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THESIS TITLE

Sinus mucosa thinning and perforations after sinus lifting performed with different xenografts. A histological analysis in rabbits.

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Abstract:

Background: Experimental studies have shown a progressive thinning and perforations associated to sharpen edges and the cutting projections of grafts used simultaneously to maxillary sinus augmentation. Hence, the aim of the present experiment was to evaluate the damaging effects of granules of bovine xenografts of different conformations on the sinus mucosa after sinus augmentation. Methods: Twenty New Zealand rabbits received a bilateral sinus lifting using as fillers two different types of deproteinized bovine bone in granules, one processed at low temperature (low-T group), and the other at high temperature (high-T group). Thinned mucosa sites (<40 μm) and perforations were evaluated at the sinus mucosa in contact with graft granules after 2 and 10 weeks, in ten animals each period. Results: After 2 weeks of healing, the number of thinned mucosa sites were 118 in the low-T group, and 149 in the high-T group (p=0.191). At the 10-week assessment, the thinned sites increased to 237 and 195 sites, respectively. The number of sinus mucosa perforations after 2 weeks was 8 and 3 in the low-T and high-T groups, respectively. At the 10-week evaluation, the perforations increased to 19 in the low-T group, and to 14 in the high-Y group. Conclusions: The contact with bovine xenografts yielded thinning and perforations of the sinus mucosa. Despite the differences in characteristics and dimensions, no differences were found between the two xenografts in number of thinning mucosa sites and perforations. However, a trend of more events was found at the low-T compared to the high-T groups.

Keywords: animal study; sinus floor elevation; bone healing; Schneiderian membrane; histology

1. Introduction

The use of endosseous implants represents a well-known therapeutic option for the rehabilitation of atrophic jaws. Conventional implantology is able to achieve good long-term results in patients who have an adequate bone volume. Nevertheless, the lack of sufficient bony vertical or horizontal dimension may convey a challenge to the clinician.

Advanced bone resorption may occur after tooth loss following extractions, trauma, infections or in case of strongly pneumatized maxillary sinuses; the severity of bone resorption can reach an extent such to impair the placement of implants. Several bone augmentation techniques have been described, such as the sinus lift, onlay bone grafts, Le Fort I osteotomy with interpositional grafts and even microsurgical free flaps (Fig. 1, 2, 3).

For over 30 years bone grafts, positioned either before or simultaneously to implants, have been an everyday tool in the hands of oral surgeons.

To increase the volumes in the posterior segment of the maxilla a sinus floor augmentation procedure is often applied to allow the placement of dental implants aiming to rehabilitate that region. This technique has been proved to have a high success rate [1,2]. One of the most common complication during surgery is the perforation of the sinus mucosa [3,4]. Perforations have been also observed during the placement of biomaterial or implants in the subantral space after sinus mucosa elevation [5-7]. Even though these perforations might heal spontaneously [6], sinusitis have been associated to extruded biomaterial [8-10], event that required the removal of the graft remnants from the sinus cavity [9-10].

Perforations have been also observed over time, triggered by both biomaterials and implants. In experiments on maxillary sinus lifting in rabbits [11-13], a progressive thinning of the sinus mucosa and a progressive increased number of perforations were observed over time. Thinning mucosa and perforations were seen on sharpen edges and cutting projections of grafts made of deproteinized bovine bone mineral (DBBM) [11,12]. However, when autogenous bone was used as filler, no perforations, and few thinning mucosa sites were observed after 7 and 40 days of healing [11]. Thinning sinus mucosa regions and perforations were also reported on implant apex and threads in experiments in rabbits [11,13].

The characteristics and rate of resorption varies for different grafts [11,14] and the dimensions of the graft granules might influence the number of sinus mucosa thinned sites and perforations [12]. It seemed rational to perform studies aiming to evaluate differences on the effect of various grafting biomaterial on the sinus mucosa. Hence, the aim of the present study was to evaluate the damaging effects of granules of

xenografts with different characteristics on the sinus mucosa after sinus augmentation.

2. Materials and Methods

2.1 Ethical statements

The experimental protocol was submitted to and approved by the Ethical Committee of the Faculty of Dentistry of Ribeirão Preto, University of São Paulo (protocol No 2019.1.113.58.1; April 8, 2019). The article was written according to the ARRIVE guidelines. The Brazilian guidelines for animal care were accurately followed.

