Author’s response to reviews

Title: Impregnation of bone chips with alendronate and cefazolin, combined with demineralized bone matrix: a bone chamber study in goats.

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Author’s response to reviews: see over
Dear Sir/Madam,

Hereby I send you the revised manuscript with the following formatting changes:

**Reviewer: Jorgen Baas**

**Minor Essential revisions:**
1. A table of the histomorphometric comparisons conducted between the groups and the exact p-values stated in a separate table (new bone and allograft bone) to ease data overview
   ➔ A table of the histomorphometric comparisons between groups for new bone and allograft bone with the exact p-values has been added. (Table 2A and 2B)
2. The safety of the use of topical cefazolin should be discussed in closer light of the statistical difference of the groups w/wo cefazolin, since the study was powered to show differences, not equality between groups. Also, the dose of cefazolin should be discussed, as the graft was rinsed after adding cefazolin.
   ➔ In the methods section (under ‘bone graft preparation’), the used dose of cefazolin has been explained (p8-9). We agree with the reviewer that our study was powered to show differences, not equality between groups. In the discussion section (p17) the issues regarding the safety of the use of topical cefazolin has been clarified (p18).

**Reviewer: Magnus Tagil**

**General comments:**
1. Methodologically I miss evidence that the antibiotics ever would stay in the graft after rinsing and impaction. It should not be that difficult to repeat the impregnating procedure and measure the amount of remaining antibiotics.
   ➔ In the methods section (‘bone graft preparation’), the used dose of cefazolin is explained (p8-9). We repeated the impregnation procedure and measured the amount of remaining antibiotics. Cefazolin was present on the bone chips after rinsing and impaction. No difference was found between impregnating and rinsing the graft before and after impaction.
2. The other major concern is that measuring the amount of new bone that remains after remodeling, not only reflects how much new bone have formed but rather how effective the antiresorptive treatment is to prevent the new-formed bone to resorb. So you cannot claim you measure anabolism but rather catabolism. Rewrite section on p15.
   ➔ We agree with the reviewer. The section on page 15 has been rewritten.

**Specific points:**

**Abstract**
- The aims could be sharper defined. That antibiotics prevent infection might be generally known but I am not sure every reader understands why inhibiting the osteoclastic response prevent instability.
→ The aims have been sharper defined and the inhibition of osteoclasts has been explained more so that the reader understands why inhibition prevents instability (p2).

**Background**

- Also here the hypothesis and the aims are missing. I found them partly in the beginning of the discussion and the first sentences there could be moved to the introduction forming the aims.
  → The aims were missing and are now defined in this part of the article (p6).
- The hypothesis of bisphosphonates stalling resorption thereby providing better support during healing of a hip prosthesis is not very easy to understand as it is written now.
  → The hypothesis of bisphosphonates stalling resorption and thereby providing better support during healing has been explained better in the background section (p4-5)
- Further there is no mechanistic hypothesis why Cef would influence bone healing, whether this could be stopped by the proposed drugs and how this could be measured.
  → A hypothesis (with a reference to an in vitro study) why cefazolin would influence bone healing and how we could measure that has been added (p6).
- Further there is no mechanistic hypothesis why DBM is used in the experiment more than “This critical period after reconstruction with bone impaction grafting might be shortened when demineralized bone matrix (DBM) is used.” Please state what do you expect DBM to do and how you will measure it?
  → The hypothesis for the addition of DBM has been rewritten (p6).
  → The introduction has been partly rewritten to include hypothes and aims (p4-6).

- Minor things- I dislike the phrase catabolic effect on osteoblasts. Decreased anabolism or toxic effect?
  → The phrase catabolic effect on osteoblasts is replaced by decreased anabolism or even a toxic effect (p5).
- “Although bisphosphonates efficiently block resorption” Do BPS really block resorption or rather postpone it?
  → Bisphosphonates are known to suppress resorption by apoptosis of the osteoclasts, and therefore postpone resorption until other osteoclasts resorb the bone. This has been changed in the background section (p4).

**Methods**

- I cannot find what dosis you used when combining DBM and bisphosphonate.
  → The dosis of bisphosphonate when combined with DBM has been added in the methods section (p7).
- Please describe briefly the random order of the chambers so the readers understand how you randomized the sites.
  → The methods section explains briefly the random order of the chambers: “The side and position of implantation of the 8 chambers were alternated with a random start (p7).
- Please mention shortly the results of the sample size estimation/power analysis what effect that was expected, the measurement accuracy and how many animals that were needed.
A short sample size calculation has been added to the methods section (p8).

- Bisphosphonates bind tightly to bone- what about cefazolin? Please expand. Do we know whether there are any bone-bound cef or does it go away when rinsing? References? Or what is your hypothesis?
  - The cefazolin part of the methods is expanded (p8-9), including a reference which explains that cefazolin binds to bone and that the cefazolin does not do away when rinsing with saline.

- Does the bisphosphonate concentration matter most or is it the soaking time? References?

- In rats twelve weeks is a long time and the differences are greater at six weeks. Why 12 weeks and why only 12 weeks and not for example 6 and 12 weeks. With 200 chambers and 25 samples in each maybe you could have done two time points?
  - We agree that in rats 12 weeks is a long time and that difference are more pronounced after 6 six weeks. In goats, however, bone ingrowth requires more time and in previous studies we also used implantation times of 12 weeks (refs). Although we chose to evaluate after 12 weeks, we agree that it would have been interesting to evaluate at an additional (earlier) time point.

