Tomographic, Histological, and Immunohistochemical Evidences on the Use of N-Butyl-2-Cyanoacrilate for Onlay Graft Fixation in Rabbits

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ABSTRACT

Background: The bone tissue responses to Cyanoacrylate have been described in the literature, but none used N-butyl-2-cyanoacrilate (NB-Cn) for bone graft fixation.

Purpose: The aims of the study were: (a) to analyze the bone grafts volume maintenance fixed either with NB-Cn or titanium screw; (b) to assess the incorporation of onlay grafts on perforated recipient bed; and (c) the differences of expression level of tartrate-resistant acid phosphatase (TRAP) protein involved in bone resorption.

Materials and Methods: Eighteen New Zealand White rabbits were submitted to calvaria onlay grafting on both sides of the mandible. On one side, the graft was fixed with NB-Cn, while on the other hand the bone graft was secured with an osteosynthesis screw. The computed tomography (CT) was performed just after surgery and at animals sacrifice, after 1 ($n = 9$) and 6 weeks ($n = 9$), in order to estimate the bone grafts volume along the experiments. Histological sections of the grafted areas were prepared to evaluate the healing of bone grafts and to assess the expression of TRAP protein.

Results: The CT scan showed better volume maintenance of bone grafts fixed with NB-Cn ($p \leq 0.05$) compared with those fixed with screws, in both experimental times (analysis of variance). The immunohistochemical evaluation showed that the TRAP expression in a 6-week period was significantly higher compared with the 1-week period, without showing significant difference between the groups (Wilcoxon and Mann–Whitney). Histological analysis revealed that the NB-Cn caused periosteum damage, but provided bone graft stabilization and incorporation similar to the control group.

Conclusion: The perforation provided by screw insertion into the graft during fixation may have triggered early revascularization and remodeling to render increased volume loss compared with the experimental group. These results indicate that the NB-Cn possesses equivalent properties to titanium screw to be used as bone fixation material in osteosynthesis.

KEY WORDS: bone graft fixation, cyanocrylate, healing bone, histologic evaluation, immunohistochemistry, onlay bone graft, tomographic evaluation

INTRODUCTION

Onlay bone grafts have become a routine procedure when bone volume increases are necessary prior to the installation of dental implants.[1,2] After harvested, these grafts are fixed with screws so osteosynthesis can occur in the region to be reconstructed. However, the use of metallic osteosynthesis devices for the fixation of bone

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DOI 10.1111/j.1708-8208.2010.00321.x

grafts in atrophic edges brings many disadvantages and potential problems, such as artifacts on computed tomography (CT) scans and magnetic resonance imaging, corrosion, allergic reactions, infection, loosening of screws, heightened sensitivity for cold, among others.[3–9] An additional disadvantage with metal osteosynthesis is the necessity to remove due to interference with the planned implants. Recently, absorbable plates and screws made from a biodegradable copolymer of polylactic and polyglycolic acids have been developed to help prevent some of the problems associated with the use of metal plates and screws.[3,5–7] Although the use of absorbable fixation systems is an attractive alternative, the costs are still high, and plates and screws for this type of system often exhibit delicate and difficult handling as well as an unpredictable resorption process resulting in swelling and pain in the covering soft tissue. All these drawbacks have stimulated interest in finding alternate methods for rigid fixation of grafts in this type of surgery. Thus, it would be extremely advantageous to discover a new method of bone fixation that offered stability comparable to the use of metallic screws and absorbable devices, but without the drawbacks inherent to these materials.

Experimental studies in animal models have examined the response of bone tissue and stability in fracture sites fixed with Cyanoacrylate glue.[3,4,6,8,9] However, there are no studies evaluating the remodeling process of onlay grafts in the presence of Cyanoacrylate and neither in the fixation of these grafts promoted by the glue and its counterparts.

When used as a means of bone fixation, Cyanoacrylate adhesive has some potential advantages over the use of metallic and absorbable fixation devices. Among these advantages, one may cite its low cost, ease of use, speed of implementation, do not produce image artifacts in CT scan or magnetic resonance exams, and because they are bioabsorbable, they do not demand secondary surgical procedures to remove the fixation devices. There are several different forms of this compound, and, among them, the N-butyl-2-Cyanoacrylate (NB-Cn) has been approved for clinical use since 1996 and is becoming a popular method for closing skin wounds.[10] It is a polymeric adhesive belonging to the new generation of Cyanoacrylates, which are long-chain derivatives, and therefore more biocompatible than the first-used Cyanoacrylates.[11] Some experimental studies on bone tissue responses to Cyanoacrylate can be found in the literature, however, no evaluative histological response on the fixation of onlay bone grafts with NB-Cn has been carried out. The aim of the proposed study is to (a) compare the maintenance of bone graft volume when it is fixed to the mandible of rabbits using NB-Cn or titanium screws (asses through tomography); (b) histologically analyze the dynamics of the graft incorporation process to the recipient bone bed; and (c) evaluate graft-remodeling process through immunohistochemical methods.

MATERIALS AND METHODS

Eighteen adult male New Zealand White rabbits, weighing between 3.5 and 4.0 kg, were used for this research. The experimental protocol was approved by the local Ethics Committee (08.1.998.53.1).

