Angiogenesis and Osteogenesis at Incorporation Process of Onlay Bone Graft

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Purpose: The roles of angiogenesis and osteogenesis in autologous and allogenic bone grafts and the use of platelet-rich plasma (PRP) as a modifier were investigated.

Materials and Methods: Forty rabbit mandibles received onlay grafts of fresh autologous and frozen allogeneic bone. PRP was added on the right side. After intervals of 3, 7, 14, 28, and 56 days, the animals were euthanized. Hematoxylin and eosin staining was used to measure the quantity and area of osteoblasts. Sections stained with toluidine blue showed newly formed bone area. In sections with Weigert-van Gieson staining, the number of vessels and their lumens was quantified. The quantity and area of cellular arrangements expressing CD31 and the area of vessels were obtained.

Results: Quantities of osteoblasts and their areas, newly formed matrices, and vessels and their lumen areas were obtained and identified by immunomarking with CD31. In general, values for these were higher in rabbits with allogeneic bone grafts and on the sides where PRP had been added. There was a variable significance between categories and days. It was confirmed that osteogenesis was intensified when angiogenesis was consolidated.

Conclusions: Angiogenesis was important for greater osteoblast differentiation and bone matrix synthesis, ensuring consolidation of onlay grafts with the receptor bed. Allogeneic grafts and PRP intensified these processes.

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Onlay grafting for increasing alveolar ridge height or width can be performed with autologous and allogeneic bone grafts. Although autologous grafts present no immunologic importance, the use of another surgical approach is necessary to harvest the bone graft. Therefore, in recent times, frozen allogeneic grafts have received increasing attention.[1–5]

Irrespective of the nature of the bone grafted, its incorporation into the maxilla and the maintenance of its volume require angiogenesis in the microenvironment of healing. Angiogenesis assures the presence and proliferation of preosteoblasts. After their differentiation into osteoblasts, these cells contribute to a more intense new bone formation around these grafts.[6–8]

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When considering these aspects, it is plausible to suggest that the addition of platelet-rich plasma (PRP), an autologous source of growth factors, to onlay grafts could accelerate their incorporation into the receptor bed.[9]

The aim of this study was to investigate the presence of osteoblasts and the intensity of bone matrix secretion by these cells, correlated with angiogenesis, when onlay bone grafts were placed in the rabbit mandible. This behavior was studied in allogeneic and autologous grafts associated with and without PRP.

Materials and Methods

SURGICAL PROCEDURE

The protocol of this research was approved by the ethics committee on animal care of the Bahia Foundation for Science Development (Salvador, Bahia, Brazil; protocol number 02/2006). Forty nonisogenic, adult, male, albino New Zealand rabbits weighing 2.5 to 3.0 kg were used in this study. Of these, 20 animals received fresh autologous grafts and 20 received allogeneic bone that had been frozen at −70°C for 120 days (Table 1).

The sample was empirically determined according to the bioethical principles of the 3Rs (replacement, refinement, and reduction). Animals were randomly selected to form the entire sample and groups of analysis. The same experienced surgeon performed all surgical procedures in all rabbits.

Surgical procedures were performed under sterile conditions. Before surgery, atropine (2 mg/kg) was administered. The animals were anesthetized with acepromazine (1 mg/kg) and ketamine (10 mg/kg). Enrofloxacin (10 mg/kg) was used for antibiotic prophylaxis. After the shaving and asepsis procedures, bupivacaine 0.5% with adrenalin (1:200,000) was infiltrated into the surgical bed. From the wing of the right ilium, a circular bicortical block of bone was harvested using a trephine burr. Then, the bone block was cut through the cancellous portion into 2 corticocancellous fragments.

Table 1. DIAGRAM OF STUDY GROUPS

Graft Nature	Day of Observation	PRP Used	Without PRP
Autologous (20 rabbits)	3 (4 rabbits)	right side	left side
	7 (4 rabbits)	right side	left side
	14 (4 rabbits)	right side	left side
	28 (4 rabbits)	right side	left side
	56 (4 rabbits)	right side	left side
Allogenous (20 rabbits)	3 (4 rabbits)	right side	left side
	7 (4 rabbits)	right side	left side
	14 (4 rabbits)	right side	left side
	28 (4 rabbits)	right side	left side
	56 (4 rabbits)	right side	left side

Abbreviation: PRP, platelet-rich plasma.

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The fragments were measured, onlay grafted on each side of the mandible, and fixed by the lag screw technique. The mandibular cortex in the surgical bed had been perforated previously. The cancellous portion of the graft was placed facing toward the perforation. On the right side, 500 μL of PRP was applied on the graft. On the left side, no adjuvant was added.

Each group was divided into 5 subgroups that differed by the interval at which they were euthanized after surgery (ie, days 3, 7, 14, 28, and 56).

TREATMENT OF GRAFTS FOR PLACEMENT IN RECEPTOR BEDS

During the same surgery, autologous grafts were harvested and then placed in the receptor bed. Bone fragments remained immersed in 0.9% saline solution during divulsion of mandibular soft tissues.

Allogeneic grafts were obtained from rabbits subjected to autologous grafting after general anesthesia and before excessive administration of general anesthetic and then euthanized. Bone fragments were frozen at −70°C for 120 days. Before being placed in the receptor bed, the fragments were washed with 100 mL of 0.9% saline solution and immersed in 40% gentamicin solution for 40 minutes.

OBTAINING PRP

Nine milliliters of whole blood was collected from each rabbit by cardiocentesis using a syringe containing 1.5 mL of ACD-a (JP Pharmaceutic, Sao Paulo, Brazil). The centrifugation protocols used were those proposed by Efeoglu et al[3] and Andrade et al.[10] Whole blood was centrifuged (Biofuge Stratos; Haereus Institute, Osterode, Germany) at a relative centrifugal acceleration of 300*g* for 10 minutes at a constant temperature of 22°C. Plasma was pipetted up to 1 mm from the interface of red cells and centrifuged at a relative centrifugal acceleration of 5,000*g* for 5 minutes at a temperature of 22°C. PRP coagulation was obtained by 15 μL of 10% calcium chloride to 500 μL of PRP.

