Histomorphometric Analysis of Rabbit Calvarial Bone: Storage in Saline Solution Versus Storage in Platelet-Poor Plasma

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Purpose: *In reconstructive procedures harvested bone grafts are often temporarily stored in extraoral media while the recipient site is prepared. The aim of the present study was to analyze histomorphometrically osteocytes in calvarial bone grafts stored in either physiologic saline solution or platelet-poor plasma (PPP).* **Materials and Methods:** *Calvarial bone fragments were obtained from 12 rabbits and fixed immediately in formalin (control) or stored in PPP or in saline solution for 30 minutes prior to fixation. All specimens were decalcified and embedded in glycol methacrylate. A differential osteocyte count (normal osteocytes, abnormal osteocytes, and empty lacunae) was performed for the sections and submitted to analysis of variance (ANOVA) and the Fisher least-square-difference (LSD) test.* $P < .05$ *was considered significant.* **Results:** *The histomorphometric analysis demonstrated statistically significant differences among the groups (control, PPP, saline) for all analyzed parameters ($P < .05$). The median number of normal osteocytes observed was 31.8 ± 2.3 for the control group, 29.7 ± 4.2 for the PPP group, and 19.1 ± 4.6 for the saline group. The median number of abnormal osteocytes observed was 14.7 ± 3.9 for the saline group, 8.7 ± 2.3 for the PPP group, and 7.0 ± 2.3 for the control group. The median number of empty lacunae observed was 12.9 ± 4.7 for the saline group, 8.5 ± 2.4 for the PPP group, and 6.9 ± 2.3 for the control group.* **Conclusion:** *The current study has shown that PPP is a better storage medium for osteocyte preservation than physiologic saline solution. The best results were observed in the control group.* (More than 50 references.) INT J ORAL MAXILLOFAC IMPLANTS 2007;22:905–910

Key words: *bone graft, osteocyte, platelet-poor plasma, platelet-rich plasma, rabbit*

Bone grafts are used in several maxillofacial procedures, including sinus floor augmentation,[1] horizontal and vertical alveolar ridge augmentation,[2] alveolar cleft reconstruction,[3] and correction of facial deformities.[4,5] Allogeneic demineralized bone, frozen bone, and freeze-dried bone have also been used for reconstruction.[6] Nevertheless, the results are not always predictable because the precise cellular mechanism underlying the grafted bone remodeling process is not fully understood.[7] Autogenous bone grafts are more predictable because of their osteoconductive and osteoinductive properties and thus are frequently used.[7–10]

Viable graft cells are responsible for the reactive and adaptative changes that cause rapid bone healing and subsequent remodeling.[9,11–14] However, osteocytes and osteoblasts under a prolonged period of ischemia may fail to participate in bone remodeling.[14,15] This is more critical when using cranial bone, as it is not possible to work simultaneously in the recipient and donor sites. Usually, preparation of the recipient sites begins only after the donor site has been surgically closed, which increases the storage time of the bone graft.

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Graft storage should be minimized to reduce ischemic conditions while the recipient site is prepared. Dechichi et al[16] described a decrease in the amount of normal osteocytes in bone grafts stored in physiologic saline solution over time. The authors suggested the need to evaluate alternative storage media to preserve these bone grafts during surgical interventions.

It has been postulated that the use of growth factors[17–19] in association with autogenous bone grafts improves bone regeneration. The addition of platelet-rich plasma (PRP), an autologous concentrate of blood platelets, to a graft site offers improved quality and faster healing for both hard and soft tissues.[19–21]

PRP is derived from whole blood through a process of gradient density centrifugation.[17,18,21–24] After a double blood centrifugation, 2 separate phases are obtained: PRP at the lowest level of the tube and platelet-poor plasma (PPP) at the top.[23] PPP has a lower concentration of platelets and growth factors than PRP.[24–26] PPP stimulates cell proliferation in culture media[25,26]; however, it is not generally used in regenerative procedures. In procedures that use PRP, the PPP might be an alternative storage media for the bone graft.

The aim of the present study was to perform osteocyte histomorphometric analysis of rabbit calvaria bone grafts kept in either physiologic saline solution or PPP.