Surgery

All 18 animals underwent cranial surgery for the extraction of bone grafts, and bilateral surgeries in the mandibles for their fixation. The animals were anesthetized using subcutaneous 1 mg/kg acepromazine, followed by an intramuscular injection of 5 mg/kg xilazine mixed with 25 mg/kg ketamine (União Química Farmacêutica Nacional S.A., Embuguaçú, São Paulo, Brazil). Afterwards, the area to be operated was infiltrated with a local anesthetic (mepivacaine 2% plus noradrenaline 1:200,000 – DFL, Indústria & Comércio S.A, Rio de Janeiro, Brazil). Following anesthesia, the animals received 0.2 mL/kg oxi-tetracycline as prophylactic antibiotic therapy.

Graft removal was preceded by a 4 cm linear skin incision along the cranial vault over the parietal region. The muscle, galea aponeurotica, and periosteum were incised in order to expose the skull, where an 8 mm trephine was used to retrieve two bicortical bone grafts from each side.

The buccal side of the mandibular body was used as the experimental site and it was bilaterally exposed extraorally through its base. Both sides were adequately perforated. On one side, the calvarial bone graft was fixed using one titanium screw (Synthest, Solothurn, Switzerland), measuring 1.5 mm in diameter by 8 mm in length, placed in an appositional fashion in the center of the graft (Group I – control). On the other side, graft fixation was done with NB-Cn and its center registered by a radiopaque marker (Group II) (Figure 1).

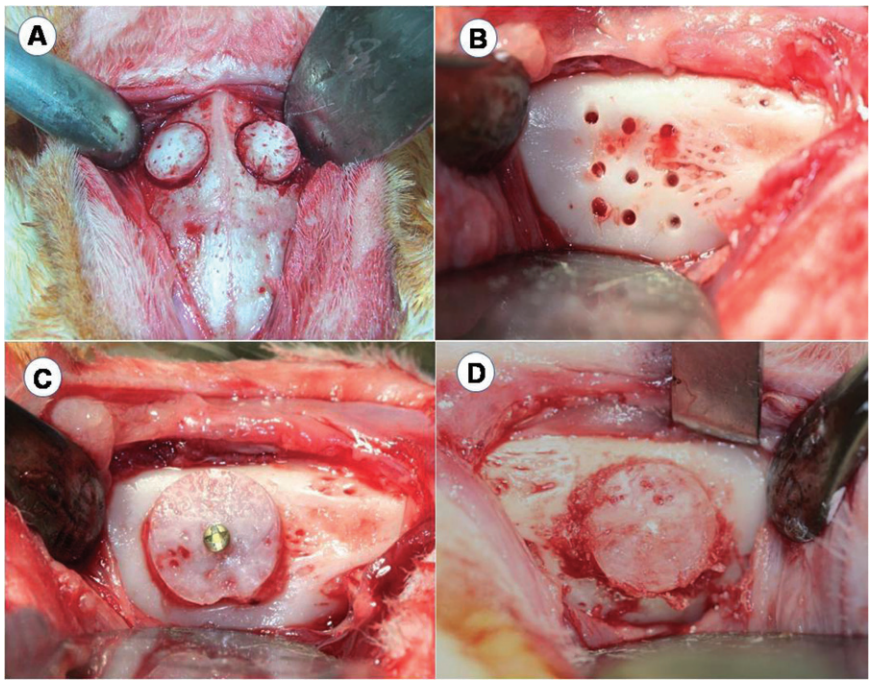


Figure 1 *A*, Two bi-cortical bone blocks measuring 8.0 mm in diameter being harvested. *B*, Mandibular recipient bed after the perforations had been finalized. Calvarial bone graft fixed in the mandible with titanium screw (C) and with NB-Cn (D).

The wound was sealed using Vicryl 4 (Ethicont, Johnson & Johnson, São José dos Campos, São Paulo, Brazil, Brazil) on the periosteum, and nylon 4-0 (Ethicont, Johnson & Johnson) on the skin.

In the immediate post-operative period, the animals received analgesic (Tramals 0.02 mg/kg, Biolab Searle, São Paulo, Brazil) and anti-inflammatory medication (Profenids 3 mg/kg, Distribuidora Farmácia Brazil LTDA, Jandira, São Paulo, Brazil) intramuscularly. From 01 to 06 weeks into the experimental period, the animals (09 rabbits for each experimental step) were sacrificed using an anesthetic overdose of 1 g/2 mL–45 mg/kg sodium thiopental, intravenously.

CT

Immediately after grafting surgery and sacrificing, the animals were subjected to a mandible CT in order to assess graft volume (Figure 2). The scanning was performed under sedation and employing a helicoidal scanner (Emotion™, Siemens, Elrlangen, Germany). The animals were positioned so to obtain axial image slices from the operated sites, using the following parameters: 110 kv and 60 mA radation; 1 second rotation time; 1 mm thick slices spaced 1 mm apart. The images were generated in a Dicom pattern and transferred to a PC workstation equipped with Dicomworks 1.3.5b software intended to perform graft volume measurements. The seven central-most axial sections in each graft were used for thickness, height, and length measurements, for volume calculation. Volume was calculated right after surgery (baseline) and at sacrifice.

