Author's response to reviews

Title: Systemic zoledronate treatment both prevents resorption of allograft bone and increases the retention of new formed bone during revascularization and remodelling. A bone chamber study in rats.

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Author’s response to reviews: see over
Re: 'Systemic zoledronate treatment both prevents resorption of graft bone and increases the retention of new formed bone during revascularization and remodelling. A bone chamber study in rats.

Dear Editor

Thank you for reviewing our article and below we answer the concerns you and the two reviewers raised on a point to point basis. The comments by you or the reviewers are in italics. Regarding your specific questions we have added details on the anesthesia and analgetics in the methods. Your second concern about originality we have commented in the reply to the first reviewer. Our study is an effort to optimize the dosing regime of a bisphosphonate- how few injections can still protect the remodeling bone as the resorption and formation front is gradually advancing into the graft. It must be an advantage to decrease the numbers of dosings and it seems enough with once weekly with zoledronate.

Best regards Magnus Tägil

Dear Dr Tägil,

Your manuscript has now been peer reviewed and the comments are accessible in PDF format from the links at the bottom of this e-mail. Please let me know if you have any problems opening the files.

Could you please address the comments both by providing us with a point-by-point response to them and by revising your manuscript accordingly, letting us know where and how it has been revised. If substantial points were raised, we will probably need to seek further advice.

The editorial office is specifically concerned about the originality of the research and clarification of the treatment of the animals. Please address these points when you return your revised manuscript.

Could you also please go through the manuscript formatting checklist, the link to which is provided at the bottom of this e-mail, and ensure that your revised manuscript conforms to all of the points. It is important that your files are correctly formatted.

We hope you can return your revised manuscript within three weeks (i.e. by 23 May 2006). If you imagine that it will take longer to prepare (for example, if the reviewers have requested further studies that would take longer than this time), please give us some estimate of when we can expect it.

First Reviewer's report

Title: Systemic zoledronate treatment both prevents resorption of graft bone and increases the retention of new formed bone during revascularization and remodelling. A bone chamber study in rats.

Version: Date: 1 20 April 2006

Reviewer: Dominique P Pioletti

Reviewers's report:

Systemic zoledronate treatment both prevents resorption of graft bone and increases the retention of new formed bone during revascularization and remodeling. A bone chamber study in rats by Åstrand et al.
**General comments**

This is a concise and well written manuscript which checks the hypothesis that systemic zoledronate can influence the remodeling following a bone graft. The only major aspect is related to the originality of this work as similar studies were performed by the authors with only the bisphosphonate being different (zoledronate instead of alendronate). It would be necessary to justify more the need to perform the present study.

Since it is a more potent bisphosphonate also the dosing is different. Is it possible to dose a bisphosphonate less frequently in an osteonecrotic situation as the current trend is regarding osteoporosis treatment? We cannot translate results from osteoporosis treatment directly to the treatment of osteonecrosis since there is a huge relevant difference between the two conditions; osteoporotic bone is still vascularized but in osteonecrosis (and graft healing) we try to reach an avascular part with a drug transported via the blood stream. In clinical situations this is a highly relevant question—how do we get the drug there? Do we have to treat every day to completely reach and treat the ingrowth frontier as it advances into the necrosis/graft or is it enough once a week, once a fortnight etc? Alendronate was more potent than clodronate and now zoledronate offers a higher potency than alendronate. We think we have to test the new drugs to know more since they do differ and the bone chamber is a apt tool for test them in.

The text has been altered at the end of “Background” and a reference added.

**Specific comments**

Minor aspects concern technical points.

From the 10 donor rats, twenty grafts were obtained. Were the grafts obtained from the same rat used systematically as control and treated in the hosts?

Correct, the grafts are frozen in pairs and one graft is assigned to the control group and the other to experiment group.

2) The way the graft is placed in the chamber is not clearly described. Were the grafts placed in similar position each time as the contact surface between the graft and bone may influence the remodeling process.

We keep the direction of the graft the same as situated before graft harvest and try to assure a good contact of the graft to the bottom of the chamber. The problem is rather the position of the chamber in the bone once implanted, which could differ and interfere with the position of the ingrowth holes. This adds to the variation but should be equal in the controls and experiments.

3) In the results, it is not easy to understand what the authors means by the remodeled area.

That is major criticism since this is important to the whole message of the study. We have tried to clarify and rewritten the whole results section. Only the remodeled bone is afflicted by the drug and only remodeled bone is measured.

4) Conclusion should be replaced by Discussion.

Changed, misunderstanding of instructions.
5) As it could have been expected, the results obtained confirm the previous results obtained with alendronate. As previously mentioned, it would be necessary to better justify the study than only using a different bisphosphonate.

The bisphosphonate differ in mechanism on the cellular level between nitrogen containing such as alendronate and zolendronate and non-nitrogen containing such as clodronate. Further the potency differs and some speculate that side effects are not related to this difference in potency which would mean an advantage for a more potent drug. See reply in general comments above.