2.2 Study design

A split-mouth design was adopted. Maxillary sinus augmentation was carried out bilaterally in twenty rabbits. Deproteinized bovine bone grafts of different characteristics were randomly placed one each sinus.

The number and width of sites presenting sinus mucosa reduced in dimensions and the number of perforations of the sinus mucosa in contact with the filler material were assessed. The histomorphometric data describing the healing within the elevated region were reported elsewhere [15].

2.3 Experimental animals

Twenty albino New Zealand rabbits, 3.5-4 kg of weight and 4-5 months old, were used for the experiment. Two groups were obtained, ten animals each, and euthanized after 2 or 10 weeks from surgery, respectively.

2.4 Biomaterials

Two biomaterials processed at different temperatures were used.

Low-T (low temperature): Bio-Oss® (Geistlich Biomaterial, Wolhusen, LU, Switzerland) is a natural inorganic, porous hydroxyapatite from bovine cancellous bone obtained through a deproteinization process at a temperature of 300°C. Bio-Oss® has high porosity (70.5%), low level of hydrophilicity, low crystallinity and an amorphous structure [16,17]. (Lee JH. Et al , Trajkovski B. et al)

High-T (high temperature): Cerabone® (Botiss Biomaterials GmbH, Zossen, Germany) is composed of a ceramic hydroxyapatite (pentacalcium hydroxide trisphosphate) from bovine cancellous bone produced at a high-temperature process (>1200 ºC). Cerabone® possess a foam-like surface structure with low porosity (62.0%) high hydrophilicity, high crystallinity, with very low impurities level [16,17]. (Lee JH. Et al , Trajkovski B. et al)

2.5 Sample size

The sample was determined for the histomorphometric study performed on the same experiment [15]. It was calculated a sample of 10 animals each group to allow finding statistical significance differences in bone formation within the elevated space between the experimental groups. For the present study, two experimental articles were taken into consideration. In one study [18], it was shown that the particle size of high-T granules was 2.7 times larger than low-T granules. Moreover, in another study in which six animals were used for each period of evaluation [12], after 8 weeks of healing, the number of perforations of the sinus mucosa was double at the smaller (0.125-1.0 mm) compared to the larger (1-2 mm)

granules of a DBBM graft. Considering that also in the present study the biomaterials used have different sizes, one smaller than the other, ten animals were considered sufficient to disclose differences in number of perforations of the sinus mucosa.

2.6 Randomization and allocation concealment

The randomization plan was created electronically at randomization.com by an author who was not involved in the selection and handling of animals and/or surgical procedures. The treatment assignments were held in opaque sealed envelopes. After the sinus mucosa was elevated bilaterally, the envelopes were open and the treatment assignment was disclosed to the surgeon.

2.7 Clinical procedures

The anesthetic procedures included the use of 1.0 mg/kg of acepromazine (Acepran®, Vetnil, Louveira, São Paulo, Brazil) subcutaneously and a mix of 3.0 mg/Kg xylazine (Dopaser®, Hertape Calier, Juatuba, Minas Gerais, Brazil) and 50mg/kg of ketamine hydrochloride (Ketamin Agener, União Química Farmacêutica Nacional S/A, Embu-Guaçú, São Paulo, Brazil) intramuscular. After shaving and disinfection of the region, an incision of the midline of the nasal dorsum was performed by an expert surgeon (V.F.B.). The nasal bone was exposed and two antrostomies were prepared bilaterally of the nasal-incisal suture using a trephine and a round diamond drill (Figure 4A). A small screw was placed in the nasal-incisal suture as reference for the histological process. The sinus mucosa was lifted using a small elevator (718-EN1; Bontempi Strumenti Chirurgici, San Giovanni in Marignano, RN, Italy) and similar quantity of grafts were placed in the elevated spaces.

(Figure 4B). The antrostomies were subsequently covered with collagen membranes (Figure 4C; Geistlich Biomaterial, Wolhusen, LU, Switzerland).