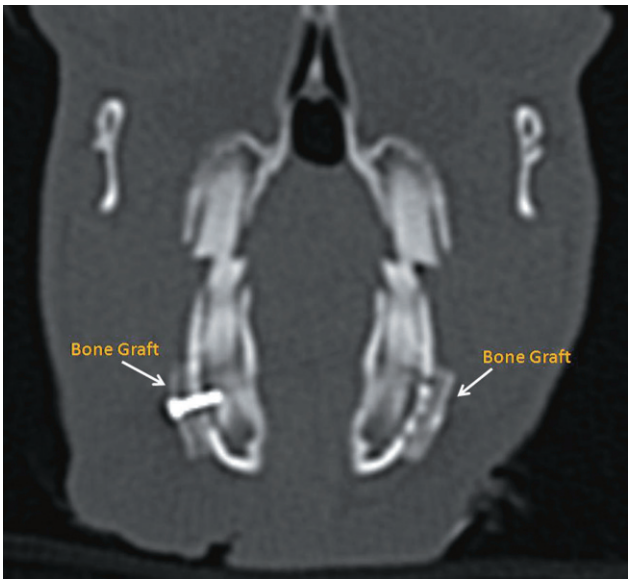


Figure 2 Computed tomography of grafted sites shown in Dicom Works software. Bone grafts fixed with titanium screw (left side) and NB-Cn (right side).

TABLE 1 Mean, Standard Deviation, and Comparison by Paired *t*-Test between Mean Graft Volume Value at Initial Time and Sacrifice in Each Studied Group

		Initial		Sacrifice		
Time	Group	Mean (mm^3)	SD (±)	Mean (mm^3)	SD (±)	*p*
1 week	Screw (I)	113.20	11.48	112.76	11.44	0.863 ns
	NB-Cn (II)	122.63	17.34	126.14	17.45	0.527 ns
6 weeks	Screw (I)	123.85	22.13	119.19	28.31	0.153 ns
	NB-Cn (II)	114.65	18.00	120.21	25.28	0.330 ns

NB-Cn, N-butyl-2-cyanoacrilate; ns, difference was not statistically significant.

Histology and Immunohistochemistry

Bone blocks containing the mandible grafted area from the rabbits was removed, fixed in 10%-buffered formaldehyde at 7.4 pH for at least 24 hours. Following fixation, the samples were decalcified using 4% ethylenediaminetetraacetic acid. Next, the blocks were dehydrated using increasing concentrations of alcohols (from 50% to 100% every 2 hours). Following dehydration, the samples were diaphanized using Xylol immersion until transparency was achieved. The bone blocks were included in paraffin for histological slides preparation.

The immunohistochemical labeling was carried out using the immuno-peroxidase detection method of anti-trap antibody. The biotynilated donkey anti goat IgG (705-065-147; Jackson Immuno research Laboratories, West Grove, PA, USA) was used as a secondary antibody at the titling of 1:200. Avidin and biotin (Vector Laboratories, Burlingame, CA, USA) was used in order to amplify the signal of the reaction that was revealed using Diaminobenzidina (Sigma Aldrich, St Louis, MO, USA) as a chromogene. The intensity of labeling in each glass slide was assessed using a semi-quantitative rank ranging from 0 for no labeling to 3 for intense labeling. This subjective quantification was carried out at the surface of the bone graft and at the recipient bone/bone graft interface, on a light microscope (Leica DMLB; Leica, Bensheim, Germany).

Statistical Analysis

Tomographic data were analyzed through normality tests using Shapiro–Wilk methodology. *t*-Tests were used to verify graft volume at the initial and sacrifice stages at different experimental times for each treatment. Variance analysis was used to compare graft volume variation at different experimental times and the two types of fixation methods employed. Nonparametric tests were used to analyze immunohistochemical staining. Wilcoxon test was used to compare different grafting sides, and when time was also a factor, Mann–Whitney test was utilized. In all statistical studies, a significance level of 5% ($p < 0.05$) was used. All statistical analysis was performed under Statistica for Windows software version 5.1 (Statsoft Inc., Tulsa, OK, USA)

RESULTS

All animals tolerated the surgical procedures well and the postoperative period was uneventful.

CT

Graft Volume. Based upon the obtained results, it was observed that in both studied groups, bone graft volume did not alter significantly between initial and sacrifice stages in both experimental times (Table 1).

No statistically significant difference was observed when it came to graft volume mean value variation throughout the experimental times for each type of treatment, at 1 and 6-week treatment periods, and neither between time variation and treatment types. However, there was a statistically significant difference for volume variation between two treatment types (screw × NB-Cn; $p < 0.05$), in which group II (NB-Cn) showed improved graft volume maintenance when compared with group I (screw), in both experimental times, exhibiting an experimental mean value variation increase of 3.81% against a mean value of –2.15% from group I. Data were pooled in a table comprising mean values and standard deviation for the cited volume (Table 2) and presented in a graph (Figure 3).

TABLE 2 Percentage of Graft Mean Value Variation (Δ Volume) between Sacrifice and Initial Stages for Each Experimental Time and for Both Studied Groups

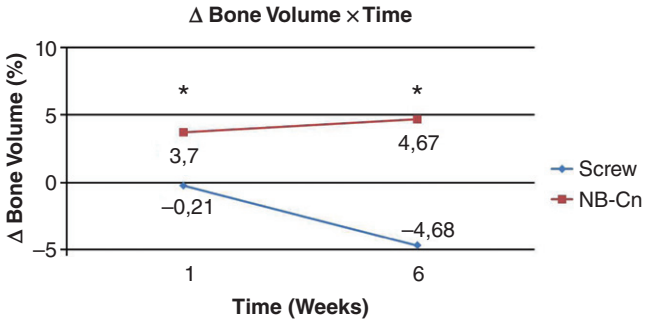
	NB-Cn		Screw	
Time	Mean (%)	SD (±)	Mean (%)	SD (±)
1 week	3.70[a]	13.47	–0.21[b]	6.60
6 weeks	4.67[a]	13.33	–4.68[b]	8.52

Equal letters, not statistically significant; NB-Cn, N-butyl-2-cyanoacrilate.