HISTOLOGIC PROCESSING

After euthanasia, fragments of the mandible containing the graft and the soft tissue were removed. The material was fixed in 10% buffered formalin solution for 48 hours. The tissues were decalcified in 10%, ethylenediaminetetraacetic acid (1 mol/L; pH, 7.2). The material was processed and embedded in paraffin. Sections were obtained so that the soft tissue, the graft, and its interface with the receptor bed could be studied. Sections of 5 μm were stained with hematoxylin

and eosin, toluidine blue, and the Weigert-van Gieson (WvG) method.

Immunohistochemical study was conducted of 4-μm sections assembled on slides treated with 6% organosilane. Antigenic retrieval was obtained with Tris-ethylenediaminetetraacetic acid (pH, 9.0) for 30 minutes. Endogenous peroxidase was blocked with hydrogen peroxide, and a protein inhibitor (DAKO, Hamburg, Germany) prevented nonspecific reactions. Primary antibody anti-CD31 (or platelet endothelial cell adhesion molecule-1 [PECAM-1]; 1:100; clone JC/70A mouse/antihuman; DAKO) was incubated at 4°C for 12 hours. The secondary antibody was an anti-mouse antibody conjugated with peroxidase for 30 minutes at 30°C (Envision LLC, St Louis, MO). The color was achieved with diaminobenzidine (DAKO). Hematoxylin was used for counterstaining. As a positive control, sections of rabbit spleen fixed in 10% formol were used. As a negative control, the test tissue was used without primary antibody incubation.

HISTOLOGIC ANALYSIS

Histologic analysis was performed under a Motic B5 Professional Series microscope (Motic, Richmond, British Columbia. Canada). Digitalized images were quantified using Motic Image Advance 3.2 software. The examiner was blinded to the animal and group being quantified. The quantity and area occupied by the osteoblasts present at the periphery of the bone graft matrix were quantified in sections stained with hematoxylin and eosin. Toluidine blue staining was used to measure the area of newly formed bone, which was more intensely impregnated by the dye. The number of vessels and the area of their lumens were evaluated using the WvG method. In sections marked with anti-CD31 antibody, the quantity and area of cell arrangements expressing this antigen and the area of vessels were calculated.

STATISTICAL ANALYSIS

Data were analyzed by SPSS 19 (SPSS, Inc, Chicago, IL). The differences between the amount of graft and PRP received were weighed by the Student t test. The Tukey test was used to determine the statistical significance of the difference among the type of graft, PRP use, and the effect of time on the major variables. For the variables resulting from immunomarking by the anti-CD31 antibody, the Kruskal-Wallis statistical test was used, because the data were not normally distributed. Correlations that presented biological plausibility were obtained by the Pearson test; however, when variables from the anti-CD31 antibody were involved, the Spearman test was used. Linear regression was used to evaluate the best adjustment for the relation between the parametric variables using the r^2 parameter and 95% confidence interval. All levels of significance were considered at 5%. Data were described by comparisons between allogeneic and autologous bone grafts on each side of the mandible. The use of PRP was evaluated by the nature of each graft. The effect of time was analyzed by simultaneously stratifying the type of graft and PRP use.

Results

The grafts had a mean thickness of 0.177 ± 0.018 cm and width of 0.902 ± 0.018 cm. The rabbits were grafted with a similar amount of bone irrespective of the type of graft or side of the mandible ($P > .05$). The PRP platelet count was similar among animals (autologous, $2{,}171.95 \pm 973.79$; allogeneic, $2{,}519 \pm 855.25$; $P = .237$).

EVALUATION OF OSTEOBLASTS

The quantity and area occupied by the osteoblasts (Fig 1) were evaluated in sections with similar bone matrix values. The mean of this matrix area was 0.043 ± 0.009 mm^2 and there was no statistical difference ($P > .05$) between the different categories of all the groups studied.

QUANTITY OF OSTEOBLASTS

On day 3, the groups were homogeneous and there was no difference among them. On the other days, the quantity of osteoblasts was larger on the side with PRP or around the allogeneic graft ($P > .05$). The quantity of osteoblasts increased from day 3 to day 14 and then decreased until day 56. A statistically significant difference between graft types was observed only on day 14 on the right and left sides of the mandible ($P < .05$). On the right and left sides of the mandible and in animals with autologous graft, day 3 differed from all other days ($P < .05$). For the allogeneic grafts, day 14 differed significantly from day 7 ($P < .05$) and from the last 2 days ($P < .05$) on the right and left sides of the mandible (Table 2).

OSTEOBLAST AREA

Up to day 7, the groups were very similar ($P > .05$). In general, from day 14, there was an increase in the area of osteoblasts in the allogeneic grafts and on the side with PRP. A statistical difference between grafts appeared only on day 14 and on the side with PRP ($P < .05$; Table 2). In the autologous bone, the osteoblast area on day 3 differed statistically from that on days 7 and 14 on the side without PRP ($P < .05$) and from day 3 to day 14 and from day 14 to day 56 ($P < .05$) on the other side. In the allogeneic bone, day 3 differed significantly from all other days on the side with PRP ($P < .05$), but this occurred only from day 14 on