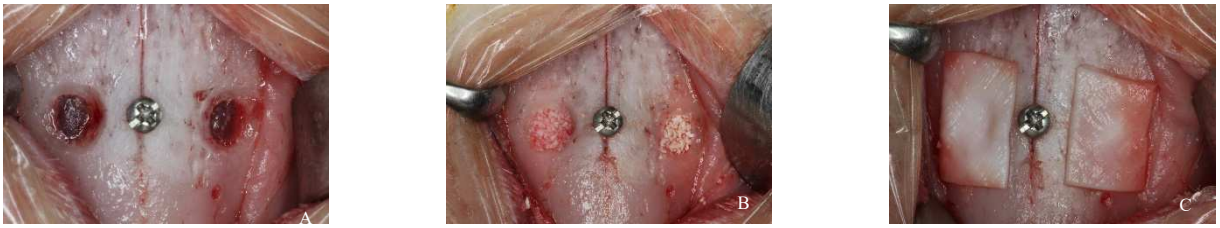

Figure 4. Clinical overview of the surgical procedures. A, antrostomy preparation; B, elevated space filled with grafts; C, collagen membrane covering the antrostomies.

2.8 Euthanasia

The rabbits were anesthetized with xylazine (3.0 mg / kg IM, Anasedan®, Sespo Indústria e Comércio LTDA, Paulínia, São Paulo, Brazil) and ketamine (50.0 mg / kg IM, Dopaser®, Sespo Indústria e Comércio LTDA, Paulínia, São Paulo, Brazil). Euthanasia was performed in a closed transparent acrylic box containing gas carbon dioxide (CO2).

2.9 Housing and husbandry

The animals were kept in individual cages in a climatized room with access to food and water ad libitum. The biological functions and the wounds were checked daily by specialized operators for the whole period of the experiment.

2.10 Histological preparation

The specimens containing the experimental regions were kept in formalin and then dehydrated and embedded in resin (LR White™ hard grid, London Resin Co Ltd, Berkshire, UK) and polymerized. Two grounds sections were obtained using a cutting and grinding equipment (Exakt®, Apparatebau, Norderstedt, Germany) and stained with either Stevenel's blue and alizarin red or toluidine blue.

2.11 Calibration for histometric evaluations

All measurements were carried out by a well-trained assessor (K.A.A.A.). The intra-rater reliability in the measurements of the sinus mucosa width and perforation dimensions was K >0.90.

2.12 Histological analyses

The pristine mucosa was measured at the medial and lateral sinus walls in regions not included in the elevated area. A mean value of the two measurements was used for analysis.

The number and width of the sinus mucosa in close contact TO the graft granules was measured, and all measurements <40 μm were recorded. The number and dimensions of the sinus mucosa perforations at the graft granules were assessed.

2.13 Experimental outcomes and statistical methods

Prism 9.1.1 (GraphPad Software, LLC, San Diego, CA, USA) was used for statistical analyses. The normal distribution of the data was assessed with the Shapiro-Wilk test for both paired and unpaired variables. Either a paired t test or a Wilcoxon test was used to evaluate differences between low-T and high-T groups. Differences between the two periods of healing were evaluated using either an unpaired t test or a Mann-Whitney test.

3. Results

3.1 Clinical outcomes

One sinus mucosa of the low-T group in the 2-month period presented a small perforation during surgery and was protected with a small piece of collagen membrane. The collagen membrane, still present after 2 weeks, was hindering a large part of the mucosa from any contact with the biomaterial granules. For this reason, this sinus was excluded from analysis together the contralateral sinus. No animals presented problems during healing so that the sample size was n=9 for the 2-week period, and n=10 for the 10-week period.

The anatomical situation after 10 weeks of healing is illustrated in a microCT image (Figure 5A).