Histology

Titanium Screw (Group I). In the 7-day experimental period, the bone grafts appeared to be almost completely preserved with rare spots of bone resorption. At the graft–receptor bone interface, one has observed a clear new bone deposition (woven bone) within a loosely organized collagen fiber matrix, joining the graft to the resident bone, as well as on the lateral margins of the grafted site right below the periosteum (Figure 4). After 6 weeks, it was possible to observe an intense remodeling process in the graft itself as well as bone neoformation from the endosteum, also showing internal cortical bone resorption and its union with the external cortical bone through mature bone trabeculae. In the graft/receptor site interface, mature bone tissue with lamellar organization was found. The same findings were observed in the lateral margins of the grafted site below the periosteum, making it evident that the graft had been properly incorporated (Figure 5).

NB-Cn (Group II). In the 7-day experimental period, the bone grafts appeared to be completely preserved. At



* = Statistically significant.

Figure 3 Graft volume mean value variation (Volume at sacrifice – initial volume) for each studied group in 1- and 6-week periods. NB-Cn = N-butyl-2-cyanoacrilate.

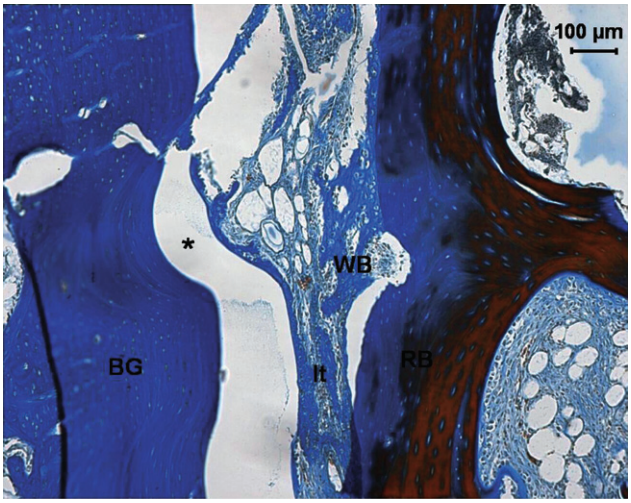


Figure 4 Woven bone apposition (WB) in a loosely organized collagen fiber matrix uniting the bone graft (BG) to the recipient bone (RB). It = Interface; * = artifact separation. (100×). Mallory's trichrome.

the graft–receptor bone interface, one has observed in some situations a clear new bone deposition (woven bone) generally located near the perforations of the receptor bed. This immature bone formation at times seemed to have come from the graft, at others from the receptor site (Figure 6). In other cases, one has observed only a fibrous conjunctive tissue at the interface, which was sometimes dense, and at others loosely organized. At the edges of the graft, the biological glue was found along the margins of the graft and the underlying periosteum (Figure 7). However, most of the time, marginal graft periosteum was already found destroyed. After 6 weeks, grafts were still vital and with a well-preserved architecture, exhibiting bone neoformation in their interior. The internal cortical, however, presented an intense resorption process allowing the graft medullar content to communicate with the interface (Figure 8). At this experimental period, the incorporation process of the graft to the receptor site was clearly evident, being characterized by mature bone bridges and organized in osteonic lamella uniting these structures (Figure 9). Loosely organized collagen fibers completed the interface portrait. Similarly, on the graft lateral margins and below the periosteum, it was possible to observe intense mature bone tissue formation, an area where the incorporation process of the graft to the mandibular receptor bone seems to have initialized from (Figure 10). The periosteum was found

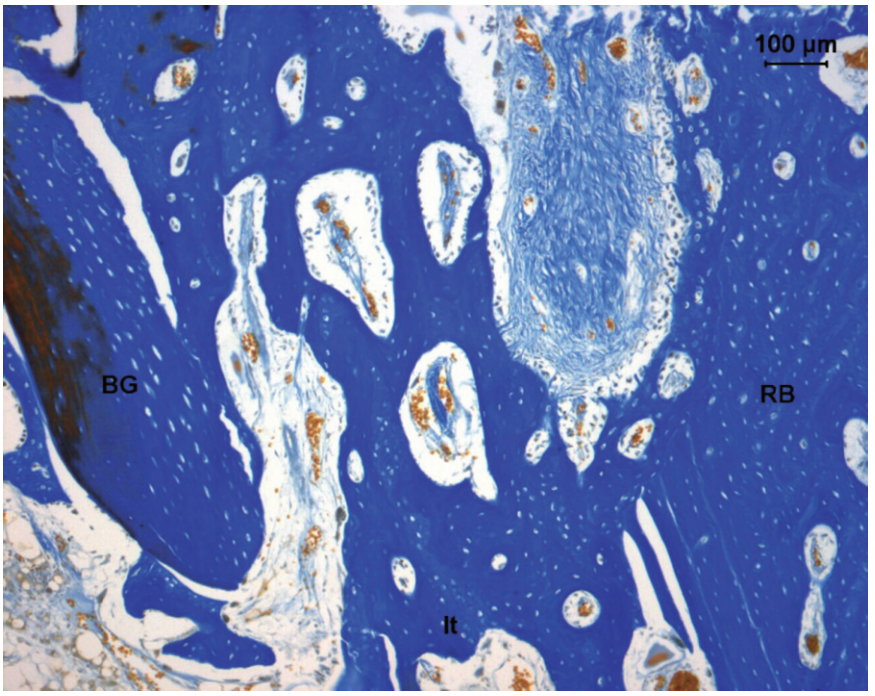


Figure 5 In the graft/recipient bed interface, lamellar mature bone was observed, making it evident that the graft had been incorporated. BG = bone graft; It = interface; RB = resident bone. (200×). Mallory's trichrome.