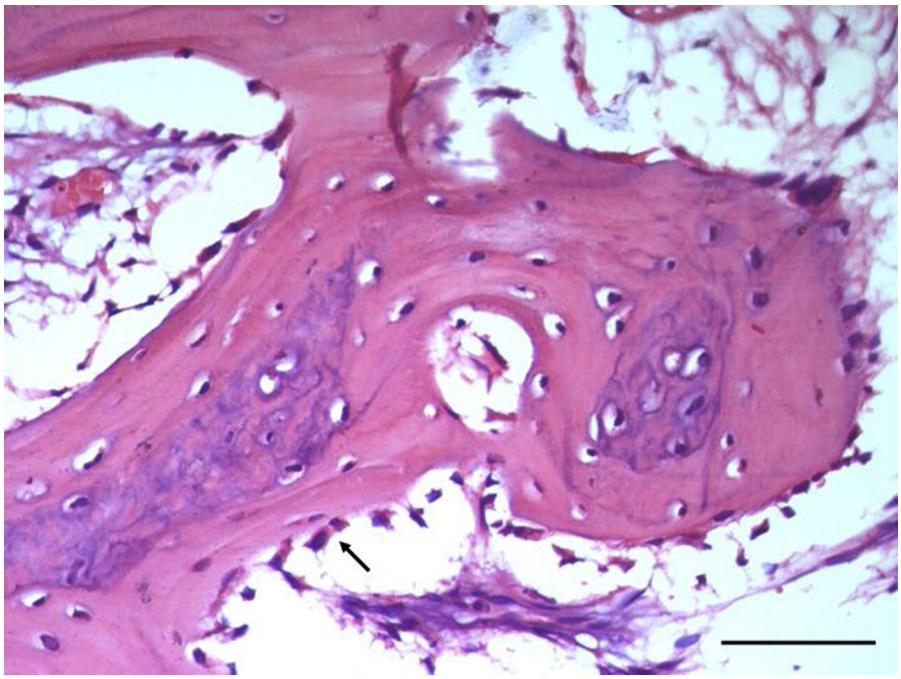

FIGURE 1. Active osteoblasts (*arrow*) on the periphery of the graft bone matrix. Allogeneic bone with platelet-rich plasma, day 28 (hematoxylin and eosin stain; magnification, ×400; scale bar, 0.1 mm).

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the side without PRP ($P < .05$). The peak of the osteoblast area verified on day 14 was different from the peak on the other days on both sides of the allogeneic graft ($P < .05$; Table 2).

EVALUATION OF OSTEOGENESIS

Newly formed area (Fig 2) also was measured in sections with similar quantities of bone matrix. The general mean of bone matrix present in all groups was 0.062 ± 0.012 mm^2 and the small difference found in each subgroup was not statistically significant ($P > .05$).

NEWLY FORMED BONE AREA

The side without PRP had a larger area of newly formed bone, except for days 14 and 56, independent of the type of graft. Autologous grafts were associated to a higher osteogenesis area when compared with allogeneic grafts until day 14. Afterward, they were very similar. The differences between groups were not statistically significant ($P > .05$; Table 3).

When the effect of time was evaluated, different grafts and different sides behaved in a similar manner. In autologous grafts, only the side with PRP showed a statistical difference on day 14 compared with all other days ($P < .05$). In allogeneic grafts, the side with PRP on day 3 presented a statistically significant difference compared with other days and on day 7 compared with day 14 ($P < .05$; Table 3).

ANALYSIS OF WVG STAIN

Allogeneic grafts (Fig 3) were found to have more vessels than autologous grafts, irrespective of the side of the mandible. This behavior was statistically significant only on days 3 and 7 on the side without PRP and on day 14 on the side with PRP ($P < .05$; Table 4).

The vessel lumen evaluated by this method was wider on the side with PRP in autologous bone. This pattern was very similar for the allogeneic graft. In general, the vessel lumens in the region of the allogeneic graft were larger compared with those in the autologous graft. The small differences observed between groups were not statistically significant ($P > .05$; Table 4).

The quantity of vessels and their lumens was constant during the different days of observation ($P > .05$). However, a small decrease in the quantity of vessels occurred from day 3 to day 14 in the allogeneic graft region on the side without PRP ($P < .05$; Table 4).

IMMUNOLABELING OF CD31/PECAM

Concerning the quantity of CD31$^+$ groups (Fig 4) in the graft microenvironment, several significant differences were observed for the type of graft and the use of PRP ($P < .05$). The quantity of CD31$^+$ groups throughout the study was very similar, with a slight trend toward a decrease on day 56. However, there was significant difference among days in each group ($P < .05$). The area occupied by CD31$^+$ cells presented

Table 2. QUANTITY OF OSTEOBLASTS AND AREA OCCUPIED BY THESE CELLS IN A MATRIX ACCORDING TO TYPE OF GRAFT, PRP ADDITION, AND PERIOD OF OBSERVATION

	Quantity of Osteoblasts/1,000				Osteoblast Area (mm^2) × 10			
	Autogenous Graft		Allogenous Graft		Autogenous Graft		Allogenous Graft	
Period	PRP	No PRP	PRP	No PRP	PRP	No PRP	PRP	No PRP
3 days	3.20 ± 2.00	2.90 ± 2.00	4.50 ± 2.00	3.40 ± 2.00	0.45 ± 0.12	0.33 ± 0.12	0.53 ± 0.12	0.41 ± 0.12
7 days	41.60 ± 6.00[†]	27.00 ± 6.00	49.25 ± 6.00[†]	22.95 ± 6.00	9.86 ± 1.53[†]	5.15 ± 1.53	10.34 ± 1.53[†]	5.27 ± 1.53
14 days	46.90 ± 6.00[*,†]	40.25 ± 6.00[*,†]	81.25 ± 6.00[*,†,‡]	72.75 ± 6.00[*,†,‡]	6.81 ± 1.53[*,†]	8.45 ± 1.53[†]	16.70 ± 1.53[*,‡]	12.11 ± 1.53[†,‡]
28 days	39.00 ± 6.00[†]	33.15 ± 6.00[†]	46.85 ± 6.00[†,§]	37.85 ± 6.00[†,§]	5.13 ± 1.53	4.26 ± 1.53	7.20 ± 1.53[†,§]	5.96 ± 1.53[§]
56 days	29.80 ± 6.00[†]	17.70 ± 6.00[†]	48.85 ± 6.00[†,§]	27.95 ± 6.00[†,§]	4.07 ± 1.53	2.34 ± 1.53[§]	9.70 ± 1.53[†,§]	5.11 ± 1.53[§]

Note: Data are presented as mean ± standard deviation.

