Author's response to reviews

Title: Novel use of cranial epidural space in rabbits as an animal model to investigate bone volume augmentation potential of different bone graft substitutes

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Author's response to reviews:

Dear editor and reviewers:

First, we want to thank you for reading and commenting our text. We have carefully considered your observations. Herein, we explain how we revised the manuscript based on your comments and recommendations point by point. We want to extend our appreciation for taking the time and effort necessary to provide the guidance. You can find your comments with our answers immediately below.

In relation to comments and observations from reviewer # 1

Reviewer #1: The authors present an interesting pilot study. However, before publishing some topics have to be addressed.

- How was the area of access prepared? Was a shaving done? What agent was used for disinfection?

Shaving was done, and the surface was cleaned carefully with iodized alcohol. The excess was removed with sterile gauze. This information was added to the materials and methods section (pag 5, lines 18-19).

- How was the volume of 0.5 cc measured?

The sequence of instruments (osteotomes) used was intended to create a space for biomaterial insertion. However, your question is very important because we made a mistake in the transcription. The biomaterial volume inserted was 0,15 cc, not 0,5. The commercial presentation of biomaterial used (GUIDOR easy-graft) is provided in syringes with 0.15, 0.25 or 0.4 cc. We used the first (0.15 cc) with particles around 0.5- .63 mm diameter for CLASSIC and 0.45-0.63 mm diameter for CRYSTAL (these technical aspects about the biomaterial were not included in the text). Consequently, we made the corrections in the methods section (pag 6, lines 1 and 18).

- Was a postoperative antibiosis administered? How was postoperative analgesia performed?

In the postoperatory, antibiotherapy by oxytetracycline (1ml/kg subcutaneously every 12 hours for three days) and analgesia using meloxicam (0.2mg/kg once daily for three days) were administered to each animal. This information was added to the materials and methods section (pag 6, line 19-21).

- Were the sections (0.2 mm) thinned after BS-SEM evaluation for toluidin blue staining?

We think our text was not clear in this part. The measurement indicated (0.2 mm) corresponds to circular saw thickness. We improve this part of the text. In relation to the treatment of samples included in resin to obtain histological view by toluidine blue stain, each one was processed by wear and polish to obtain a thin sheet of 30-40 microns thickness which were subjected to staining processes. This information was added to the materials and methods section (Histomorphometric analysis, pag 8, lines 1-2).

- Discussion

In my opinion, one major setback is the fact that only sham-operated controls were used in the study. The authors want to create a new model for the evaluation of biomaterials under physiological pressure like e.g. in the maxillary sinus. It is a good idea. However, the authors should have thought about using autologous bone (which could have been easily obtained from the access) as control. For example, in the sinus maxillaris nobody would think about doing a sinus lift without inserting any space holder. The authors should at least discuss the possibility of using autologous bone.

The sites with sham operated controls were performed to demonstrate that the space created between meninges and internal cranial surface (called "bursa") will collapse if it is not filled with biomaterials (or autologous bone), because of the cerebrospinal liquid pressure on meningeal structures (as it is shown in the figure 2A, 2B and 4). Considering this objective, we think that the use of an additional specimen with autologous bone will be taken into account in future experimental protocols, but on this occasion may be less necessary. In this form, our study follows the principles of animal experimentation, in relation to the reduction of the number of specimens used and the refinement of technic. However, to clarify this aspect, we have modified the initial lines of discussion as follow:

“In our study, the bursa created in the epidural space was maintained when it was filled with biomaterial, even with the hydrodynamic intracranial pressure produced by cerebrospinal fluid and the natural meningeal vascularization. While, the bursa created in the sham operated side collapsed and the meninges returned to contact with the intracranial bone surface (fig 2A, 2B and 4)” (pag 9, lines 10-14).

Also, we added a paragraph in the discussion about the advantages/disadvantages of the autogenous bone use as a bone graft in maxillary sinus lift and as a further study in this new model.

“In the absence of bone graft, the dura mater tends to return to its original position contacting the bone surface. In similar form, sinus tends to recover its original shape after sinus lift because of the air pressure [1]. Consequently, the graft behaviour in the site of application is a relevant aspect to consider for maintaining the space created. Autologous bone has been considered as the “gold standard” for grafting the maxillary sinus floor [35] due to it has a higher rate of newly formed bone at the application site when it is compared to other bone substitutes [36,37]. However, other study showed no significant differences after nine months comparing autologous bone with others grafts considering the total bone volume gained [27,38]. Also, some disadvantages have been reported about the autogenous bone as an early resorption rate and a greater shrinkage of the elevated sinus floor space when compared with alloplastic materials [39,40]. A new protocol using epidural space that includes autologous bone is required for contributing the discussion.” (pag 12, lines 3-14).