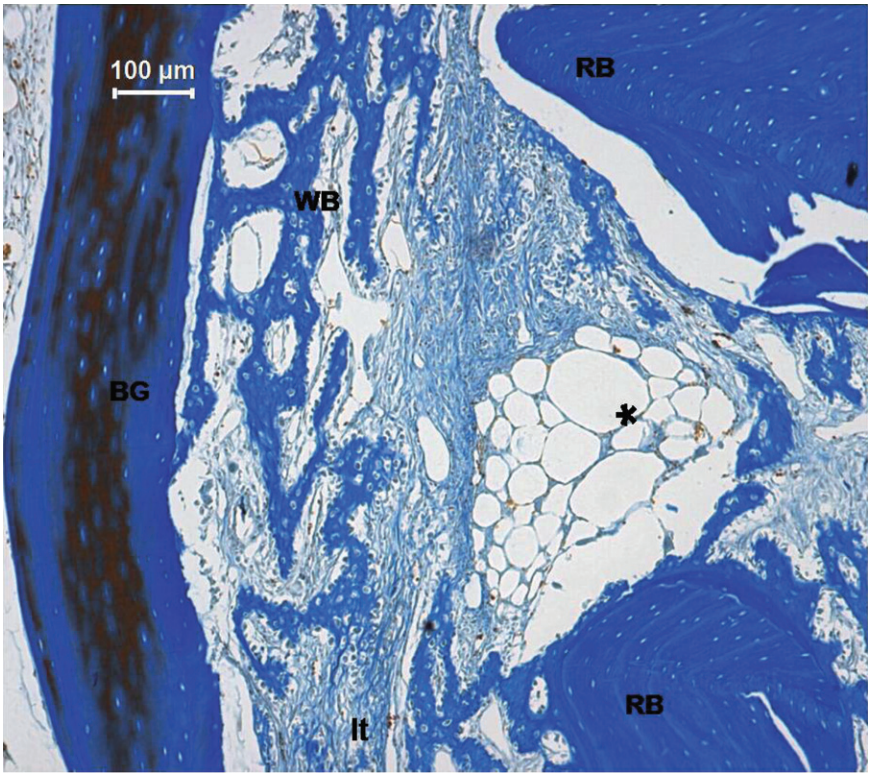


Figure 6 Histological photomicrography featuring the resident bone (RB), bone graft (BG), and the new bone (WB) near a perforation (*) at 7 postoperative days. (100×). Staining: Mallory's trichrome.

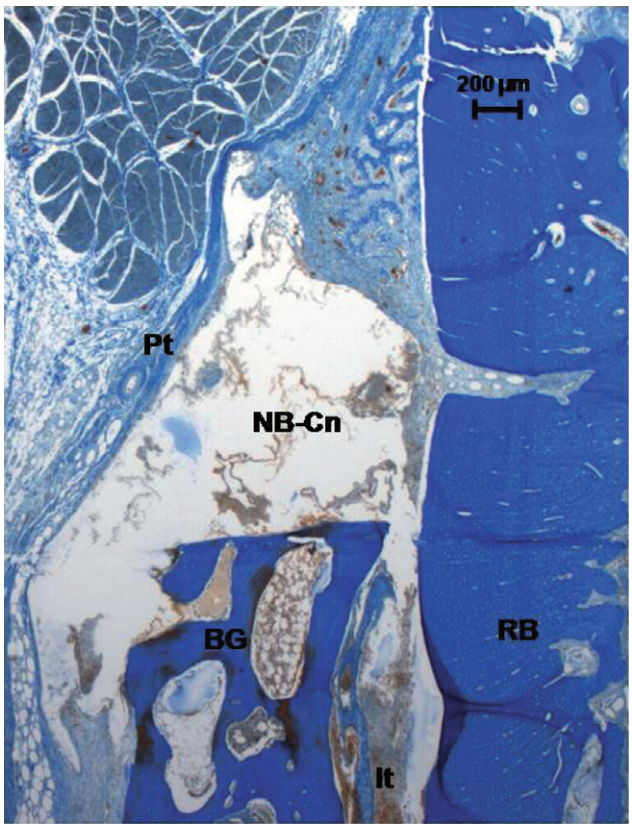


Figure 7 At the graft margins (BG) it was possible to observe the presence of the adhesive material (NB-Cn) and the underlying periosteum (Pt). Note the immature bone formation below the periosteum next to the recipient bed (RB). It = interface. (25×). Mallory's trichrome.

regenerated, differently from what had been observed at the 1-week period when the periosteum was mostly destroyed.