Abbreviation: PRP, platelet-rich plasma.

* $P < .05$ by Tukey test for differences between graft types on each side.

† $P < .05$ by Tukey test for the difference between day 3 and other days for each side of the mandible and each graft.

‡ $P < .05$ by Tukey test for the difference between day 7 and other days for each side of the mandible and each graft.

§ $P < .05$ by Tukey test for the difference between day 14 and other days for each side of the mandible and each graft.

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a similar behavior with respect to the type of graft and the use of PRP ($P < .05$). When comparing the grafts on each side of the mandible, the allogeneic bone induced more CD31+ cell differentiation than the autologous bone on the side without PRP on day 3 ($P < .05$) and on the side with PRP on day 7 ($P < .05$). The effect of time on the type of graft and on each side of the mandible was important ($P < .05$; Table 5).

The endothelium identified by this immunomarker showed that PRP induced larger vessel lumens around the allogeneic grafts on days 7, 28, and 56 ($P < .05$). Conversely, around the autologous grafts, at the side without PRP, vessels lumem presented large area, but only on day 28 ($P < .05$). On the side with PRP, the allogeneic grafts induced larger vessels compared with the autologous grafts only on day 28 ($P < .05$). On the side without PRP, there was no difference in the type of graft ($P > .05$). With respect to time, vessel lumen areas increased until day 7 and then began to decrease. There was a significant difference among days only for the allogeneic bone and on the side with PRP ($P < .05$; Table 6).

CORRELATION BETWEEN VARIABLES

Only the quantity of osteoblasts showed a positive statistical correlation with the newly formed area ($P < .0001$). The area of osteoblasts showed a statistically significant positive correlation with the newly formed area ($P < .0001$) and with the area of $CD31^+$ arrangements ($P < .03$). The newly formed area showed a positive correlation with the quantity of vessels evaluated by the WvG method ($P < .01$) and the quantity of $CD31^+$ arrangements ($P < .013$). The other correlations were not significant. The only linear interaction observed between the variables occurred between the quantity of osteoblasts and the newly formed area ($r^2 = 0.059$). All other correlations analyzed exhibited very weak interactions between variables (<5%; Table 7).

Discussion

In a bone defect, angiogenesis is a necessary factor for osteogenesis to occur.[7,11–13] Because the literature lacks information with regard to the mechanism by which onlay grafts are incorporated into mandibles, this article makes a relevant contribution by analyzing this process and its association with angiogenesis. Angiogenesis is currently considered the most important research pathway toward a better understanding of osteogenesis.[6]

PRP is recognized as a healing stimulator, intensifying the proliferation of preosteoblasts and endothelium.[6,14,15] Allogeneic grafts have been highlighted in clinical practice because of their benefits associated with surgical advantages, in addition to greater resistance to absorption and greater osteogenic

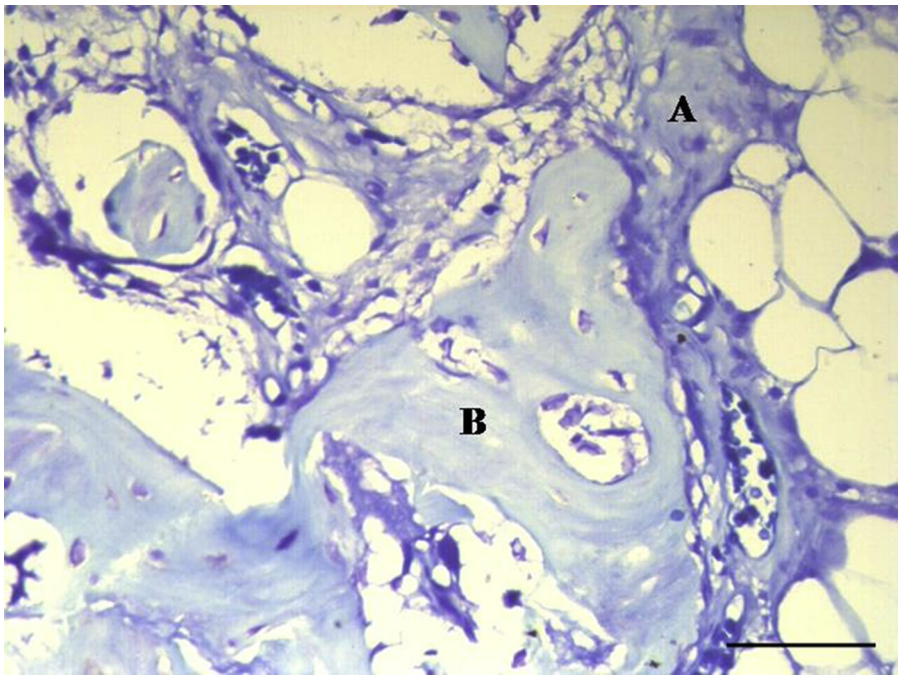

FIGURE 2. New bone formation (*A*) surrounding the graft matrix (*B*). Autologous graft with platelet-rich plasma, day 7 (toluidine blue stain; magnification, ×400; scale bar, 0.1 mm).

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potential.[16,17] Therefore, these 2 graft types and PRP were studied as factors that would modify post-surgical behavior.

