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Immunological aspects of fresh-frozen allogeneic bone grafting for lateral ridge augmentation

Key words: bone allograft, bone autograft, ELISA, immunological evaluation, ridge augmentation

Abstract
Objectives: To present some immunological aspects of fresh-frozen allogeneic bone grafting for lateral bone augmentation, based on the quantitative evaluation of IL-10, IL-1β, IFN-γ and TNF-α in patients sera.

Material and methods: Thirty-three partially or totally edentulous patients received fresh-frozen allogeneic bone (AL – 20 patients) or autologous bone onlay block grafts (AT – 13 patients) prior to oral implant placement. Blood samples were collected from each patient at various time-points during a 6 month-period (baseline, 14, 30, 90 and 180 days postoperatively). Quantitative evaluation of IL-10, IL-1β, IFN-γ and TNF-α was performed by enzyme linked immunosorbent assay (ELISA).

Results: For all evaluated markers and at all evaluated periods, inter-group comparisons showed no statistically significant differences between the groups, while the observed values were within normal levels. For AL-treated patients, intra-group evaluation showed statistically significant increase of TNF-α from baseline to 90 (P < 0.001) and 180 (P < 0.01) days, and from 14 to 90 (P < 0.01) and 180 (P < 0.05) days. IFN-γ showed intercalated results, with a decrease from baseline to 14 days (P < 0.05), and increase from 14 to 90 days (P < 0.001) and 180 (P < 0.05) days. No differences between the periods of evaluation were found for the AT group.

Conclusions: AL grafting for lateral bone augmentation, similar to AT grafting, does not seem to challenge the immune system significantly.

Adequate bone volume is a prerequisite for installation of oral implants of appropriate size and in the correct three-dimensional position, facilitating good esthetics and increased implant survival rates (Stanford 2002; Renouard & Nisand 2006). Inadequate amounts of bone for proper implant installation may be due to disease, trauma or congenital anomalies, various reconstructive techniques – such as bone grafting – are employed with the aim to restore the missing bone volume [Chiapasco et al. 2006].

Autologous bone (AT) is considered the gold standard in grafting materials, especially when relatively large amounts of bone need to be restored [Misch & Misch 1995]. AT has the advantage of rapid incorporation and consolidation with the host bed [Burchardt 1987], and there are no immunologic concerns regarding its use [Williams & Szabo 2004]. However, disadvantages associated with the harvesting procedure itself, like morbidity of the donor site and increased costs, as well as the extended and unpredictable resorption of the graft, make the pursuit of alternative grafting materials necessary [Zerbo et al. 2003].

Throughout the years, a variety of materials have been suggested as an alternative to autologous bone [Bayerlein et al. 2006; Theler 2011], among those, fresh-frozen allogeneic bone graft [AL] has gained renewed attention during recent years [Eppley et al. 2005]. AL has the obvious advantages of being easily obtainable in large quantities [from certified bone-tissue banks] and of reduced morbidity and surgical time due to absence of a second surgical donor area, making thus AL an...
attractive clinical alternative to AT. The increased use of allogeneic bone during recent years is directly linked to the establishment of strict guidelines for donor bone processing [Palmer et al. 1999] increasing the safety of its use. Risk of disease transmission through AL grafting is thus considered to be low nowadays and no report of cross-contamination with serious diseases (e.g. hepatitis or HIV) has been published so far [Waasdorp & Reynolds 2010].

Comparing to AT grafts, however, AL grafts show slower incorporation and remodeling [Waasdorp & Reynolds 2010, Spin-Neto et al. 2011a]; in addition, lower survival rates of oral implants placed in association with AL grafts have been reported [Carinci et al. 2010]. In this context, limited information exists on the possible relationship between immunological reactions and the fate of bone grafts [Friedlaender 1987, Bauer & Muschler 2000]. In fact, there is evidence of osteoblast-related vital cells escaping the freezing process during AL preparation [Simpson et al. 2007]; these cells might be related to specific immune responses, such as increased reactivity against AL bone proteins [VandeVord et al. 2005], which in turn may lead to graft resorption, incomplete incorporation, or fracture/non-union (Hubble 2002). No information exists in the literature on the impact of AL grafting for maxillofacial indications on the immune system. Thus, the aim of this study was to evaluate the influence of AL grafting for lateral ridge augmentation on systemic inflammatory markers, in comparison with AT grafting.

