Bioactivation of Bovine Bone Matrix Using Argon Plasma: An Experimental Study for Sinus Augmentation in Rabbits

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Purpose: To evaluate the influence of bioactivation with argon plasma of a xenograft used as graft material for sinus floor augmentation. Materials and Methods: Sinus floor elevation was performed in 20 rabbits using a deproteinized bovine bone mineral as graft material. The xenograft used in the test sites was bioactivated with argon plasma (plasma group), while that used in the control sites was left untreated (control group). Collagen membranes were used to cover the antrostomy. The rabbits were euthanized after 2 and 10 weeks, in groups of 10 each. A histomorphometric analysis was performed in various regions of the elevated space. Results: After 2 weeks of healing, a similar pattern of healing was observed in both groups. New bone fractions were 5.2% ± 2.9% in the plasma group and 5.0% ± 3.5% in the control group (P = .795). In this period, higher amounts of new bone were found in the region close to the sinus bone walls. After 10 weeks of healing, the amounts of new bone within the elevated space increased to 23.5% ± 7.0% and 21.3% ± 7.3% (P = .176) in the plasma and control groups, respectively. The only statistically significant difference was found in the central region, with 20.4% ± 9.7% in the plasma group and 13.2% ± 10.5% in the control group (P = .037). Conclusion: The bioactivation of a xenograft using argon plasma improved bone formation within an augmented sinus, especially in the central regions, far from osteogenic sources. Int J Oral Maxillofac Implants 2020;35:731–738. doi: 10.11607/jomi.8385

Keywords: animal study, argon plasma, bone healing, sinus floor elevation, xenograft

Several materials were described in the literature for regeneration of bone defects around teeth and implants. From a biologic point of view, bone regeneration could be didactically divided into different phases. Once the fibrin network is organized and the endothelial cell is recalled, osteoregenerative cells compete with fibroblasts and epithelial cells for the invasion of the space to be regenerated. However, the use of bone graft depends greatly on the interactions between the bony substitutes and the host bone cells. In fact, together with the individual bone regenerative potential and the defect morphology, the healing of bone defects is influenced by bio-chemical-physical properties of the biomaterial surface. In fact, the cell adhesion seems to be strictly related to the graft material adsorption of bone attractant factors (fibronectin, vitronectin, actin, vinculin).

Different studies described the effect of plasma technology in increasing the surface energy of different titanium surfaces. In fact, the plasma of argon treatment, while functionalizing the surface at the atomic and molecular levels, seems to activate the oxide layer interacting with proteins and cells of contiguous tissues. The consequence of this surface change is represented in an increased wettability and protein absorption, which was described to result in boosted cell adhesion. The secondary microscopic effect of this process (bioactivation) is represented by the removal of organic traces of the former industrial process.

On the other hand, the biologic benefit of this bioactivation is qualitative, expressed by a higher number of adhered cells.

At the same time, the advantage of titanium that has undergone plasma of argon treatment is also expressed quantitatively, represented by a spread cell arrangement with an increased number of focal points of adhesion. Additionally, this bioactivation was described to even promote cell differentiation, increasing the expression of cellular markers of activity and regulation of adhesion-related proteins. These findings seemed to be related to the change of the electric mantel of the material, which becomes attractive for charged proteins and osteoblasts.
From a clinical point of view, the bioactivation of titanium implant surfaces through plasma of argon was demonstrated to increase the osseointegration process in animals. At the same time, translated to the abutment, this advantage results in stronger fibroblast adhesion even in the initial stages of the treatment, as demonstrated by the presence of pseudopodia, with the indirect consequence of marginal bone level stabilization. Following these observations, bioactivation through plasma of argon was adopted to increase the surface bio-physical-electric characteristics of bone graft materials. Preliminary in vitro results demonstrated that this process is able to increase cell adhesion on various scaffolds (from synthetic hydroxyapatite, biphasic calcium phosphate, and cancellous and cortical xenogeneic bone matrices) at the early stage.

However, to date there is a lack of in vivo studies in the literature on the effect of bioactivation induced by plasma of argon. Hence, the aim of the present experiment was to evaluate the influence of bioactivation with argon plasma of a xenograft used as graft material for sinus floor augmentation. The hypothesis was that the treatment with argon plasma might increase bone formation of the xenograft surface.

**MATERIALS AND METHODS**

**Ethical Statement**
The Ethical Committee of the School of Dentistry, of Ribeirão Preto, University of Sao Paulo (USP), Brazil, approved the protocol 2018.1.10.58.7 on March 21, 2018. The ARRIVE checklist for animal studies was followed. The Brazilian guidelines for animal experiments were accurately respected.