In the present work, the number of osteoblasts was larger on the side where PRP was added to tissue. These cells were equally more exuberant in the region of the allogeneic graft. The growth in the osteoblast population from day 3 to day 14 is compatible with the chronology of bone repair.[18] From the earlier moment of inflammation, undifferentiated mesenchymal cells arrived at the tissue and came closer to the microenvironment of the onlay bone.[13] Once the tissue had an abundance of growth factors for bone deposition, differentiation of the pluripotent cells into preosteoblasts and osteoblasts was expected.[6,7,18,19]

Moreover, it is logical that the quantity of matrix secreted by these cells increased in the same proportion. When osteoprogenitor cells were in close contact with the graft, they were completely differentiated. Thus, they began to release bone matrix, which promoted

Table 3. AREA OF NEWLY FORMED MATRIX ACCORDING TO TYPE OF GRAFT, PRP ADDITION, AND PERIOD OF OBSERVATION

	Newly Formed Matrix (mm^2) × 100			
	Autogenous Graft		Allogenous Graft	
Period	PRP	No PRP	PRP	No PRP
3 days	1.73 ± 0.10	2.45 ± 0.12	0.85 ± 0.04	1.17 ± 0.09
7 days	2.68 ± 0.14	3.24 ± 0.16	2.00 ± 0.10	2.19 ± 0.14
14 days	4.01 ± 0.14*,†	3.14 ± 0.11	3.54 ± 0.15*,†	3.17 ± 0.10
28 days	2.40 ± 0.10‡	2.70 ± 0.10	2.63 ± 0.12*	2.76 ± 0.10
56 days	2.60 ± 0.14‡	2.44 ± 0.12	2.65 ± 0.09*	2.36 ± 0.11

Note: Data are presented as mean ± standard deviation.
Abbreviation: PRP, platelet-rich plasma.
* $P < .05$ by Tukey test for the difference between day 3 and other days for each side of the mandible and each graft.
† $P < .05$ by Tukey test for the difference between day 7 and other days for each side of the mandible and each graft.
‡ $P < .05$ by Tukey test for the difference between day 14 and other days for each side of the mandible and each graft.

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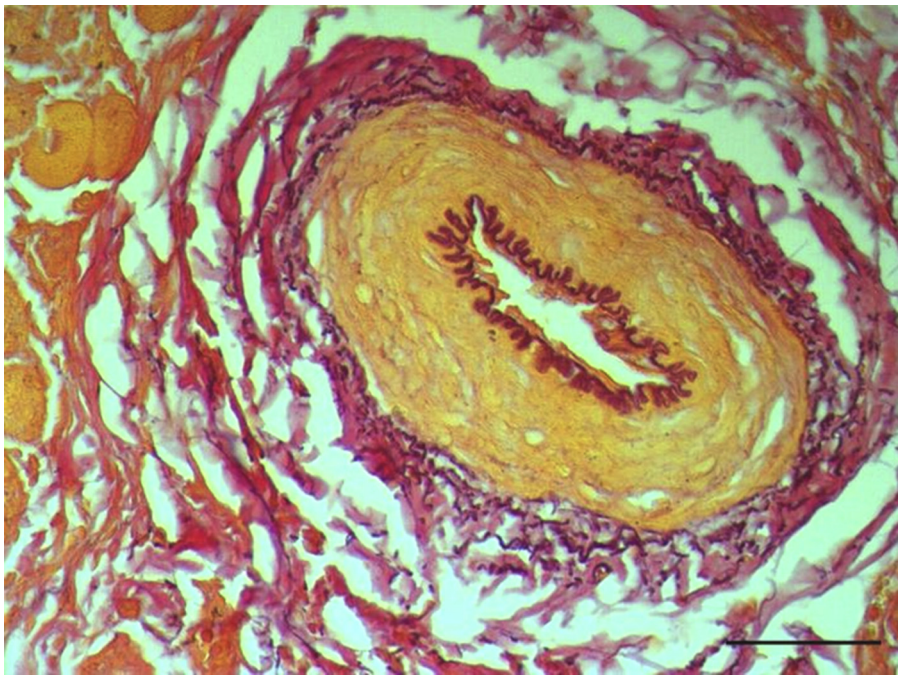

FIGURE 3. Vessel shown by Weigert-van Gieson staining. Allogeneic bone without platelet-rich plasma, day 3 (magnification, ×400; scale bar, 0.1 mm).

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consolidation of the graft with the mandibular cortex.[7] Hence, there was a progressively increasing quantity of newly formed matrix surrounding the onlay grafts, so that the correlation between the quantity of osteoblasts or their area and the newly formed matrix area was positive.

The most exuberant osteogenesis on day 14 was necessary to guarantee that the onlay graft would adhere to its receptor bed. On subsequent days, the need for osteogenesis decreased and it occurred basically to ensure the osteoconduction phenomenon. The resident osteoprogenitor cells were still active, so that on the last days of the experiment, they continued to release bone matrix, especially to replace the graft matrix. Thus, the remodeling phase was progressing and the graft that was placed at the time of surgery was modified to new bone.[7]

Some features are relevant when discussing new bone formation. On day 3, the cancellous portion of the graft was poorly organized and rare

Table 4. QUANTITY AND AREA OF VESSEL LUMENS MEASURED USING WEIGERT-VAN GIESON STAIN ACCORDING TO TYPE OF GRAFT, PRP ADDITION, AND PERIOD OF OBSERVATION