Figures

The magnification should be mentioned in the figure legends as the magnification in figures 2 and 3 seem to be different.

The text of Figure 3 was changed to emphasise the magnification compared with Figure 2 (pag 23, lines 1-7). Additionally, the measurement bar in each image in figures 3 and 4, allows a better reference to the reader.

In relation to comments and observations from reviewer # 2

- Reviewer #2: The manuscript „Novel use of cranial epidural space in rabbits as an animal model to investigate bone volume augmentation potential of different bone graft substitutes" deals with an important and interesting research topic. The multivariate interaction of pressure on bone substitute materials, their degradation and the new bone formation was investigated with a novel animal model. The discussion is particularly noteworthy, since all findings and effects are compared with each other very well.

- In principle, there are some points to be named or explained more precise. Starting with the Abstract, where the topic of pressure - which is the main focus in the discussion - is not mentioned in the background part.

Thanks for your appreciation. For improving the abstract following your comments, we have emphasised the text in relation to the influence of cerebrospinal fluid pressure on biomaterial in the bursae created. The Background was modified as follow:

“Background: The success of bone augmentation to a major degree depends on the biomechanics and biological conditions of the surrounding tissues. Therefore, an animal model is needed providing anatomical sites with similar mechanical pressures for comparing its influence on different biomaterials for bone regeneration. The present report describes the new bone formation associated to biomaterial in a bursae created in the epidural space, between dura mater and cranial calvaria, under the constant pressure of cerebrospinal fluid” (pag 3, lines 4-9).

- The conclusions part of the abstract emphasizes in its second sentence the different information to be gained from this new defect model. This is quite ambitious as the degradation of the material and the pressure in this defect model cannot be separated from each other. Furthermore the new bone formation is depending on the degradation without a clear positive or negative correlation, as shown by the selected materials. Therefore the gained information on biomaterials should be limited to the very special application as void fillers under (comparatively low) pressure.

The conclusion was corrected and summarized in only one phrase:

“Conclusion: The experimental bursae created bilaterally in the epidural space allows comparing objectively bone formation in relation to biomaterials for bone regeneration under permanent physiological forces from cerebrospinal fluid pressure” (pag 4, lines 1-3).

- Please check, whether it is possible to explain the problem of permanent pressure on biomaterials (as done in the second paragraph of the discussion) in the background/introduction part of the manuscript. Please emphasize in the abstract/introduction that it is the aim to create a space and preserve it with the formation of new bone.

The background and introduction were corrected as suggested.

Anteriorly we described the modification made in the Background where we explain how the similar mechanical pressures are important for comparing its influence on different biomaterials for bone regeneration (pag 3, lines 4-9).

In the introduction, we have added the following text at the end of the second paragraph:

“In this anatomical site, biomaterial particles could be inserted in bursae atraumatically opened between the dura mater and the cranial vault surface at a distance of the bone defect created for accessing. This model may be an advantage to analyse if a specific biomaterial can maintain the space created being subjected to the constant hydrodynamic pressure of cerebrospinal fluid, which is transmitted through the dura mater to the biomaterial” (pag 4, línea 22; pag 5, lines 1-5).

Additionally, by the background (pag 3, lines 4-9) and the final paragraph of the introduction (pag 5, lines 6-8), we have reinforced the aim of the space created between dura mater and internal cranial surface.

- The consequence of the more mature bone recognized for BCP should be discussed more in detail.

- So far, it is not clear whether the lower bone formation in case of beta-TCP, which is directly connected to its degradation, is advantageous/disadvantageous compared to BCP, which seems to be not resorbed at all. A long time remaining BCP might be problematic for later implant stability, even when it is integrated into bone.

We have put together these comments for the following response:

We understand the reviewer's concern. However, we think that our text was unclear. It was corrected, and an explanation of resorption rates was added in the discussion section. The β-TCP showed a higher quantity of newly formed bone when compared to BCP (as stated in the results section and Table 1). However, an increase of mature bone was observed related to BCP. The literature report successful results with β-TCP as well as BCP in maxillary sinus floor elevation procedures.