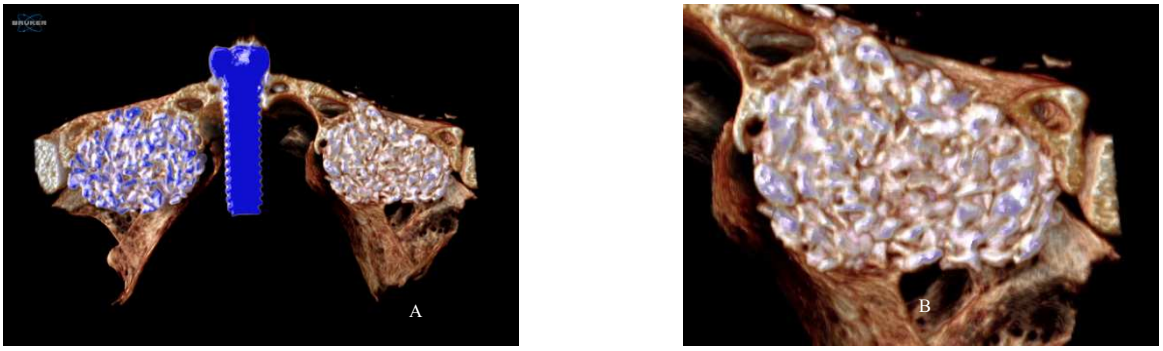

Figure 5. MicroCT images showing: A, the location of the elevated spaces with respect to the nasal cavities; B, graft granules presenting cutting edges protruding beyond the elevated space profile.

3.2 Descriptive histological evaluation

More than one hundred thinned mucosa sites (<40 μm) were found in both groups, and the number increased at the 10-week compared to the 2-week evaluations. The thinned mucosa sites were associated with granules, mainly at surfaces protruding beyond the dome profile of the

elevated spaces (Figure 5B). The mucosa was found in tight contact to the granules surface and the tissues contained in the submucosa were affected by this contact. The degree of damage showed a progressive trait. In the first stages, a dislocation of vessels and mucous glands was observed, while the pseudostratified epithelium was not affected yet (Figures 6A and 7A). However, at the thinnest sites, the epithelium became involved in the damage, presenting a progressive decrease of width and a loss of cilias and globet cells (Figures 6B and 7B). Finally, only a very thin layer of soft tissues was laying on the graft surface (Figures 6C and 7C).

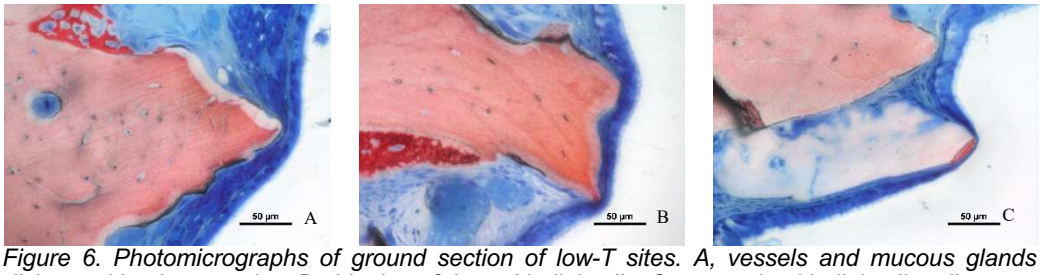

Figure 6. Photomicrographs of ground section of low-T sites. A, vessels and mucous glands dislocated by the granules; B, thinning of the epithelial cells; C, tapered epithelial cells adjacent a very thin remnant of sinus mucosa. Stevenel's blue and alizarin red stain.

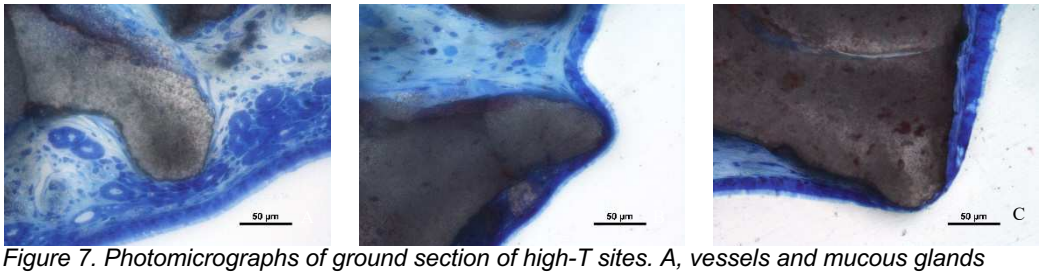

Figure 7. Photomicrographs of ground section of high-T sites. A, vessels and mucous glands dislocated by the granules; B, thinning of the epithelial cells still presenting cilias; C, tapered epithelial cells adjacent a very thin remnant of sinus mucosa. Stevenel's blue and alizarin red stain.