MATERIALS AND METHODS

Twelve New Zealand adult male rabbits were used in this study. General anesthesia was induced by intramuscular injection of ketamine (10 mg/kg of body weight), 2% xylazine (4 mg/kg), 0.2% acepromazine (0.15 mg/kg),[27] and midazolam (0.2 mg/kg). The study was performed in agreement with the rules of the Brazilian College of Animal Experiments (COEBA).

Protocol to Obtain PPP
Ten milliliters of blood was collected from each animal in two 5-mL Vacutainer tubes (Beckton Dickinson, Franklin Lakes, NJ) containing 0.5 mL of 3.8% sodium citrate as an anticoagulant.[28] A medial auricular vein puncture was made using a 21-G gauge needle and a cannon that adapted the gauge to the tube. The blood was collected up to 4 hours prior to the surgical procedure. A protocol proposed by Sonnleitner et al[29] was used to obtain the PRP and PPP. The blood samples were processed by 2 centrifugations. The first one required 20 minutes centrifugation at 160g, after which all the plasma above a line 6 mm below the "mist" was separated and submitted to a second cen-

trifugation of 15 minutes at 400g. The precipitate formed in the tube by this second centrifugation was the PRP used for another experiment and the superficial part was the PPP used in this study.

Surgical Procedure in Rabbit Calvaria and Experimental Design
The surgical protocol was adapted from that described by Alberius et al.[30] A midline incision from the frontal area to the occipital protuberance was made down to the osseous surface of the skull, and a full-thickness flap was raised to expose the calvarial surface on both sides of the midline. Thereafter, 3 standardized bone fragments were obtained using a trephine with an inner diameter of 5 mm mounted on a handpiece at 2,000 rpm under abundant saline solution irrigation. The fragments consisted of both the outer and inner calvaria cortical bone, which was approximately 3 mm thick with a diameter of 5 mm. From each rabbit 1 fragment was retrieved and immediately fixed in a 10% neutral buffered formalin (control group). The other two fragments were stored in either PPP (PPP group) or physiologic saline solution (saline group) at room temperature for 30 minutes and then immersed in the same fixative solution. All fragments were decalcified in 10% EDTA for 30 days and embedded in glycol methacrylate (Historesin; Leica, Heidelberg, Germany). One hundred representative nonconsecutive sections 3 μm thick were cut from each specimen, and 10 histologic fields were randomly selected. The peripheries of the fields were excluded from the analyses, and counting proceeded from the center of each field.

Histomorphometric Analysis
For each of the 3 groups (control, PPP, and saline) a differential osteocyte count (normal osteocyte, abnormal osteocyte, empty lacunae) was performed for each section. The osteocytes were classified in agreement with morphological criteria established by Dechichi et al[16]: those that occupied more than 50% of their lacunae were considered normal, and those that occupied 50% or less of their lacunae were considered abnormal. Empty lacunae were also counted (Fig 1).

The sections were analyzed by a blinded examiner under a light microscope using a 40× objective. There were 10 fields per fragment, for a total of 120 fields per group, with 1,600 objects counted for each bone treatment.

The results obtained from the histomorphometric analysis were submitted to analysis of variance (ANOVA), and the Fisher least-square-difference (LSD) test was applied to compare the treatments for each parameter. Differences were considered statistically significant if P was less than .05.
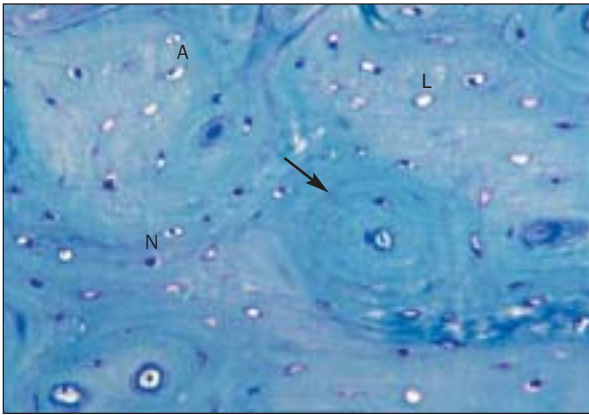
Fig 1 Rabbit calvarial bone tissue shown to illustrate the criteria used to classify osteocytes. An osteon is indicated by the arrow. A normal osteocyte (N), an abnormal osteocyte (A), and an empty lacuna (L) can also be seen.
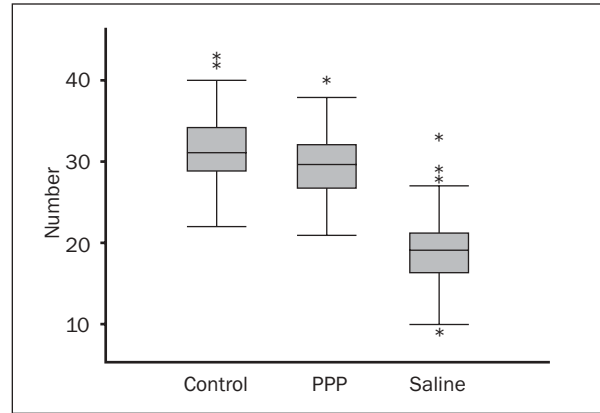