Materials and methods

The research protocol of this controlled case series was approved by the Araraquara School of Dentistry Ethics Committee (CEP-FO/Car) and by the National Research Ethics Committee (CONEP-MS) under the protocol number 36/08, and it is in accordance to the World Medical Association Declaration of Helsinki (2002). All treatments were performed in the Department of Periodontology, Araraquara Dental School (UNESP – Univ. Estadual Paulista), Araraquara, São Paulo, Brazil.

Patient selection
A total of 33 partially or totally edentulous patients [12 men/21 women; average age: 47 years; range 27–69 years], who desired oral rehabilitation with titanium implants and had at least one site with severe bone deficienci (i.e., <4 mm alveolar ridge width) not allowing the direct placement of a regular size implant, were treated from May to December 2009. Demographic information on this population is presented in Spin-Neto et al. [2011b]. Alveolar ridge width was determined on the cross-sectional image section view of CBCT (i-CAT Classic, Imaging Sciences International, USA) generated images (DICOM-based data sets) with a resolution of 96 dpi, 14-bits gray scale and 0.25 mm voxel size. The CBCT unit was set at 120 kVp, 5 mA, with a 20 s exposure time. None of the patients presented with systemic diseases affecting bone turnover, or were pregnant or lactating, or had habits that could interfere with treatment (for example: smoking, alcoholism and drug use).

Based on the clinical screening examination and the CBCT examination, and depending on a subjective judgment of the amount of bone resorption and/or number of sites requiring reconstruction in each patient, it was decided whether patients could be considered for AT grafting. Patients judged not to possess adequate amounts of donor bone were treated with AL grafts, after an informed signed consent was obtained. Following Brazilian regulations, documents regarding the allogeneic biomaterial request were filled-out and sent to the registered bone bank that supplied the allografts (Unios, Marília, Brazil). The fresh-frozen allogeneic bone was collected and processed according to the American Association of Tissue Banking (AATB) guidelines (Troyer 2008). Thus, 20 patients were treated with AL and 13 with AT grafts; approximately 85% of the cases regarded the maxilla.

Surgical procedures

The surgical procedures have been previously described in detail [Spin-Neto et al. 2011b]. Briefly, under local anesthesia a full thickness flap was raised and any reminiscent soft tissues were removed from the bone surface. Host cortical bone was penetrated with delicate burs to enhance vascularization toward the base of the grafts. In the AT group, grafts of adequate size according to defect dimensions were retrieved from the mandibular ramus. In the AL group, standard size cortico-cancellous bone blocks [15 x 10 x 6 mm] were used. Both types of blocks were trimmed to fit the defects. The blocks were fixed with the cancellous bone side facing the host bone using 1.5 mm in Ø x 10 or 12 mm long titanium screws [Neodent, Cambira, Brazil] (Fig. 1). The grafts were covered with a collagen membrane (Genius Baumer, São Paulo, Brazil), and the flaps were repositioned and sutured with interrupted nylon 4-0 single sutures for primary intention healing.

As a post-surgical protocol, all patients received antibiotics [Amoxicillin 500 mg] three times daily for 7 days, non-steroidal anti-inflammatory treatment [Nimesulide 100 mg] two times daily for 5 days, and analgesics [Acetaminophen 750 mg] according to individual needs. Patients rinsed with chlorhexidine digluconate 0.12% for 7 days postoperatively. Sutures were removed 14 days after surgery.

Blood sampling and evaluation

From each of the 33 patients, 5 ml of venous blood were collected at 7 days prior to grafting [baseline], and at day 14, 30, 90 and 180 postoperatively. The blood was collected in vacuum tubes containing EDTA/K3, always in the morning after 12 h of fasting, and with the patient in a seated position. The blood was stored at 4°C immediately after collection and transported to the laboratory within 30 min. The blood was centrifuged under refrigeration at 1800 g for 10 min, for sera separation. The sera were inactivated by placing them into a 56°C bath for 30 min, and then separated in 350 µl samples, identified and frozen at –80°C.

Immunological analyses were conducted through enzyme linked immunosorbent assay (ELISA) using specific reagent-set kits (BD OptEIA ELISA Sets, BD Biosciences Pharmingen, San Diego, USA) for interleukins 10 (IL-10) and 1β (IL-1β), interferon γ (IFN-γ), and tumor necrosis factor α (TNF-α). All tests [e.g. for all patients and time points] were performed in triplicate, using 100 µl of sera for each well. Following the specifications of the reagent-set kit manufacturer, results were obtained using a spectrophotometer, with readings being performed at 450 nm within 30 min with 0.5 correction 570 nm.