Perforations of the sinus mucosa were observed in both periods and biomaterials (Figures 8A-C and 9A-C). Granules were found trespassing the sinus mucosa and the perforations were bordered by a tapered epithelium. In some instances, the regions presented any or few inflammatory cells (Figures 8A,B). However, in other cases, an inflammatory infiltrate was encompassing the granules (Figure 8C).

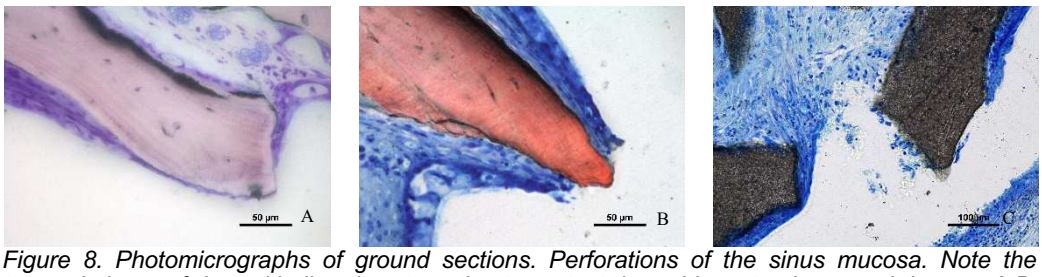

Figure 8. Photomicrographs of ground sections. Perforations of the sinus mucosa. Note the tapered shape of the epithelium in a tentative to protect the subjacent submucosal tissues. A,B, inflammatory cells were rare; C, inflammatory reaction; note the epithelium trying to isolate the granule on the left side of the image.

Some granules were found expelled through the sinus mucosa (Figures 9A-C).

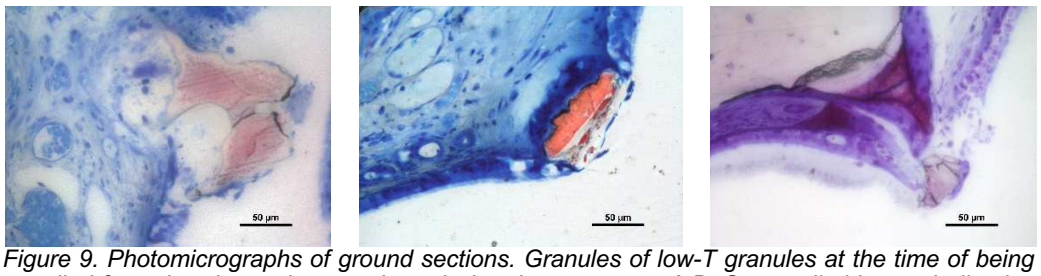

Figure 9. Photomicrographs of ground sections. Granules of low-T granules at the time of being expelled from the elevated space through the sinus mucosa. A,B, Stevenel's blue and alizarine red stain; C, toluidine blue stain.

3.3 Histometric assessments

The width of the pristine sinus mucosa was similar in both groups and periods (mean values range 54 µm and 61; Table 1). After 2 weeks of healing, in the low-T group, the sites of the sinus mucosa presenting a width <40 µm were 118, eleven of which were <10 µm (Table 2). The minimum width registered was 5 µm. Eight perforations were found in the low-T group in six sinuses. The largest perforation was about 2.4 mm in dimension, while five perforations were comprised between 100-500 µm, and two were <100 µm.

In the high-T group, after 2 weeks of healing, 149 sites presented a mucosa width <40 µm, 16 of which were <10 µm, and 2 µm was the lowest

value registered. Three perforations in three sinuses were found, presenting dimensions of about 700 μm, 300 μm, and 40 μm, respectively. In the 2-week period, no statistically significant differences were found between groups for any variable analyzed.

Table 1. Histometric data.

	2 weeks			**10 weeks**		
	low-T	high-T	P value	low-T	high-T	P value
Pristine mucosa in μm	61 ±21	63 ±14	0.617	62 ±12	54 ±8	0.107
Thinned mucosa in μm	26 ±3.2	26 ±5.3	0.789	19 ±3.0	21 ±4.5	0.065
No. sinus with thinned mucosa	8	9	>0.9999	10	10	NA
No. thinned mucosa zones	118	149	0.191	237	195	0.090
No. of sinuses with perforations	6	3	0.375	9	7	0.625
No. of perforations	8	3	0.188	19	14	0.898

Table 2. Number of sites of thinned mucosa categorized according to the width.