1. Danesh-Sani SA, Wallace SS, Movahed A, El Chaar ES, Cho SC, Khouly I, Testori T. Implant Dent. Maxillary Sinus Grafting With Biphasic Bone Ceramic or Autogenous Bone: Clinical, Histologic, and Histomorphometric Results From a Randomized Controlled Clinical Trial. Implant Dent. 2016 Aug 10. [Epub ahead of print]

2. Giuliani A, Manescu A, Mohammadi S, Mazzoni S, Piattelli A, Mangano F, Iezzi G, Mangano C. Quantitative Kinetics Evaluation of Blocks Versus Granules of Biphasic Calcium Phosphate Scaffolds (HA/β-TCP 30/70) by Synchrotron Radiation X-ray Microtomography: A Human Study. Implant Dent. 2016 Feb;25(1):6-15.

3. Gorla LF, Spin-Neto R, Boos FB, Pereira Rdos S, Garcia-Junior IR, Hochuli-Vieira E. Use of autogenous bone and beta-tricalcium phosphate in maxillary sinus lifting: a prospective, randomized, volumetric computed tomography study. Int J Oral Maxillofac Surg. 2015 Dec;44(12):1486-91.

Consequently, the paragraph dealing with this part has been modified as follows:

"The content of newly formed bone observed in the specimen with the bilateral application of biomaterial was significantly higher with β-TCP compared to the site with BCP (Table 1). This result also could be explained by the different resorption characteristics of β-TCP particles compared to BCP. The β -TCP bonds to bone directly, which granules are rapidly dissolved and newly formed bone fill in the area of dissolved granules [42]. A higher degree of resorption may provide more space inside the β-TCP particles that can be occupied by newly formed bone

tissues. Oppositely, the BCP are composed of β-TCP and hydroxyapatite (HA) granules, observing that HA adheres directly to bone through a collagen-free layer interface with no preferred orientation [42], which is more stable than β-TCP and delaying its resorption. Concerning the samples with BCP, the results showed a close integration between the newly formed bone tissue and the biomaterial (Fig. 3C-D). As depicted in figure 3D, the organisation of osteocytic lacunae around the particles is compatible with the lamellar bone formation. At 90 days of observation, both experimental sites show remnants of the biomaterial particles in contact with osseous trabeculae constituted by woven and lamellar bone and a scarce quantity of chondroid tissue" (pag 13, lines 1-15)

- Results and discussion seem to focus the biomaterials and not the novelty of the animal model. A comparison of this model to the most relevant animal models with comparable pressures and/or materials might be of interest.

We have added the following text in the discussion about other animal models for incorporating this point.

"Other experimental models for sinus floor augmentation have been developed mainly for larger animals, such as pigs, sheep or dogs due the their anatomical size [1,2,3]. Nonetheless, the anatomy of these large animal models still differ significantly from the human situation and do have other critical drawbacks such as costs, increased demand for housing space and maintenance, as well as ethical considerations" (pag 10, lines 16-20).

- Is the bone formation in Fig. 4 physiological, physiological relevant, and relevant at all?

This reaction is because of the separation of dura mater from the bone surface. It is a physiological response, but we do not have information about the persistence of this bone layer in the anatomic site. For this reason, in the text only was described the phenomena, without delving into it (pag 11, lines 18-19; pag 23, lines 10-12).

- All in all, a following study seems essential to enforce the ambitious statement of introducing a new animal model, since soft materials f.i. collagenous sponges/scaffolds, granular bone substitutes, gels, autologous bone from drilling debris, etc. have not be investigated so far. As mentioned by the authors more time points are necessary as well.

We agree with these comments and have added to the text as part of the projections and limitations of the study.

"Within the limitations of the study, only sham operated sites were used as a control. Furthermore, a small number of rabbits and a single sacrifice period were used. However, the main objective of this study was to describe the first results of the use of epidural space in rabbit to explore the new bone formation associated to biomaterial for bone regeneration in a protected site and subject to permanent hydraulic pressure. Thus, further studies with larger samples, others biomaterials, autogenous bone and more time points could lead to improving the predictability and interpretation of model results" (pag 13, lines 16-22).

- Please check the usage/spelling of SI units: f.i. cc = cm^3; add a value for mmHg and mmH2O in Pascal.

We agree with your comment. The measurements were transformed in SI units as was suggested.

- Please check following spelling suggestions:

Page3 Line 11: where "it" is

P3L20: developed"."

P3L39: "generation "of" new

P4L33: toward"s" the dorsal

P4L50: hardening "cement" of biphasic

P6L31: in the observed "area"

Fig. 3: Change A+B with C+D in accordance with the order BCP and beta-TCP are named in the figure legend.

Thank you for these suggestions. We have corrected the text and images according to your observations.

Finally, we hope we have adequately answered your comments. We look forward to your news.

Sincerely,

The authors.