crystals in bovine graft bone substitutes may promote the biomaterial-PRP interaction. Within the limits of this study, the treatment with a combination of PRP and Laddec® bovine graft seems to lead to significantly favourable and fast bone regeneration after grafting enucleated mandibular cyst cavities, however further studies are necessary to assess the long-term effectiveness of PRP-Laddec® association, and a larger sample size is recommended.

CONCLUSIONS

The results of this study showed that Laddec® in association with platelet rich plasma has a potential for routine clinical use for regeneration of cystic bony defects.

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