Immunohistochemistry

The semi-quantitative analysis of immunolabeling was performed subjectively using scores. The results are shown in Table 3. Tartrate-resistant acid phosphatase protein (TRAP) is an enzyme that is present in large quantities at the corrugated margin of osteoclasts, being released in the gap between this margin and the underlying bone during bone resorption. According to the results obtained, it was observed that TRAP yielded more intense labeling for the screw-fixed grafts; however, no statistically significant difference was attained when compared with the group fixed with NB-Cn, at both experimental times (1 week: $p = 0.109$ and 6 weeks: $p = 0.285$). Nevertheless, results analysis have showed that there were statistically significant differences between two times at sacrifice, both for NB-Cn ($p = 0.007$) and screw ($p = 0.03$), in which labeling intensity for the 6-week period was significantly more pronounced when compared with the 1-week time. TRAP-positive cells were predominantly located in the graft interface (Figure 11).

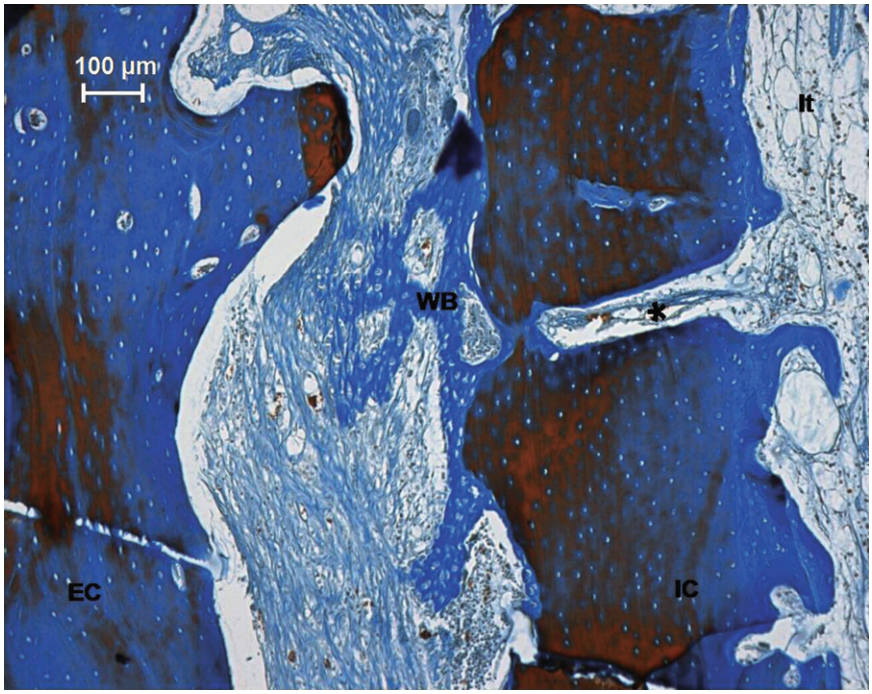


Figure 8 Immature bone formation (WB) in the interior of the graft. Evident* internal cortical resorption (IC), connecting the marrow content with the interface (It). EC = External Cortical. (100×). Mallory's trichrome.

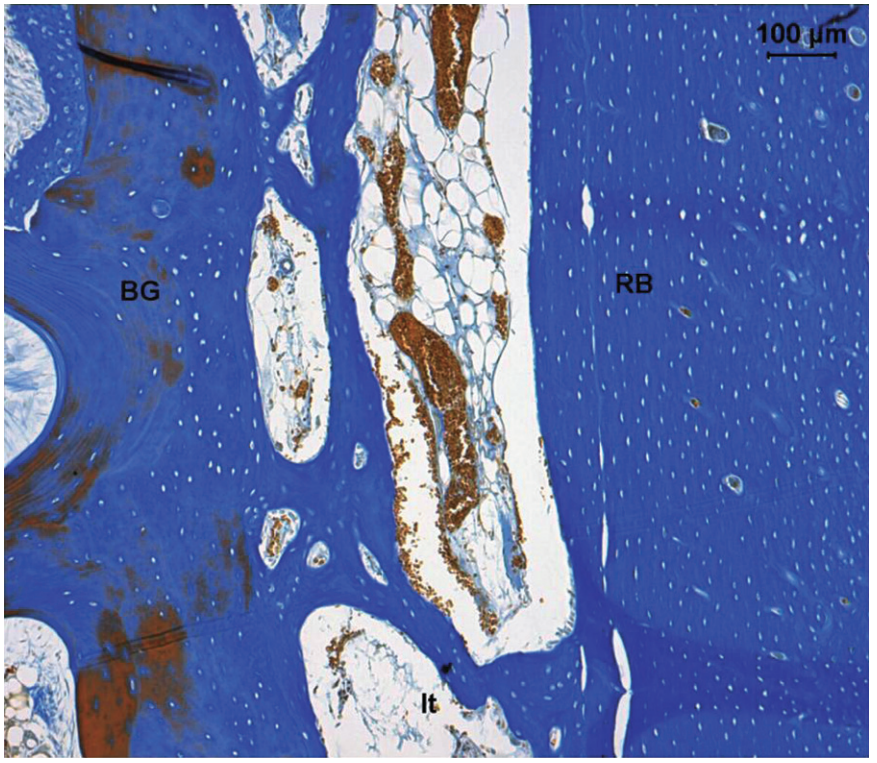


Figure 9 Bone graft incorporation process (BG) to the recipient bed (RB), characterized by mature bone bridges and organized with osteonic lamellae uniting these two structures. It = interface. (100×). Mallory's trichrome.