Fig 2 The number of normal osteocytes in the control, PPP, and saline groups. Midline indicates the median; the upper and lower edges of the box indicate the interquartile range; the asterisks indicate outliers. *P* < .05 for all comparisons.
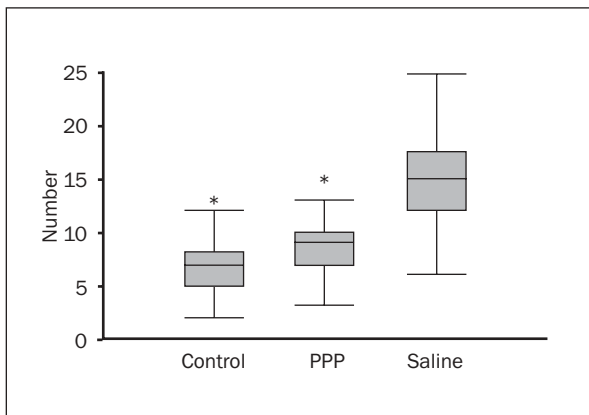

Fig 3 The number of abnormal osteocytes in the control, PPP, and saline groups. *P* < .05 for all comparisons.
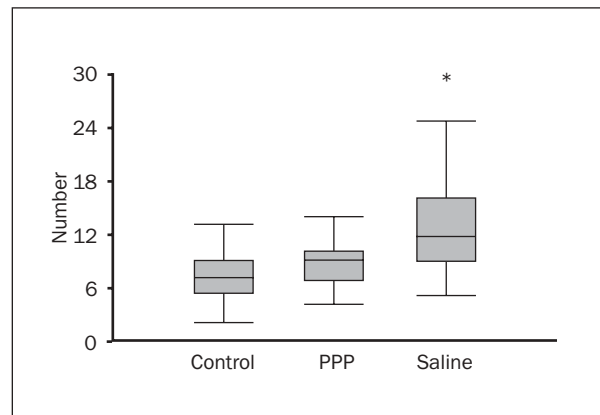

Fig 4 The number of empty lacunae in the control, PPP, and saline groups. *P* < .05 for all comparisons.

RESULTS

The histomorphometric analysis demonstrated significant differences among the groups for all analyzed parameters (*P* < .05). The median number of normal osteocytes (Fig 2) was greatest in the control (31.8 ± 2.3), followed by PPP (29.7 ± 4.2) and saline group (19.1 ± 4.6; *P* < .05 for all comparisons). The median number of abnormal osteocytes (Fig 3) was greater in the PPP (8.7 ± 2.3) and saline (14.7 ± 3.9) groups compared to the control group (7.0 ± 2.3; *P* < .05 for all comparisons). Specimens stored in saline solution were found to have a significantly higher number of abnormal osteocytes compared to those stored in PPP (*P* < .05). The number of empty lacunae (Fig 4) was greatest in the saline group (12.9 ± 4.7), followed by the PPP (8.5 ± 2.4) and control groups (6.9 ± 2.3; *P* < .05 for all comparisons).