	Quantity of Vessels				Area of Vessel Lumens (mm^2) × 100			
	Autogenous Graft		Allogenous Graft		Autogenous Graft		Allogenous Graft	
Period	PRP	No PRP	PRP	No PRP	PRP	No PRP	PRP	No PRP
3 days	1.35 ± 0.58	1.20 ± 0.52*	2.10 ± 1.74	2.65 ± 1.53*	0.71 ± 0.50	0.38 ± 0.20	0.78 ± 0.50	0.43 ± 0.15
7 days	1.60 ± 1.31	1.30 ± 0.80*	1.80 ± 1.43	2.30 ± 1.34*	0.65 ± 0.35	0.57 ± 0.45	0.97 ± 0.50	0.62 ± 0.45
14 days	1.15 ± 0.36*	1.40 ± 0.82	1.80 ± 1.00*	1.40 ± 0.68	0.77 ± 0.45	0.60 ± 0.40	0.68 ± 0.30	0.74 ± 0.35*,†
28 days	1.75 ± 0.86	1.60 ± 0.68	2.80 ± 1.96	1.85 ± 1.08	0.49 ± 0.30	0.45 ± 0.15	0.58 ± 0.20	0.67 ± 0.25
56 days	1.75 ± 0.86	1.60 ± 1.09	2.00 ± 1.65	1.70 ± 0.97	0.76 ± 0.55	0.72 ± 0.35	0.56 ± 0.25	0.38 ± 0.01

Note: Data are presented as mean ± standard deviation.

Abbreviation: PRP, platelet-rich plasma.

* $P < .05$ by Tukey test for differences between graft types on each side.

† $P < .05$ for difference between day 3 and other days for each side of the mandible and each graft.

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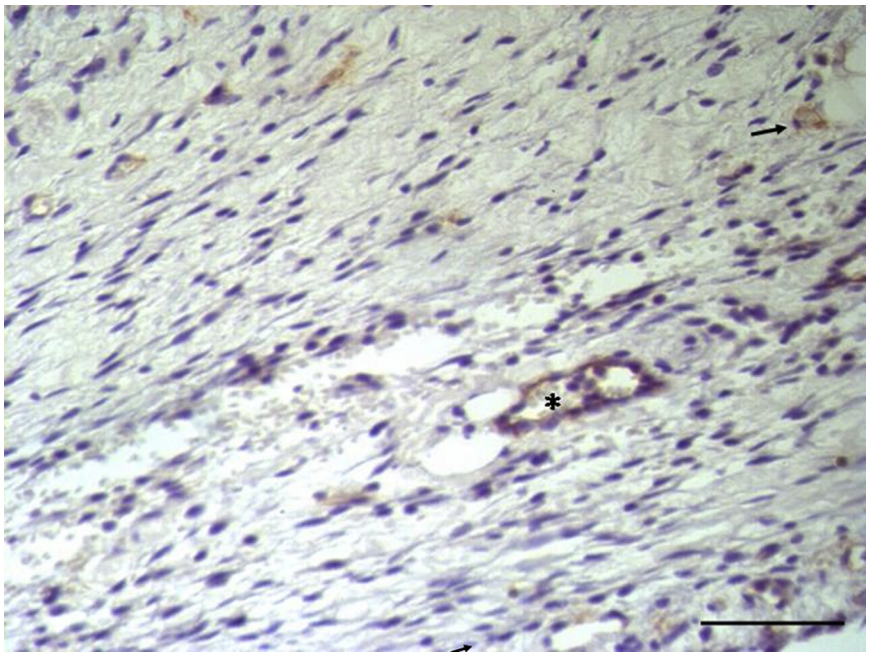

FIGURE 4. Immunomarking for CD31 showing vessels (*asterisk*) and capillary sprouts (*arrows*). Autologous bone without platelet-rich plasma, day 3 (magnification, ×400; scale bar, 0.1 mm).

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osteoblasts were shown in the graft, which were probably derived from the donor region. Even with a small population of osteoblasts, there was a matrix that was more intensely stained by toluidine blue on day 3. This matrix probably had been secreted by native osteoblasts from the ilium before graft removal. The smaller quantity of matrix found for the allogeneic grafts is compatible with this analysis, because freezing, to which they had been subjected, decreased the quality of collagen, possibly altering their behavior toward in response to the dye.[10] From day 7, PRP promoted more extensive or greater intercellular contact between the graft and the preosteoblasts, an essential event for their proliferation and differentiation into osteoblasts and matrix deposition.[6]

The use of PRP with onlay grafts seemed to have inhibited angiogenesis during the initial days. Some theories have defended the concept that a growth factor rate that exceeds the physiologic rate, as occurs with PRP, can act in an inhibitory manner.[20] However, the analysis of the other variables showed that there was no compromise of bone consolidation in the receptor bed.

Table 5. QUANTITY AND AREA OF CD31$^+$ GROUPING ACCORDING TO TYPE OF GRAFT, PRP ADDITION, AND PERIOD OF OBSERVATION

	Quantity of CD31$^+$ Grouping				Area of CD31$^+$ Grouping (mm^2)			
	Autogenous Graft		Allogenous Graft		Autogenous Graft		Allogenous Graft	
Period	PRP*	No PRP*	PRP	No PRP*	PRP*	No PRP*	PRP*	No PRP*
3 days	4.60 ± 2.20‡	6.30 ± 5.16‡	2.80 ± 1.28†,‡	10.65 ± 5.09†,‡	0.69 ± 0.46	0.69 ± 0.42‡	0.54 ± 0.23†	1.70 ± 0.83†,‡
7 days	2.80 ± 1.60	5.45 ± 4.95	5.10 ± 3.69	4.15 ± 5.67	0.54 ± 0.49†,‡	1.56 ± 0.95†	1.20 ± 0.58‡	1.50 ± 0.80
14 days	4.70 ± 4.60	3.70 ± 2.22	3.95 ± 2.28	4.65 ± 6.34	0.56 ± 0.47	0.57 ± 0.34	0.10 ± 0.35	0.54 ± 0.55
28 days	4.00 ± 1.94	3.15 ± 1.81	2.95 ± 1.46	3.70 ± 2.47	0.53 ± 0.43	0.76 ± 0.42	0.62 ± 0.50	0.80 ± 0.42
56 days	2.05 ± 1.09‡	1.45 ± 0.60	3.70 ± 1.59†,‡	1.15 ± 0.36†	0.23 ± 0.12	0.39 ± 0.13	0.17 ± 0.11†	0.34 ± 0.14†

Note: Data are presented as mean ± standard deviation.
Abbreviation: PRP, platelet-rich plasma.
* $P < .05$ by Kruskal-Wallis test for differences among days.
† $P < .05$ by Kruskal-Wallis test for PRP use versus no PRP use for each graft.
‡ $P < .05$ for differences between graft types for each side.