DISCUSSION

Autogenous bone grafts are largely used for the reconstruction of atrophic alveolar ridges and it is believed that it must be firmly fixated to the receptor site when used for height and width gains. Several studies have showed that the internal screw fixation reduces onlay bone graft resorption when compared with steel wire fixation or even when no fixation is used at all.[12–14] Based upon these evidences, the use of screws and titanium plates has become the most widespread bone graft fixation method nowadays, for it promotes improved stability. However, the use of these metallic devices for bone graft fixation brings about some disadvantages and problems amply discussed anteriorly. Among the aforementioned problems, one may cite the generation of artifacts during imaging exams, the high cost of the used materials, and the risk of screw exposure leading to infection, among others. Thus, Cyanoacrylate adhesives can be considered a possible replacing alternative to the osteosynthesis methods, which traditionally employ metallic materials for bone fixation, promoting a less rigid attachment without compromising graft incorporation and its volumetric maintenance. Experimental studies have showed similar results between bone segments fixed with plates

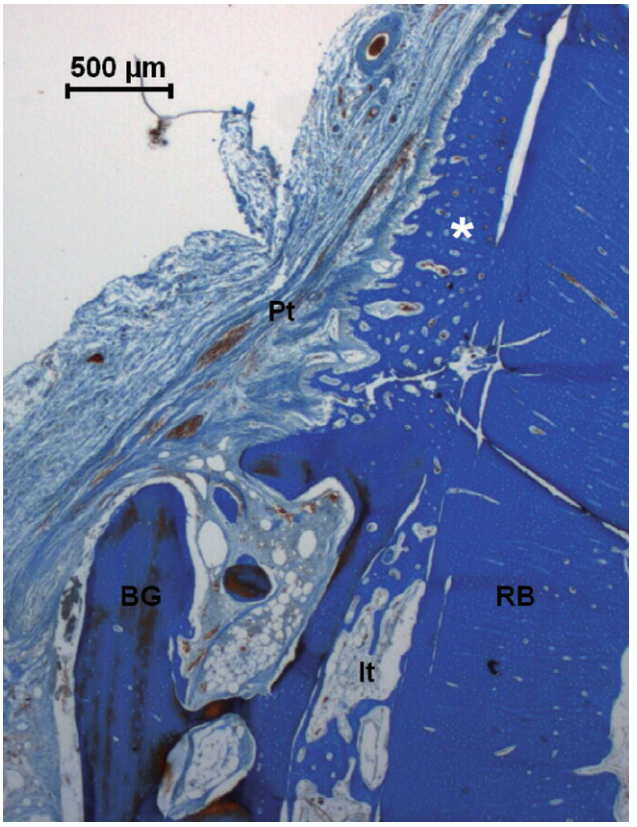


Figure 10 Graft's lateral margin (BG) laying underneath the periosteum (PT), where intense mature bone formation is observed (*), as well as at the interface (It) between the graft (BG) and recipient bed (RB). (25×). Mallory's trichrome.

TABLE 3 Labeling Intensity for Tartrate-Resistant Acid Phosphatase Protein for Both Treatments and at Both Times at Sacrifice

Time	NB-Cn			Screw			*p* (between treatments)
	Median	Mean	SD	Median	Mean	SD	
1 week	0.00	0.33	0.52	1.00	0.67	0.52	0.109 ns
6 weeks	2.00	2.00	1.10	2.50	2.50	1.64	0.285 ns
p (between times)		0.007			0.030		

NB-Cn, N-butyl-2-cyanoacrilate.

and titanium screws and those fixed with Cyanoacrylate glue.[4,8] Nevertheless, no study has evaluated the use of NB-Cn for the fixation of onlay bone grafts and its histological, immunohistochemical, and tomographic impacts in the incorporation and maintenance of graft volume when compared with metallic screws. In the present study, metallic-screw-attached grafts showed a negative volume variation in both experimental times (1 and 6 weeks) when compared with graft installation volume. Adversely, the group in which the adhesive was used showed positive volumetric graft variation in the same periods. Saska and colleagues,[15] in a histometric study in rabbit calvarias, have also found increased volume preservation in grafts fixed with ethyl-Cyanoacrylate when compared with titanium screws. Blocks fixed with the screw also suffered increased bone volume loss and the authors have attributed this effect to the internal pressure generated by the screw over the graft. Notwithstanding, in the ethyl-Cyanoacrylate groups, bone grafts had not been entirely incorporated into the receptor site, even after 120 days following their placement. Differently from our study, the adhesive was applied in the whole extension of the graft/receptor site interface and the receptor site was not perforated. Furthermore, the presence of adhesive material even after 120 days in Saska's study may have interfered with the revascularization and incorporation of bone grafts, as it is well known that ethyl-Cyanoacrylate degradation only