**Study Design**
Two groups of 10 rabbits each were planned; one group was euthanized after 2 weeks and the other after 10 weeks. Sinus floor elevations were performed bilaterally, and a xenograft, either activated or not with argon plasma, was used to fill the elevated space.

**Bioactivation with Argon Plasma**
The xenograft granules were removed from the sterile vials with a standardized small spoon (Fig 1a) and immediately inserted through a sterile cup (Fig 1b) in the argon plasma reactor (10 W, 1 bar for 12 minutes, Plasma R, Diener).

**Experimental Procedures**
Acepromazine 1.0 mg/kg (Acepran, Vetnil, Louveira) subcutaneously and xylazine 3.0 mg/kg (Dopaser, Hertape Callier) and ketamine hydrochloride 50 mg/kg (Ketamin Agener, União Química Farmacêutica Nacional S/A, Embu-Guaçu) intramuscularly (IM) were injected for anesthesia. Anesthesia was injected locally.

All surgeries were carried out by a specialized maxillofacial surgeon (E.R.S.). After a dermoperiosteal incision, the nasal bone was exposed and a trephine of 3.5-mm diameter was used to prepare a rounded antrostomy on both sides of the nasal-incisal suture. The bony window was removed, and the sinus mucosa was elevated. Similar amounts (~125 mg; 250 mL) of deproteinized bovine bone mineral soaked with saline (DBBM; Bio-Oss, granules 0.125 to 1 mm, Geistlich Biomaterials), either bioactivated (test site; plasma group) or not bioactivated (control group), were used to fill the elevated space (Fig 1c). Both antrostomies were covered using a membrane in collagen (Fig 1d; Bio-Gide, Geistlich Biomaterials).

**Euthanasia**
After sedation, the animals were euthanized with a lethal dose of sodium thiopental (1.0 g, 2 mL, Thiopentax, Cristália Produtos Químicos Farmacêuticos, Itapira).

**Experimental Animals**
Twenty New Zealand rabbits of approximately 3.5 kg of weight and 5 to 6 months of age were chosen for the experiment.

**Housing and Husbandry**
The animals were kept in individual cages maintained in rooms with a controlled environment located at the experimental facilities of the School of Dentistry, USP, Ribeirão Preto (Brazil). Wounds, pain, infections, and biologic functions were monitored for the full period of the experiment. Food and water were provided without restrictions.

Oxytetracycline dehydrate (40 mg/kg, IM, Terramicina LA, Zoetis Indústria e Produtos Veterinários) was injected in all rabbits. After the surgery, ketoprofen (3.0 mg/kg, IM, Ketofen, Merial) and tramadol hydrochloride subcutaneously (Tramadol 2%, 1.0 mg/kg, Cronidor, Agener União Saúde Animal) were administrated.

**Sample Size**
Given that no data were available on similar treatment on xenograft with argon plasma, a difference of 10% of bone formation within the elevated space was considered of clinical relevance. Using a standard deviation of 10% with a power of 0.8 and an $\alpha = .05$, a sample of 10 animals in each group was considered enough to disclose differences.

**Randomization and Allocation Concealment**
The randomization was made by a researcher not included in the present article using the website www.randomization.com. Sealed opaque envelopes were...
Histologic Preparation and Analyses

Biopsy specimens were obtained in blocks and fixed in 10% buffered formalin. Dehydration and inclusion in resin were performed, and two ground sections were prepared following a procedure reported in a previous article.21 The ground sections were stained with either Stevenel's blue and alizarin red or toluidine blue. High-resolution photomicrographs (100× magnification) were digitally captured using a digital video camera (Digital Sight DS-2Mv, Nikon Corporation) connected to an Eclipse Ci microscope (Nikon Corporation) and using an EK14 motorized stage (Nikon Corporation). The morphometric evaluation was carried out by an expert examiner (K.A.A.A.) in both histologic slides of each sinus with the software NIS-Elements D 5.11 (Laboratory Imaging, Nikon Corporation) applying a lattice composed of 16 × 10 squares of 75 microns (1,200 × 750 microns; 0.9 mm²) in dimensions superposed to the images. The sinus was evaluated for tissue components, and mean values were obtained for each tissue. Moreover, the tissue content of the specific areas, i.e., near the bone walls (bone wall region) and in the most central area of the elevated space (central region) were also evaluated separately. The central region was defined as the area equally distant from the periphery of the elevated area. The following tissues were assessed: new mineralized bone, soft tissues, xenograft, vessels, osteoclastic zones, and inflammatory infiltrate.