DISCUSSION

The rabbit model was used for this study because the bone repair process of rabbits is physiologically similar to that of humans.[31,32] Furthermore, rabbits are useful models for preparation of platelet concentrates because they have a sufficient volume of blood.[22]

The use of cranial bone for grafting purposes is common. This donor site has some important advantages, such as the absence of a visible scar, short hospitalization, and a higher amount of surviving bone graft,[33] which has been attributed to the embryologic, morphologic, and physiologic similarity of this bone to host bone in the maxillofacial region.[31,32] Additionally, cranial bone harvesting is a safe procedure,[34] with a low morbidity compared to iliac crest bone harvesting.[33]

In the current study, bone blocks were obtained carefully to avoid overheating. Moreover, the storage media (PPP and saline solution) were kept at the same room temperature in order to reduce the number of variables. During histologic prepare of bone fragments, the tissue undergoes changes that can induce alterations in the cell morphology. According to Marotti et al,[35] the delayed fixation of the bone does not significantly interfere in the osteocyte preservation. The use of EDTA brings about slow bone tissue decalcification, which reduces the number of artifacts. Moreover, the temperature of the processing method (glycol methacrylate) decreases inhomogeneous distortion of the osteocytes inside their lacunae.[36] Therefore, the histological method used allows morphological analysis of the cells.[16]

Osteocyte viability can be assessed in conventional decalcified specimens by experienced histologists. This is the most frequently used method to confirm the clinical diagnosis of osteonecrosis, which is characterized by empty lacunae and osteocytes with pyknotic nuclei.[37] Although the remains of an apoptotic osteocyte can persist for a long time, they are easily dislodged during section preparation. In this case, an apparently empty lacuna is a reliable sign of osteocyte death.[38] Abnormal osteocytes are recognizable under conventional light microscope by their contracted aspect and the presence of a pyknotic nucleus. Thus, the morphologic criteria used in this study were relevant as an analysis method.

It has long been recognized that donor cells participate in osteogenesis at the host site.[33] Fresh grafts seem to revascularize earlier due to the presence of surviving cells.[9,11,12,15,39] According to Stevenson[8] and Kruyt et al,[40] the presence of a potentially osteoinductive extracellular matrix is insufficient to achieve bone formation; the presence of living cells is required. Thus, lack of osteocytes or the presence of abnormal osteocytes could disturb bone remodeling.[38,41] The absence of osteocytes at graft probably does not interfere directly with the osteoconductor potential of the graft, but it compromises osteoinduction[7] and increases the resorption of graft.[7,14,41] Osteocytes were analyzed in the current study because of their importance to the mechanisms described and to bone metabolism.[41–44]

Little has been published about the effects of storage media on bone grafts.[11,13,16,45,46] A favorable bone graft storage media should be able to preserve as many osteocytes as possible. Saline solution is often used to store bone grafts in clinical surgical procedures, and osteocytes maintained in such solutions have shown morphologic changes.[16] In the current study, PPP demonstrated better results as storage solution than saline solution. This difference could be the result of the presence of growth factors in PPP, which might favor the maintenance of the cell balance.[47]

PPP contains the same growth factors found in PRP at lower concentrations.[26] These growth factors induce biologic changes in cultures of osteoblasts,[19,25] fibroblasts,[25,48] and chondrocytes.[47] Transforming growth factor-β (TGF-β), one of the growth factors found in PPP,[26] regulates cell proliferation, differentiation, motility, and apoptosis.[49,50] This cytokine activates specific receptors on the osteocyte's membrane,[49] leading to biologic effects even at low concentrations.[50] The transient lack of nutrition during storage may lead to hypoxia of the osteocytes,[51] which might initiate a napoptotic signal transduction.[52] Growth factors could act on the production of cell survival factors regulated by anti-apoptotic genes,[49,50] which would probably increase cell survival time.

Further investigations into the effect of PRP and whole blood as conservation media of bone grafts should be undertaken, as well as the effect of these findings in the repair process of grafts. It is also necessary to evaluate the advantages of using these products exclusively as a storage media for grafts.

The creation of PPP does not involve new or complex techniques, since it is the most superficial part of the centrifuged blood obtained during the preparation of PRP. In cases in which PRP will be used to improve bone healing, the concomitant use of PPP could be an interesting alternative for bone graft storage.

CONCLUSION

The current study has shown that PPP is a better storage medium for osteocyte preservation than physiologic saline solution. However, the best results were observed in the control group.

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