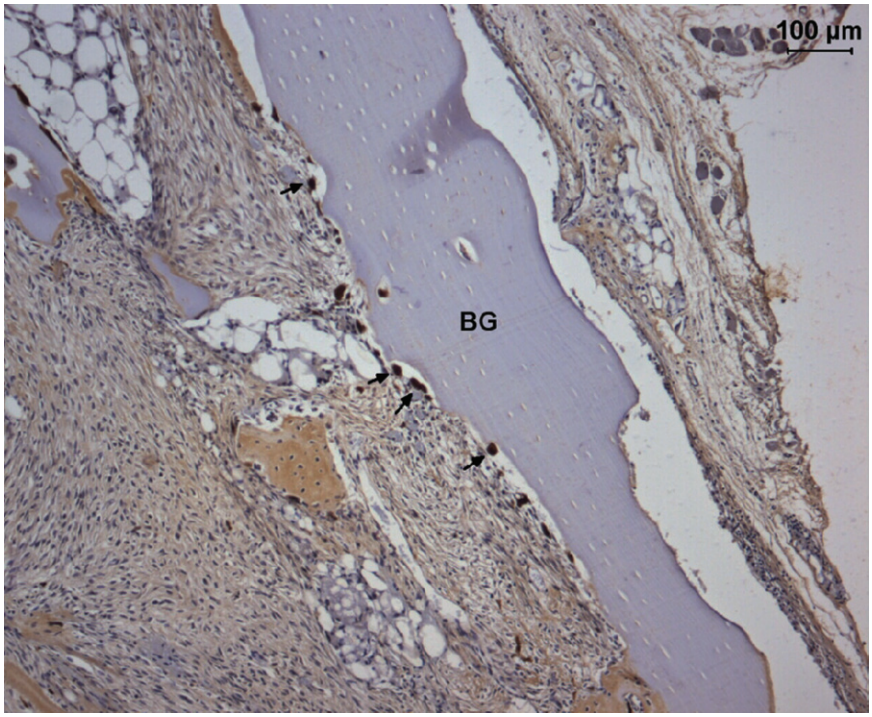


Figure 11 Bone graft (BG) right down below the periosteum, where tartrate-resistant acid phosphatase-positive cells were observed (arrows). (100×). Group I – 6 weeks.

takes place after 12 months, which may partially explain the results obtained by the authors. In our study, the adhesive material was only applied in the margins of the graft, and no presence of NB-Cn was detected after 42 days, which suggests that NB-Cn formula would be considerably more adequate for osteosynthesis procedures using adhesive materials.

Histological analysis revealed that in both groups graft incorporation process to the receptor site was already noticeable at a 1-week period and that this process was practically concluded at a 6-week period, with the presence of lamellar bone tissue in the bone interface. Bone apposition in both groups initiated from the graft margins as a result of a "periosteal reaction" and for the proximity between graft margins and receptor site. This histological picture has been described by other authors in similar studies[16–19] and affirms the importance of these two factors in the incorporation of onlay bone blocks. Bone graft revascularization occurred at the cost of its internal cortical being resorbed in both groups, allowing communication between the interface and its medullary content, thus contributing to increased bone neoformation and its remodeling at the 6-week period. On the other hand, this remodeling process was further intensified in the control group at the end of the evaluated period. The NB-Cn group was frequently related with periosteum degeneration at the 1-week period, suggesting some form of reaction to the adhesive material presence. Such affirmation may partially explain the remodeling delay in the bone blocks fixed with the adhesive material.

Resorption origin in onlay bone grafts is not entirely known. Recent tomographic and immunohistochemical studies carried out by our group have showed significant differences in favor of the calvaria studies when compared with the iliac crest in terms of graft volume maintenance,[18,19] confirming the importance of graft microarchitecture for remodeling and volume maintenance in the long run.[20] In one of these studies, Pedrosa Jr. and colleagues[19] published a study in which tomographic, histological, and immunohistochemical data on the incorporation of calvarial bone grafts were compared against the presence or absence of perforations on the receptor site. Based on these results, the authors concluded that calvarial bone graft volume is more adequately maintained when the receptor site is perforated, probably due to improved graft revascularization and osteogenesis. Therefore, it seems that the microarchitecture of the graft and an adequate preparation of the recipient bed are paramount for graft volume maintenance. In this present study, the same methodology employed in our previous study was utilized. All the grafts were harvested from the same cranial region; they were standardly trimmed and adapted to the perforated recipient bed, only varying their attachment method. It is reasonable to ponder that this difference in terms of fixation methods might have contributed to the remodeling discrepancies and loss of volume in the group where the screw was used, as other variables such as graft microarchitecture and preparation of the recipient bed were previously standardized and controlled. Thus, graft and recipient bed perforation for screw installation might have caused a premature revascularization pathway, which did not occur in the adhesive material group. The acute revascularization process might have led to more intense bone remodeling, leading to loss of volume especially at later periods. The fact that the scores for TRAP proteins were higher in the screw group at the 1-week period corroborates with this affirmation. In our study, the internal cortical of the graft in the NB-Cn group was previously submitted to resorption so the graft was revascularized, which might explain a less intense bone remodeling process in this group. Nevertheless, more specific studies are necessary to determine the influence of the fixation screw in the remodeling of onlay grafts.

The fixation granted by the adhesive material, even though less rigid when compared with the screw, allowed enough stability to the graft/recipient bed combination so that bone neoformation could occur in the interface and margins of the bone block. One disadvantage is that Cyanoacrylate adhesives led to the destruction of the periosteum in the 1-week period, despite the fact that this did not jeopardize revascularization and graft incorporation. Several clinical studies have shown the use of Cyanoacrylate in mouth surgeries,[10,21–23] as well as its utilization in vascular embolism, skin wounds, as a hemostatic agent, among others.[24–26] Nonetheless, in none of these studies was any toxic effect of Cyanoacrylate-based products reported. NB-Cn is a long chain compound, belonging to a new generation of Cyanoacrylate compounds, which are more biocompatible than those initially used.[11] However, Pelissier and colleagues,[11] in a study using NB-Cn to seal dorsal wounds in rats, observed edema and a moderate acute inflammatory reaction after histological analysis of the

wounds. Therefore, additional studies are necessary to better investigate the biocompatibility of NB-Cn when used for bone fixation methods.

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