Calibration for Histometric Evaluations

An expert examiner not included as an author, and masked about the allocation of the treatments, did all histologic assessments, after having performed a calibration with another expert, also not included as an author (see Acknowledgments). A K > 0.90 interrater agreement was reached.

Experimental Outcomes

The primary variable was the percentage of new bone within the elevated space and in the various regions evaluated. The other tissues were considered as secondary variables.

Statistical Methods

The Wilcoxon test was used to estimate differences between the plasma (test) and the control groups. The software IBM SPSS Statistics (IBM) was used for statistical analyses. An α = 5% was applied.

RESULTS

During the surgery, only one small perforation of approximately 0.5 mm was detected in the 2-week control group, which was protected with a small piece of collagen membrane.
At the end of the study, no complications were reported for any animal and all biopsy specimens and histologic slides were available for analysis, so n = 10 was achieved for both periods included in the study.

**Histologic Analysis in the Elevated Region**

After 2 weeks of healing (Fig 2a), similar amounts of new bone were found within the elevated area, both for the plasma group (5.2% ± 2.9%) and the control group (5.0% ± 3.5%). A high amount of xenograft was found, in similar proportions in the two groups, with 49.1% ± 5.8% and 48.1% ± 7.4% in the plasma and control groups, respectively. No differences were found for any of the parameters evaluated (Table 1).

After 10 weeks of healing (Fig 2b), only a tendency of higher content of new bone was assessed in the plasma group (23.5% ± 7.0%) compared with the control group (21.3% ± 7.3%). However, the difference was not statistically significant. The xenograft percentages decreased compared with the previous period of healing to 38.0% ± 4.1% and 36.0% ± 5.6% in the plasma and control groups, respectively. No differences were found for any of the parameters evaluated (Table 1).

The area and height of the elevated space were 17.8 ± 4.3 mm² and 4.3 ± 0.6 mm for the plasma group and 18.2 ± 2.3 mm² and 4.2 ± 0.6 mm for the control group, respectively. No statistically significant differences were found between groups.

**Histologic Analysis in the Bone Walls and Central Regions**

Two different regions were also evaluated separately: the bone walls and the central regions. After 2 weeks of healing, the highest amount of new bone was found in the bone wall regions in similar amounts in both groups (Figs 3a to 3d). Bone was sprouting from the sinus bone walls toward the elevated space, often incorporating the closest xenograft particles. Small amounts of new bone were instead found in the central region in both groups. No statistically significant differences were found for any of the parameters assessed between the plasma and control groups (Table 2; Fig 4).

After 10 weeks of healing, bone increased in both regions examined, again with the highest proportions in the bone wall region. The only statistically significant difference was found in the central region (Figs 5a to 5d), in which higher amounts of new bone were found in the plasma group (20.4% ± 9.7%) compared with the control group (13.2% ± 10.5%), while no difference was found in the bone wall region (Table 3; Fig 4).

**Table 1** Tissue Components in Elevated Area in the Plasma and Control Sites After 2 and 10 Weeks of Healing

<table>
<thead>
<tr>
<th></th>
<th>New mineralized bone</th>
<th>Soft tissue</th>
<th>Xenograft</th>
<th>Vessel</th>
<th>Osteoclast</th>
<th>Infiltrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 weeks</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>5.2 ± 2.9 (3.4; 7.0)</td>
<td>42.5 ± 3.9 (40.0; 44.9)</td>
<td>49.1 ± 5.8 (45.5; 52.7)</td>
<td>2.2 ± 1.5 (1.3; 3.1)</td>
<td>0.7 ± 0.4 (0.5; 1.0)</td>
<td>0.3 ± 0.9 (–0.2; 0.8)</td>
</tr>
<tr>
<td>Control</td>
<td>5.0 ± 3.5 (2.8; 7.1)</td>
<td>43.5 ± 7.1 (39.1; 47.9)</td>
<td>48.1 ± 7.4 (43.6; 52.7)</td>
<td>1.8 ± 1.1 (1.1; 2.5)</td>
<td>0.8 ± 0.5 (0.5; 1.0)</td>
<td>0.8 ± 2.3 (–0.6; 2.2)</td>
</tr>
<tr>
<td>P</td>
<td>.760</td>
<td>.978</td>
<td>.878</td>
<td>.608</td>
<td>.674</td>
<td>.285</td>
</tr>
<tr>
<td>10 weeks</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>23.5 ± 7.0 (19.1; 27.8)</td>
<td>35.4 ± 7.2 (30.9; 39.9)</td>
<td>38.0 ± 4.1 (35.4; 40.5)</td>
<td>2.9 ± 1.1 (2.2; 3.5)</td>
<td>0.2 ± 0.1 (0.2; 0.3)</td>
<td>0.1 ± 0.2 (–0.1; 0.2)</td>
</tr>
<tr>
<td>Control</td>
<td>21.3 ± 7.3 (16.8; 25.8)</td>
<td>39.2 ± 10.1 (33.0; 45.5)</td>
<td>36.0 ± 5.6 (32.5; 39.5)</td>
<td>3.1 ± 1.3 (2.3; 3.9)</td>
<td>0.3 ± 0.2 (0.1; 0.4)</td>
<td>0.1 ± 0.2 (0.0; 0.2)</td>
</tr>
<tr>
<td>P</td>
<td>.169</td>
<td>.066</td>
<td>.053</td>
<td>.441</td>
<td>.777</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Mean values ± standard deviation and 95% confidence interval (upper; lower) in percentages.
None of the differences was statistically significant.
The area and height of the elevated space were 17.4 ± 2.3 mm² and 4.6 ± 0.5 mm for the plasma group, and 17.7 ± 1.9 mm² and 4.6 ± 0.5 mm for the control group, respectively. No statistically significant differences were found between groups. Moreover, no statistically significant differences were found in changes of areas and height between 2 and 10 weeks of healing.