Data analysis

Data from the triplicate analyses were averaged to represent the sample unit. Data for each marker at each observation period for the AL and AT groups were expressed as means, medians and standard deviations. Commercially available software [GraphPad Prism 5.0 for Windows, GraphPad Software Inc., USA] was utilized for comparisons between groups and for drawing the graphics. Data was subjected to normality test analysis (D’Agostino & Pearson omnibus normality test). For all evaluated parameters, data were not normally distributed. The Mann-Whitney
test was used for paired comparisons, while the Kruskal–Wallis test followed by Dunns post-test was used for multiple comparisons (longitudinal data). It is acknowledged that use of a test for dependent data would be more appropriate for evaluating the data from the various time-points. However, in several instances individual patients missed one of the blood collection examinations, thus, if we only included patients with complete data sets, as a dependent data test requires, we would lose about 25% of the available information. Statistical significance was set at 5% ($P < 0.05$).

### Results

During the postoperative period, in the AL-treated patients, one block graft became exposed (registered during the 30-day control) and in four patients some of the grafts were lost due to surgical complications, i.e., lack of proper fixation during placement, apparently leading to block graft mobility and lack of incorporation. The exposed block graft was inserted in one of the four patients who lost a block graft. The patient with the exposed block was instructed to apply chlorhexidine 1% gel over the exposed area twice a day for 14 days; after this period the graft was again covered by soft tissue, and no clinical signs of inflammation were observed until the time of implant placement. The non-incorporated block grafts were surgically removed.

Although the values of laboratory markers of these four patients did not seem to differ substantially from the rest of the group (data not shown), it was decided to exclude these four patients from the statistical analysis. A post hoc power calculation based on the included patients showed the power of the study at 60%, considering differences of 15% among evaluated groups.

The results of the laboratory analyses are presented in Table 1 and 2 and Fig 2 and 5. All values of interleukins 10 (IL-10, Fig. 2) and 1β (IL-1β, Fig. 3) and the inflammatory markers interferon γ (IFN-γ, Fig. 4) and tumor necrosis factor α (TNF-α, Fig. 5) found herein (Table 1) were within those accepted as normal in literature. For all evaluated markers, and in all evaluated periods, inter-group comparisons (based on Mann–Whitney test) showed statistically similar results for both groups. Regarding intra-group comparisons (based on Kruskal–Wallis tests), no differences among the various observation periods for any of the evaluated parameters were found in the AT group. Similarly, regarding IL-10 and IL-1β, no differences among the various observation periods were found within the AL group. In contrast, tumor necrosis factor α (TNF-α) showed a statistically significant increase from baseline to 90 ($P < 0.001$) and 180 ($P < 0.01$) days, and from 14 to 90 ($P < 0.01$) and 180 ($P < 0.05$) days in the AL group. IFN-γ showed an initial decrease from baseline to 14 days ($P < 0.05$), but then increased statistically significantly from 14 to 90 days ($P < 0.001$) and 180 ($P < 0.05$) days, no differences were observed between baseline and 90 or 180 days.

To assess whether the number of block grafts received by the patient might have influenced the immunological response, averages for IFN-γ and TNF-α levels according to the number of AL and AT blocks grafted in...
Discussion

During recent years, the number of studies regarding the use of fresh-frozen bone allografts (AL) as an alternative to autologous bone (AT) for maxillofacial indications has increased. AL is indeed an attractive alternative to AT especially when relatively large amounts of bone are needed, mainly because there are no concerns associated with a second surgical donor site, e.g., morbidity, limited available quantity from intraoral donor sites, increased surgical time and costs (Wadsorp & Reynolds 2010). However, there is still limited information on the behavior of AL grafts for maxillofacial indications and the results are controversial. Although some studies have reported results equivalent to those achieved with AT after the use of AL (Contar et al. 2009), other studies found higher resorption rates (Spin-Neto et al. 2011b) and slower incorporation (Spin-Neto et al. 2011a) of AL grafts when compared with AT grafts. In addition, in a recent publication on 287 implants inserted in AL augmented maxillae, a larger marginal peri-implant bone resorption than what usually occurring after AT grafting was observed in 60% of the cases after a 48-month period (Carinci et al. 2010).