	<40	<30	<20	<10
2 weeks low-T (n=9)	118	73	38	11
2 weeks high-T(n=9)	149	98	58	16
10 weeks low-T (n=10)	237	198	137	46
10 weeks high-T (n=10)	195	148	96	44

After 10 weeks of healing, in the low-T group, all sinuses presented thinned mucosa, reaching 237 sites in total (Table 1). The mean width was 19 μm and 46 sites were <10 μm, the lowest being 2 μm (Table 2). Nineteen perforations, distributed among 9 sinuses, were found in this period of healing. Six of these perforations presented dimensions included between 100-200 μm, while the remaining thirteen were <100 μm, of which seven were <50 μm.

In the high-T group, after 10 weeks of healing, 195 thinned sites were found in ten sinuses. The mean width was 21 μm, and 44 sites presented a width <10 μm, being 2 μm the lowest width observed. Fourteen perforations were seen in seven sinuses, nine of which exhibiting dimensions between 100-700 μm and five <100 μm. In the 10-week period,

no statistically significant differences were found between groups for any variable analyzed.

The difference between the width of the pristine and thinned mucosae were statistically significant for both groups and periods. Considering the difference between periods, only the width of the thinned mucosa, and the number of thinned sites of the low-T xenograft were statistically significant.

4. Discussion

The aim of the present study was to evaluate the damaging effects of granules of bovine xenografts of different conformations on the sinus mucosa after sinus augmentation. Both xenograft granules yielded damages to the sinus mucosa, dislodging the soft tissues in the submucosa region, decreasing the mucosa thickness associated to loss of cilia and, finally, perforating the sinus mucosa. The number of thinned sites (<40 μm of width) and perforations increased over time. No statistically significant differences were found between the two xenografts. However, a tendency of presenting more thinning sites and perforations was observed for the low-T group compared to the high-T group. The reason of this tendency might be attributed to the larger size of high-T graft compared to the low-T [18] that increased the frequency of contacts between grafts and sinus mucosa.

After 2 weeks of healing, six perforations in 3 sinuses in the low-T group, and eight perforations in 3 sinuses in the high-T group were observed. It could be speculated that these perforations might have been procured, and not recognized, during surgery, or generated by the pressure applied to the biomaterial during the filling of the elevated space. Indeed, perforations of the sinus mucosa during the surgical procedures

had been documented both in human [9,10] and in ex-vivo [8,19,20] studies. Also in the present study one perforation was observed during elevation and protected with a collagen membrane. However, the number of perforations increased over time and this deposes against a mucosal damage only related to the surgical injury. Moreover, the dimensions of the perforations after 2 weeks appeared to be larger than that after 10 weeks. In the low-T group, in the two-week period, eight perforations were found, of which one measured 2.4 mm, five between 100-500 μm and two <100 μm. In the same group, after 10 weeks, all nineteen perforations were <200 μm. It might be hypothesized that the largest perforations of the 2 weeks period could be ascribed to surgical trauma. However, the smallest perforations, and those encountered at the 10-week period are more likely to have occurred over time, through the progressive thinning and injuring processes at the sinus mucosa discussed above. In the high-T group the perforations increased in number over time as well. However, large dimensions of the perforation were found also in the 10-week period. The difference with the low-T group in dimensions might be related to the size of the granules, being the high-T 2.7 times larger than the low-T ones [18].

Reparative processes were observed in the soft tissues at the perforations. However, these processes appeared to be a tentative of circumscribing the granules and, eventually, to expel them outwards the elevated space instead of trying to keep them in.

The decreased width of the sinus mucosa over time also provides support to the progressive increase number of perforations. Again, it might be argued that the thinning of the mucosa might have happened during surgery. However, damages such as displacement of tissues components, progressive decrease of submucosa and epithelial layers width, and loss of cilias cannot be procured by the pressure applied to the biomaterial during

the filling of the elevated space. The thinned sites increased in number over time, and this deposes once again in favor of a progressive damage to the sinus mucosa. Between 2 and 10 weeks of healing, the thinned mucosa increased by >100 sites in the low-T, and by about 50 sites in high-T groups. Moreover, in the 10-week period, 44-46 thinned sites presented a width <10 μm.