**DISCUSSION**

The present study showed that after only 2 weeks of healing, a similar pattern (P = .795) of healing was observed in a sinus elevated using untreated graft material or graft material previously bioactivated using plasma of argon. At this stage of healing, higher amounts of new bone were found in the region close to the sinus bone walls.

The area and height of the elevated space were 17.4 ± 2.3 mm² and 4.6 ± 0.5 mm for the plasma group, and 17.7 ± 1.9 mm² and 4.6 ± 0.5 mm for the control group, respectively. No statistically significant differences were found between groups. Moreover, no statistically significant differences were found in changes of areas and height between 2 and 10 weeks of healing.
After 10 weeks of healing, the amounts of new bone increased in both groups, presenting slightly higher values in the plasma group (23.5% ± 7.0%) than in the control group (21.3% ± 7.3%). However, the difference was not statistically significant ($P = .169$).

On the other hand, when bone regeneration was analyzed by regions, differences were disclosed after 10 weeks of healing. In both bone walls and central regions, higher amounts of new bone were found in the plasma compared with the untreated control group. However, only the central region presented a significant difference ($P = .037$) in favor of the plasma group (20.4% ± 9.7%) compared with the untreated control group (13.2% ± 10.5%).

The higher amounts of bone formation were found close to the bone walls in both periods examined. In fact, it has been shown that bone regeneration inside the sinus floor, once elevated to the mucosa, starts in the regions close to the bony wall both with $^{22-24}$ and without biomaterial.$^{25}$ In an animal experiment, $^{22}$ sinus augmentation was performed in 10 minipigs using either a deproteinized bovine bone mineral or an aqueous paste of synthetic nanoparticulate hydroxyapatite. Three regions were evaluated at different distances from the sinus bone wall: at a distance of 0 to 1 mm, the region where new bone grows from the parent bone; at 2 to 3 mm, defined as the region where new bone is formed on the biomaterial, and at 4 to 5 mm, the region where bone reached the maximal extension.

This allows the conclusion that the most critical regions are those farthest from the pro-osteogenic cell source. In the present study, two regions were selected to evaluate a possible effect of the plasma treatment. One region was in the area where most bone is produced: the bone wall region. This allowed evaluation of the effect of the plasma treatment in zones with high

### Table 3 Healing After 10 Weeks: Tissue Components in the Bone Walls and Central Regions in the Plasma and Control Sites