Major concerns regarding the use of AL grafts, in the past, have been their possible antigenicity and the risk of disease transmission (Palmer et al. 1999). Establishment of strict guidelines for donor bone processing has minimized the risk of diseases transmission and no case of cross-contamination after grating with AL processed according to the AATB protocols has been reported to date; thus, AL is considered nowadays as safe (Wadsorp & Reynolds 2010). In the same context, AL grafts are considered as a universal donor material with limited immunologic potential, due to the processing of donor tissue (Boyce et al. 1999). Studies have shown no marked immune response after the use of large AL orthopedic grafts (Stevenson et al. 1991, 1997). Indeed, the number of AL blocks grafted did not seem to influence the levels of TNF-α and IFN-γ in this study. Similarly, a recent study, based on the blood cell profile screening of patients grafted with AL for lateral ridge augmentation, demonstrated no impairment in the red and white blood-cell

Table 1. Interleukin 10, Interleukin 1β, Interferon-γ and Tumor necrosis factor α levels, expressed in means, standard deviations, and medians in parenthesis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>14 days</th>
<th>30 days</th>
<th>90 days</th>
<th>180 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Allograft</td>
<td>Autograft</td>
<td>Allograft</td>
<td>Autograft</td>
<td>Allograft</td>
</tr>
<tr>
<td>IL-10</td>
<td>8.47 ± 5.37</td>
<td>9.08 ± 4.03</td>
<td>7.62 ± 4.24</td>
<td>6.12 ± 4.03</td>
<td>7.30 ± 5.28</td>
</tr>
<tr>
<td>IL-1β</td>
<td>2.68 ± 3.59</td>
<td>3.08 ± 3.73</td>
<td>2.49 ± 0.84</td>
<td>3.43 ± 3.70</td>
<td>4.63 ± 4.35</td>
</tr>
<tr>
<td></td>
<td>(1.56)</td>
<td>(1.81)</td>
<td>(2.50)</td>
<td>(3.48)</td>
<td>(3.05)</td>
</tr>
<tr>
<td></td>
<td>(10.98)</td>
<td>(9.77)</td>
<td>(6.16)</td>
<td>(9.31)</td>
<td>(7.38)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.89 ± 1.22</td>
<td>2.51 ± 1.18</td>
<td>2.11 ± 1.19</td>
<td>3.42 ± 3.04</td>
<td>5.50 ± 8.32</td>
</tr>
<tr>
<td></td>
<td>(1.56)</td>
<td>(2.33)</td>
<td>(2.14)</td>
<td>(2.14)</td>
<td>(2.41)</td>
</tr>
</tbody>
</table>

IL-10, Interleukin 10 (pg/ml); IL-1β, Interleukin 1β (pg/ml); IFN-γ, Interferon γ (pg/ml); TNF-α, Tumor necrosis factor α (pg/ml).

Table 2. Average Interferon-γ and 005tumor necrosis factor α levels according to the number of AL and AT blocks grafted in each patient

<table>
<thead>
<tr>
<th>Variable</th>
<th>Blocks</th>
<th>Patients (AL/AT)</th>
<th>Baseline</th>
<th>14 days</th>
<th>30 days</th>
<th>90 days</th>
<th>180 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>1</td>
<td>4/7</td>
<td>13.11</td>
<td>15.53</td>
<td>12.72</td>
<td>12.29</td>
<td>13.60</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7/5</td>
<td>11.04</td>
<td>8.84</td>
<td>7.00</td>
<td>8.08</td>
<td>17.88</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1/1</td>
<td>15.29</td>
<td>6.59</td>
<td>8.28</td>
<td>20.71</td>
<td>6.57</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1/0</td>
<td>10.29</td>
<td>nd</td>
<td>12.00</td>
<td>nd</td>
<td>20.02</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0/0</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3/0</td>
<td>10.97</td>
<td>nd</td>
<td>8.27</td>
<td>nd</td>
<td>7.22</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1</td>
<td>4/7</td>
<td>1.47</td>
<td>1.91</td>
<td>1.88</td>
<td>5.42</td>
<td>3.11</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7/5</td>
<td>2.53</td>
<td>1.98</td>
<td>2.66</td>
<td>1.99</td>
<td>9.55</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1/1</td>
<td>0.93</td>
<td>3.06</td>
<td>2.47</td>
<td>2.17</td>
<td>3.59</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1/0</td>
<td>1.55</td>
<td>nd</td>
<td>2.08</td>
<td>nd</td>
<td>1.73</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0/0</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3/0</td>
<td>1.35</td>
<td>nd</td>
<td>1.49</td>
<td>nd</td>
<td>2.07</td>
</tr>
</tbody>
</table>

IFN-γ, Interferon γ (pg/ml); TNF-α, Tumor necrosis factor α (pg/ml); nd, not defined (absent).
population balance irrespective graft origin, something that could also be interpreted as absence of a remarkable immune response toward the AL grafts [Spin-Neto et al. 2011].