The outcomes obtained in the present study are in complete agreement with other studies in rabbits that disclosed an increased number of perforations over time, and hundreds of thinned mucosae sites [11-13]. In one of these studies [12], sinus augmentation was performed in eighteen rabbits using DBBM granules either 0.125-1 mm (small group) or 1-2 mm (large group) in dimension. The healing was studied after 2, 4 and 8 weeks, six animals each group. Between 2 and 8 weeks, the thinned sites increased from 52 to 59 at the large group, and from 55 to 74 in the small group. The perforations increased from 1 to 5 in the large group, and from 0 to 8 in the small group. These outcomes correspond to those observed in the present study in which the number of thinned mucosae and perforations increased to a higher extent in the smallest (low-T) compared to the largest granules group (high-T).

In another similar study in rabbits [11], the sinuses were augmented with either a DBBM or autogenous bone, and implants were placed simultaneously. The healing was studied after 7 and 40 days in twelve rabbits, six animals each period. In the DBBM group, between the two periods, the thinned mucosa sites in contact with the granules increased in number from 59 to 96 while, in the autogenous group, the thinned sites decreased from 14 to one. After 40 days, three perforations were found in the DBBM group while no perforations were found in the autogenous group. However, in the 40-day period of healing, owing to the different rate

of resorption of the two biomaterials, the sinus mucosa became in contact with the apex and threads of the implants to a higher extent at the autogenous compared to the DBBM groups. This condition yielded three perforations at implants in two sinuses in the autogenous group, and only one perforation in one sinus in the DBBM group while no perforations at implants was registered after 7 days of healing in any group. This, in turn, means that a biomaterial with a low rate of resorption protects the sinus mucosa from a contact with the implant apex and threads. However, at the same time, this low resorption rate exposes the sinus mucosa to thinning and perforations caused by the graft itself. From a clinical point of view, however, it should be considered that the corticalization of the new sinus floor might protect for further perforations. In fact, a total or partial corticalization of the new floor have been documented in 30% to 75% of cases after 9 months of healing [21-24]. Moreover, a perforation of the sinus mucosa might be restored with the expulsion of the granules from the elevated space, while a similar outcome is not desirable for implants.

Possible perforations of the sinus mucosa at implants were addressed in an experiment in sixteen rabbits [13]. Implants, with the surface of the test implants exposed to an argon plasma treatment, were simultaneously placed in augmented sinuses without fillers. After eight weeks of healing, the sinus mucosa collapsed onto implant apex and threads. This tight contact triggered sinus mucosa perforations at apex and threads of twenty-six out of thirty-two implants. It should be noted that perforations of the sinus mucosa were also reported for resorbable [25,26] and not resorbable [27] devices used to keep elevated the sinusal mucosa during healing.

The collapse of the sinus mucosa at the top of biomaterials and implants might be explained by the tendency of the sinus to return to his original dimensions, as documented by experimental [28-32] and clinical

studies [21-24]. The sinus mucosa will adapt its shape to that of hard tissues and implants. In the first period of healing, the post-surgical edema/bleeding will keep the sinus mucosa away from granules and implants [33]. In the present study, after two weeks, the protective action of the edema was concluded and the sinus mucosa was already adapted to the shape of the surface of the elevated space, resulting in a high number of thinned mucosa sites.

The limitation of the present study is mostly related to the model used that present a thinner width of the sinus mucosa compared that in humans [34,35] so more prone to damages by the graft granules. However, a progressive thinning of the mucosa might be expected also in humans. Another limitation of the present study is the analysis performed in only two histological slides that represent only the central region of the elevated space. An analysis of the whole surface of the elevated mucosa would have disclosed many more damaged sites. Other limitations of the present study are the faster rate of healing compared to humans [36] and the short periods of healing assessed, that did not allow a complete corticalization of the new sinus floor. Different biomaterials should be tested aiming to find those producing the lowest damage to the sinus mucosa.

5. Conclusions

In conclusion, the contact with bovine xenografts yielded thinning and perforations of the sinus mucosa. Despite the differences in characteristics and dimensions, no differences were found between the two xenografts in number of thinning mucosa sites and perforations. However, a trend of more events was found at the low-T compared to the high-T groups.

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