<table>
<thead>
<tr>
<th></th>
<th>New mineralized bone</th>
<th>Soft tissue</th>
<th>Xenograft</th>
<th>Vessel</th>
<th>Osteoclast</th>
<th>Infiltrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean values ± SD</td>
<td>95% CI</td>
<td>Mean values ± SD</td>
<td>95% CI</td>
<td>Mean values ± SD</td>
<td>95% CI</td>
</tr>
<tr>
<td>Bone wall</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>28.2 ± 7.7 (23.5; 33.0)</td>
<td>33.4 ± 7.9 (28.5; 38.3)</td>
<td>35.1 ± 7.0 (30.7; 39.4)</td>
<td>3.1 ± 1.5 (2.2; 4.0)</td>
<td>0.1 ± 0.1 (0.0; 0.2)</td>
<td>0.1 ± 0.2 (–0.1; 0.2)</td>
</tr>
<tr>
<td>Control</td>
<td>26.3 ± 8.1 (21.3; 31.4)</td>
<td>35.9 ± 11.5 (28.8; 43.0)</td>
<td>34.2 ± 5.9 (30.6; 37.9)</td>
<td>3.4 ± 1.1 (2.7; 4.1)</td>
<td>0.1 ± 0.2 (0.0; 0.3)</td>
<td>0.0 ± 0.0 (0.0; 0.0)</td>
</tr>
<tr>
<td>$P$</td>
<td>.333</td>
<td>.721</td>
<td>.959</td>
<td>.212</td>
<td>1.000</td>
<td>.317</td>
</tr>
<tr>
<td>Central</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>20.4 ± 9.7* (14.4; 26.4)</td>
<td>36.9 ± 12.8 (29.0; 44.8)</td>
<td>39.7 ± 10.6 (33.1; 46.3)</td>
<td>2.6 ± 1.8 (1.5; 3.7)</td>
<td>0.4 ± 0.4 (0.2; 0.6)</td>
<td>0.0 ± 0.0 (0.0; 0.0)</td>
</tr>
<tr>
<td>Control</td>
<td>13.2 ± 10.5* (6.7; 19.7)</td>
<td>47.0 ± 15.8 (37.2; 56.8)</td>
<td>36.1 ± 9.6 (30.2; 42.1)</td>
<td>3.3 ± 2.3 (1.9; 4.8)</td>
<td>0.3 ± 0.4 (0.1; 0.5)</td>
<td>0.0 ± 0.0 (0.0; 0.0)</td>
</tr>
<tr>
<td>$P$</td>
<td>.037</td>
<td>.086</td>
<td>.445</td>
<td>.553</td>
<td>.680</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Mean values (in bold) ± standard deviation and 95% confidence interval (upper; lower) in percentages.

* $P < .05$. 

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Fig 5 Photomicrographs of ground sections representing the healing at the central region after 10 weeks. (a, b) Plasma group. New bone was incorporating several granules of xenograft. (c) Control group; xenograft granules enclosed in new bone. (d) Control group; in some instances, only few granules were bounded by new bone, while others were bordered by a dense soft tissue. Objective $\times10$. Stained with either (a, c) Stevenel’s blue and alizarin red or (b, d) toluidine blue. Asterisks indicate examples of new bone (light blue), parent bone (light green), or xenograft granules (red).
bone productivity. In this area, the effect produced by the plasma treatment was limited, increasing the fraction of new bone by 1.9% compared with the control sites. However, in the center of the elevated zone, a more extensive effect produced by the plasma treatment was observed, increasing the bone production by 7.2% compared with the control sites. The center of the elevated sinus was the most suitable region to assess the effectiveness of the plasma treatment because of its location far from the osteogenic sources but, most importantly, because the implant is usually placed in this region. A source of new bone after sinus floor augmentation is represented by the parent bone, which will affect bone formation in the regions close to the bone walls, and the residual parent bone around the antrostomy. The sinus membrane has also shown to be a source of osteogenic progenitors, as reported by several studies.

The central region is located far from the parent bone and sinus membrane. Nevertheless, in the plasma group, a significantly higher bone regeneration rate was highlighted. This could be explained by analyzing the biophysical characteristics of the graft material after bioactivation by plasma. As demonstrated in an in vitro study, the increased surface energy results in higher wettability and therefore in an increased pattern of protein adsorption. This process was demonstrated to be even more significant in the case of porous material, such as the one used in the present study. In fact, a cancellous animal-derived bone matrix, after bioactivation, may present a much higher increased adsorption rate compared with a nonporous bioactivated scaffold.

The positive effect of a plasma treatment was also demonstrated on titanium implants in an experimental study in dogs. The surface of the implants was treated with argon plasma or left untreated. After 2 months of healing, higher amounts of bone-to-implant contact were found at the treated (72.5%) compared with the untreated implants (64.7%), with the difference being statistically significant.

As a limitation, it should be considered that the animal model used has exhibited a faster healing rate compared with humans, so any inference with the healing in humans should be first confirmed by histologic clinical studies.

An additional limitation of the present study is represented by the short follow-up. This approach, in fact, could hide whether or not bioactivation has the ability to even speed up the remodeling process of the graft material. Further studies in this direction should be performed to answer this question. Another limitation of the present study is the use of the sole histologic analysis. Further studies should be performed with sensible methods (such as immunohistochemistry) to detect a more comprehensive vision of the remodeling process.

CONCLUSIONS

The bioactivation of a xenograft using argon plasma might improve bone formation within an augmented sinus, especially in the central regions, far from the osteogenic sources.

ACKNOWLEDGMENTS

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