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Table 6. AREA OF VESSELS IMMUNOMARKED BY CD31 ACCORDING TO TYPE OF GRAFT, PRP ADDITION, AND PERIOD OF OBSERVATION

	Area of Vessels (mm^2) × 1,000			
	Autogenous Graft		Allogenous Graft	
Period	PRP*	No PRP	PRP	No PRP
3 days	2.00 ± 0.40	2.00 ± 0.20	4.00 ± 0.60	3.00 ± 0.60
7 days	6.00 ± 0.80	3.00 ± 0.20	9.00 ± 1.40†	1.00 ± 0.10†
14 days	3.00 ± 0.40	4.00 ± 0.80	3.00 ± 0.40	1.00 ± 0.10
28 days	1.00 ± 0.10†,‡	3.00 ± 0.40†	4.00 ± 0.40†,‡	3.00 ± 0.70†
56 days	1.00 ± 0.60	4.00 ± 0.90	3.00 ± 0.30†	0.90 ± 0.09†

Note: Data are presented as mean ± standard deviation.
Abbreviation: PRP, platelet-rich plasma.
* $P < .05$ by Kruskal-Wallis test for differences among days.
† $P < .05$ by Kruskal-Wallis test for PRP use versus no PRP use for each graft.
‡ $P < .05$ by Kruskal-Wallis test for differences between graft types for each side.

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Although the processes are interdependent,[13] various investigators have established that angiogenesis chronologically precedes osteogenesis.[21–23] Vessels carry essential elements to the tissues under repair for later synthesis of the bone matrix. The present results corroborate the biological suppositions established by this relation. The quantity of the $CD31^+$ groups presented a peak on day 3, whereas their area showed a significantly relevant value on day 7. It is possible to infer that the proliferation of endothelial cells in each group continued beyond day 3, increasing their area on day 7.[19]

The relevance of this finding within the context of onlay bone graft repair was confirmed by the observation that osteogenesis was intensified precisely after the peak of angiogenesis had been reached.

Table 7. CORRELATION AND LINEAR REGRESSION BETWEEN VARIABLES

Variable 1	Variable 2	*r*	95% CI	*P* Value	r^2
Quantity of osteoblasts	newly formed area*	0.2429	0.1484 to 0.3331	.0001‡	0.059010
	quantity of vessels (WvG)*	−0.0350	−0.1327 to 0.0632	.4842	0.001230
	vessel lumen (WvG)*	−0.0022	−0.1003 to 0.0958	.9641	0.000048
	quantity of $CD31^+$ arrangement†	−0.0249	−0.1228 to 0.0732	.6182	—
	area of $CD31^+$ arrangement†	0.0041	−0.0573 to 0.1385	.4134	—
	area of vessels (CD31/PECAM)	0.0176	−0.1024 to 0.1372	.7674	—
Area of osteoblasts	newly formed area*	0.2142	0.1186 to 0.3058	.0001‡	0.045880
	quantity of vessels (WvG)*	0.0150	−0.0832 to 0.1129	.7649	0.000224
	vessel lumen (WvG)*	−0.0095	−0.1075 to 0.0886	.9641	0.000094
	quantity of $CD31^+$ arrangement†	−0.0021	−0.1002 to 0.09591	.9652	—
	area of $CD31^+$ arrangement†	0.1061	0.0080 to 0.2020	.0339‡	—
	area of vessels (CD31/PECAM)	0.0168	−0.1032 to 0.1364	.7772	—
Newly formed area	quantity of vessels (WvG)*	0.1253	0.0275 to 0.2206	.0122‡	0.015690
	vessel lumen (WvG)*	−0.0139	−0.1119 to 0.0842	.7808	0.000194
	quantity of $CD31^+$ arrangement†	0.1235	0.0257 to 0.2189	.0134‡	—
	area of $CD31^+$ arrangement†	−0.0204	−0.1183 to 0.0777	.6828	—
	area of vessels (CD31/PECAM)	−0.0207	−0.1472 to 0.0993	.7276	—

Abbreviations: CI, confidence interval; PECAM, platelet endothelial cell adhesion molecule; WvG, Weigert-van Gieson.
* Pearson test.
† Spearman test.
‡ $P < .05$ by linear regression.

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Similarly, when angiogenesis decreased, osteogenesis was not a preponderant event. Concerning the behavior of angiogenesis throughout the study, the decrease in intensity of marking for CD31 from day 14 is supported by Kleinheinz et al[8] who postulated that the increase of perfusion and oxygen rate results in the delamination and apoptosis of the endothelium.

The quantity of vessels and lumens quantified in sections stained by CD31 immunomarking was larger in rabbits grafted with allogeneic bone and on the sides where PRP had been added. Therefore, the superiority of the allogeneic graft could be justified not only by its intrinsic property of stimulating the chemotaxis of preosteoblasts, but also by the larger quantity of vessels where it was present.