- Being the author that once described the compaction method I hope you are aware that it is a very dense bone graft of 65%. The density of impacted morsellized bone graft in hip revision is about 35%.
  - We are aware of the fact that 65 % is very dense and that this is less in impacted bone in hip revision arthroplasty. However, in our previous bone chamber studies in goats we used similar degree of impaction. Micro-CT analysis of unimpacted and impacted cancellous goat bone chips revealed mean graft densities of 35% and 65%, respectively.

- Why doing the impaction after giving the doses? Especially the Cef will be squeezed out?
  - We did the impaction after giving the dose, since we were interested in the development of a ready-to-use product for the orthopaedic surgeon. Based on your earlier suggestion, we performed an extra in vitro test to see whether impaction of the bone chips after impregnation had any influence on the amount of cefazolin on the
allograft bone. No significant differences between impregnation before and after impaction. The protocol of an earlier performed in vitro study was used (p8-9).

**Results**
- I would like to see better blow ups of Fig 1 to be able to see if it is living bone at the pop? In the rat chambers we have never seen such a thick fibrous tissue as in fig 1a. Interesting to see bone formation at the top due to the DBM?
  - In goats we regularly see this thick fibrous interface layer. We published othis before, although with a different research question. See for instance the publication of Hannink et al. (Hannink G, Schreurs BW, Buma P. No positive effect of OP-1 device on the incorporation of impacted graft materials after 8 weeks: a bone chamber study in goats. *Acta Orthop.* 2007 Aug;78(4):551-8). We normally see new bone formation in the part of the chamber which is close to the ingrowth openings. Even in the low magnification micrographs the red lines and the new trabecular architecture are indicative for new bone formation in the Goldner staining that is used on this particular section. However if the editor wants to have blow ups we can provide these of course. In the direct vicinity of the DBM remnants, no new bone formation is observed. The red stained particles are the decalcified DBM particles, which apparently also stained red in the Goldner stain..

**Discussion**
- Move purpose to aims?
  ➔ The purpose of this study has been better explained in the background (p4-6).
- P 14 line 3-9 Here you write it is important with a balance between formation and resorption but you only measure one side- the resorption. So we do not know where this balance is?
  ➔ This ‘balance’ has been described differently.
    “Therefore, an optimum dose regarding bone resorption is essential, since it yields a positive balance between allograft resorption and the net amount of newly formed bone (new bone which is not immediately resorbed) which results in improved fixation” (p15).
- P 14 line 10-22 Jacobsen did not rinse like you do. Is that important do you think? Or is it what is bound to the bone that matters like Agholme thinks? Jacobsen has a mechanically loaded situation and you an unloaded (even stress-shielded) hence the difference I believe. You could comment on that in the text
  ➔ The differences regarding the study of Jacobsen and our study have been mentioned in the discussion:
    “These different results might also be explained by the different rinsing method used (3 min vs 10 min) or the fact that they had a mechanically loaded situation compared to our unloaded situation” (p15).
- P 14 line 23 Agholme- as you both are evaluating the remodeled area it should be equivalent regardless of study time- even if it would be same species. But ingrowth distance i.e. the anabolic equivalent of the chamber would differ!
  ➔ The study of Agholme has been explained in more detail in the discussion (p15).
  The amount of new bone is compared to the amount of new bone in our study:
“In contrast to our study, they did not find any differences between the amount of new bone between a regular dose (with rinsing after impregnation) of bisphosphonate and an overdose amount (without rinsing the graft after impregnation). The volume fractions of new bone were comparable to the mean volume fractions of the 4 different doses in our study, although we did find differences between these doses. They stated that their results may be explained by the four-week study period in their experiments compared to the 6 weeks period in other studies (and thus the 12 week period in our study), that there was not enough time to fully resorb all allograft bone behind the bone ingrowth frontier.”

- P 14 last line Zolendronate has also been used for a decade (2002 in Sweden). Alendronate was introduced in 1995.
  ➔ The introduction of alendronate has been changed to `almost two decades´ (p16)

- P 15 Just because you find more bone it does not imply anabolism. This is important! It simply means that more or less of the new-formed bone has been resorbed- due to the BP concentration. It has nothing to do with osteoblasts- at least not until a too high toxic dose !!!!!You have to delete this passage about the amount of new bone.
  ➔ We agree. The section on `anabolism´ has been changed and parts of it have been deleted (p16).

- P 15 line 6 from the end. As written above, I would suggest you measure the amount of Cef, in the graft with or without rinsing. It is rather simple to do and would add so much to the interpretation. There is simply too much guessing as it is.
  Here are two examples of conclusions you cannot draw
  “Also, this concentration did not cause any side effects and is well above the minimal inhibitory concentration (MIC) for S. epidermidis.
  How could you tell without knowing the concentration?
  “No subinhibitory amount of the drug is left behind which can induce resistancies”
  How would you know if you do not know the concentration?
  “Cefazolin is completely eluted from the bone chips after three days.”

Please add reference or report your own data
  ➔ Concerning the comments on the cefazolin:
  The methods section on local application of cefazolin has been expanded (p8-9) and, these conclusions are now supported by literature ( references have been added) (p17). As mentioned before we repeated the experiments with Cef, rinsing and impaction. We did not find any significant differences.

- P 17-line 3 why would cement matter?
  ➔ Since cement can be used during hip revision arthroplasties, the bone chamber model might not fully simulate the clinical situation.

- P 17-line 3 osteoclasts resorb old AND NEW formed bone - which explains why you get more new bone when BP dose increases.
  ➔ We totally agree.

Best regards,

Nina Mathijssen