Theoretically, the immune response against AL may be triggered by several bone tissue components, such as collagen, fat, or matrix proteins, present in an AL graft, and can be related to mismatching of the major histocompatibility complex (or human leucocyte antigen, HLA) between donor tissue and the host [Graham et al. 2010]. HLA is subdivided into Class I and class II antigens, which both are capable of activating T-cells. Class I antigens include the HLA-A, HLA-B and HLA-C loci and are found in most nucleated cells [Nelson 1996]. Class II antigens are represented by the HLA-D locus and, except by activated T-cells, they are expressed by B-cells, reticuloendothelial system cells, cells of the macrophage and myeloid lineages, and osteoblasts [Skjødt et al. 1990]. Among these cells types, T-cells may play a vital role in both acute and chronic bone allograft rejection [Muscolo et al. 1996]. T-cells can differentiate into inflammatory CD4 T-cells (TH1) and helper CD4 T-cells (TH2) and secrete several cytokines that directly influence bone physiology. TH1 secrete TNF-α and IFN-γ, which enhance the effect of macrophages on bone [Horowitz et al. 1996]. Macrophages might infiltrate the grafts leading to osteoclastogenesis and inhibiting osteoblastic activity [Horowitz & Friedlaender 1991]. TH2 cells play also an important role in initiating allograft rejection, as they recognize antigens in association with HLA Class I molecules and can mediate cytotoxicity through more IFN-γ secretion that can induce apoptosis in their target cells [Apasov et al. 1993, Krieger et al. 1996]. Increased levels of TNF-α can lead to graft resorption by promoting osteoclastogenesis, acting directly on osteoclastic precursor cells or stimulating RANKL [nuclear factor k β ligand] expression [Lorenzo et al. 2008]. TNF-α can also inhibit osteoblastic activity both directly by inhibit collagen synthesis, but also indirectly by downregulating preosteoblast differentiation [Lorenzo et al. 2008]. Thus, although other factors could also be considered, the markers evaluated herein are considered highly representative of any possible immunological reactions due to bone grafting. In this context, the observation times chosen for analysis herein intended to evaluate possible immunological reactions throughout the incorporation/remodeling phase of the bone grafts until implant placement. An evaluation period earlier than 14 days was not chosen to avoid/minimize confounding from the surgical procedures per se. In this study, in patients receiving AL grafts, a significant increase of TNF-α compared with baseline levels was observed, IFN-γ showed a significant decrease during the early postoperative period, and then increased at normal levels during later stages of healing. These findings could in turn explain the slower incorporation and larger resorption of the AL grafts observed in the present group of patients [Spin-Neto et al. 2011a,b]. In this context, the fact that no differences between AT and AL groups were observed for any of the evaluated markers, as well as that their systemic levels observed herein are considered within the physiologic range, may indicate that even small fluctuation of TNF-α and IFN-γ levels results in significant biologic events (i.e., resorption) regarding the fate of AL grafts. In perspective, the findings of a recent literature review showed that there is evidence of an immune response against AL, which in turn could lead to graft resorption, impaired incorporation, fracture or non-union [Graham et al. 2010]. In contrast to the above, the AT grafting procedure did not challenge the immune system in a significant degree, as no differences among the various observation periods for any of the evaluated parameters were found in this study, however, this might be considered as an expected finding.

In this study, all patients received non-steroidal anti-inflammatory (NSAID) treatment for a few days postoperatively, this is a common strategy for pain control after bone grafting procedures. Small doses of NSAID, given in a short duration period postoperatively, beyond the beneficial effects – such as analgesic, antipyretic and anti-inflammatory properties – do not present deleterious effects on bone graft remodeling [Kanis et al. 2007; Nyangoga et al. 2010]. Obviously, it is impossible to evaluate how NSAID treatment in this study might have influenced the results. Nevertheless, it might be interesting to examine whether better regulation of TNF-α and IFN-γ level changes by means of NSAID could reduce AL graft resorption and/or increase their incorporation.

In conclusion, AL grafting for lateral bone augmentation, similar to AT grafting, does not seem to challenge the immune system significantly.

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References


Spin-Neto et al. Immunological aspects of allografts

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967
Spin-Neto et al Immunological aspects of allografts