The number of vessels depicted by WvG staining was uniform during the study period, which qualifies this staining as an inefficient method to evaluate new vascular formation. However, angiogenesis depends on the vascular feature of the tissue.[7,24] This process initiates from resident endothelial cells,[23] illustrating that the WvG stain can provide a basal pattern from which new vascular formation will occur. The pattern of tissue vascularization is essential to determine the correct distribution of angiogenesis in bone repair.[7,21]

Zengin et al[25] and Matsumoto et al[13] reported the existence of a cellular lineage between the tunica media and the tunica adventitia of adult vessels, which can behave as immature endothelial or osteoprogenitor cells. The increase in the quantity of $CD31^+$ cells could incite the increase of pericytes that supposedly contribute to the osteoblast population.[11] Rabie[18] determined that earlier vascularization of the graft increases the metabolism of osteoblasts. Failure of this process starts the activity of chondroblasts, osteoclasts, and the absorption of grafted tissue.

Based on the results of the present study, the authors concluded that osteogenesis and angiogenesis are intimately correlated and are crucial phenomena for the incorporation of the onlay graft into its receptor bed. The allogeneic graft used in onlay processes presented a greater osteogenic and angiogenic potential. PRP seemed to increase the osteogenesis of onlay grafts, but it did not present a linear behavior.

References

1. Aghaloo TL, Moy PK, Freymiller EG: Investigation of platelet-rich plasma in rabbit cranial defects: A pilot study. J Oral Maxillofac Surg 60:1176, 2002
2. Anitua E: Plasma rich in growth factors: Preliminary results of use in the preparation of future sites for implants. Int J Oral Maxillofac Implants 14:529, 1999
3. Efeoglu C, Akcay YD, Erturk S: A modified method for preparing platelet-rich plasma: An experimental study. J Oral Maxillofac Surg 62:1403, 2004
4. Hou CH, Yang RS, Hou SM: Hospital-based allogenic bone bank—10-Year experience. J Hosp Infect 59:41, 2005
5. Moreau MF, Gallois Y, Basle MF, et al: Gamma irradiation of human bone allografts alters medullary lipids and releases toxic compounds for osteoblast-like cells. Biomaterials 21:369, 2000
6. Arpornmaeklong P, Kochel M, Depprich R, et al: Influence of platelet-rich plasma (PRP) on osteogenic differentiation of rat bone marrow stromal cells. An in vitro study. Int J Oral Maxillofac Surg 33:60, 2004
7. Geris L, Gerisch A, Sloten JV, et al: Angiogenesis in bone fracture healing: A bioregulatory model. J Theor Biol 251:137, 2008
8. Kleinheinz J, Stratmann U, Joos U, et al: VEGF-activated angiogenesis during bone regeneration. J Oral Maxillofac Surg 63:1310, 2005
9. Wiltfang J, Kloss FR, Kessler P, et al: Effects of platelet-rich plasma on bone healing in combination with autogenous bone and bone substitutes in critical-size defects. An animal experiment. Clin Oral Implants Res 15:187, 2004
10. Andrade MG, Sa CN, Marchionni AM, et al: Effects of freezing on bone histological morphology. Cell Tissue Bank 9:279, 2008
11. Chang HN, Garetto LP, Potter RH, et al: Angiogenesis and osteogenesis in an orthopedically expanded suture. Am J Orthod Dentofacial Orthop 111:382, 1997
12. Hausman MR, Schaffler MB, Majeska RJ: Prevention of fracture healing in rats by an inhibitor of angiogenesis. Bone 29:560, 2001
13. Matsumoto T, Kuroda R, Mifune Y, et al: Circulating endothelial/skeletal progenitor cells for bone regeneration and healing. Bone 43:434, 2008
14. Kanno T, Takahashi T, Tsujisawa T, et al: Platelet-rich plasma enhances human osteoblast-like cell proliferation and differentiation. J Oral Maxillofac Surg 63:362, 2005
15. Marx RE, Carlson ER, Eichstaedt RM, et al: Platelet-rich plasma: Growth factor enhancement for bone grafts. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 85:638, 1998
16. Kluger R, Bouhon W, Freudenberger H, et al: Removal of the surface layers of human cortical bone allografts restores in vitro osteoclast function reduced by processing and frozen storage. Bone 32:291, 2003
17. Tshamala M, Cox E, De Cock H, et al: Antigenicity of cortical bone allografts in dogs and effect of ethylene oxide-sterilization. Vet Immunol Immunopathol 69:47, 1999
18. Rabie AB: Vascular endothelial growth pattern during demineralized bone matrix induced osteogenesis. Connect Tissue Res 36:337, 1997
19. Velazquez OC: Angiogenesis and vasculogenesis: Inducing the growth of new blood vessels and wound healing by stimulation of bone marrow-derived progenitor cell mobilization and homing. J Vasc Surg 45(suppl A):A39, 2007
20. Eppley BL, Woodell JE, Higgins J: Platelet quantification and growth factor analysis from platelet-rich plasma: Implications for wound healing. Plast Reconstr Surg 114:1502, 2004
21. Carano RA, Filvaroff EH: Angiogenesis and bone repair. Drug Discov Today 8:980, 2003
22. Pacicca DM, Patel N, Lee C, et al: Expression of angiogenic factors during distraction osteogenesis. Bone 33:889, 2003
23. Pezzatini S, Morbidelli L, Solito R, et al: Nanostructured HA crystals up-regulate FGF-2 expression and activity in microvascular endothelium promoting angiogenesis. Bone 41:523, 2007
24. Katsube K, Bishop AT, Simari RD, et al: Vascular endothelial growth factor (VEGF) gene transfer enhances surgical revascularization of necrotic bone. J Orthop Res 23:469, 2005
25. Zengin E, Chalajour F, Gehling UM, et al: Vascular wall resident progenitor cells: A source for postnatal vasculogenesis. Development 133:1543, 2006