6) The last paragraph is not very conclusive.

Last paragraph is essential since we point out that although we find increased amount of new bone this does not imply that more new bone has formed only that more new bone has been retained. (important since some authors describe bisphosphonates as being anabolic which is erroneous in our eyes)

What next?: Accept after minor essential revisions
Level of interest: An article of limited interest
Quality of written English: Acceptable
Statistical review: No

Reviewer 2

Reviewer's report:

General
The manuscript contains interesting material that is worth publishing. It reports about an experimental animal study in mice (well it was rats), where allogenic bone grafts were introduced in a titanium chamber and implanted in the tibia. There, graft resorption and/or new bone formation was followed over 6 weeks with or without the osteoclast inhibiting drug zoledronate. Results demonstrated that grafts resorbed faster and new bone formation was less in untreated grafts. The authors conclude that treatment with zoledronate is an effective drug to prevent undue bone resorption in clinical cases of bone defects and osteonecrosis.

This is not entirely correct. We conclude that “zoledronate can be used to decrease the resorption of both old graft and new-formed bone during bone graft remodeling”. We discuss “This might be useful in bone grafting procedure but also in other orthopedic conditions, both where necrotic bone has to be remodelled i.e. after osteonecrosis of the knee and hip and in Perthes disease, or in high load, high turnover conditions like delayed union, periprosthetic osteolysis or bone lengthening operations”.

Although the study shows potential, the manuscript shows weaknesses that need addressing before it can be published.

Comments met at a point-to-point basis, see below

Title:
The title should reflect that the authors were studying allografts and not autografts.
Previous studies with allogeneic and syngeneic animals (Thoren K, Aspenberg P. Arch Orthop. Trauma. Surg 1995;114(3):167-71) but also Tagil M, Jeppsson C, Aspenberg P. Bone graft incorporation. Effects of osteogenic protein-I and impaction. Clin Orthop Relat Res. 2000 Feb;(371):240-5) has showed that immunologic reactions in rats receiving bone grafts does not significantly influence the results in this model. A section commenting this has been added in the “Conclusion”-section and the reference has been added.

Abstract:
The description of the background does not reflect the content of the study. The authors did not prove that zoledronate reduces the resorption of necrotic bone, rather a frozen allogenic bone graft. In conclusions: The authors should limit their conclusions on bone grafts and not extend it to other orthopedic diseases, which were not part of the study. The abstract should be re-written.

Frozen allogenic bone is by most standards necrotic. Again, previous studies in the same model has showed that immunologic reactions in rats receiving bone grafts is not an issue. As replied above, we only make conclusions on the graft experiments but discuss all the other indications.

Background:
Page 5, first paragraph: Why do the authors start with the topic of osteonecrosis. Osteonecrosis is different on a pathological level than a bone matrix, where living cells have been destroyed through freezing. The implantation of allogenic, pretreated allografts should not be compared with conditions such as corticosteroids treatment, sickle cell anemia, etc. Also the subchondral bone has other problems related to overlying cartilage degeneration. The authors should clearly stay with i) (allogenic) graft survival and remodeling and ii) effect of bisphosphonates in their introduction. Although the results of this study may once be applied in cases of osteonecrosis and other diseases mentioned in the first paragraph, they are not the focus of the current study. The reviewer recommends to start with the second paragraph where bisphosphonates are introduced and then in a second paragraph summarize their findings with the first study where the authors tested obviously the effect of alendronate.

Point taken, here as well as in several comments by the reviewer 2 below. (we try to meet these comments here and just refer to this reply in the comments below). We argue that dead bone is dead regardless what killed it and that the cells that have to repair are the same. We believe that an animal model such as our chamber can be used to make at least speculations that necrotic bone might behave similarly in our chambers as well as in different clinical situations where osteoclasts are involved and to find mutual common mechanisms for cells and tissues. We use our chambers to study such basic mechanisms, then go on with maybe a surgically induced osteonecrosis or fracture animal models and finally extrapolate these into the clinical situation of disease or injury and try to prove the similarities in humans as we do with bisphosphonates at present. However we need the basic studies to learn more. We need to know whether different bisphosphonates act differently, since we now use both alendronate as well as zoledronate in those clinical studies. We need to know if expanding the intervals between the doses affects the resorption since there are differences between for example osteoporosis and osteonecrosis. Not in basic mechanisms or drug action but in the timing of the remodeling, bioavailability (trying to reach avascular bone via the blood stream) etc. Therefore we must discuss the drug and the mechanisms in the context of
both osteonecrosis and osteoporosis and maybe even fracture healing or growth plate because that is what we and others are exploring in humans at present.

There are results, extremely promising also in a randomized study that bisphosphonates can be successfully applied to clinical cases of osteonecrosis (Agarwala, Nishii et al, Lai et al). Clinical use of bisphosphonates on osteonecrosis cases are increasing and also all other indications we discuss have been reported in humans, which further increases the importance of learning more on different aspects of how and what type of bisphosphonates should be used.

**Methods:**
The authors rely on the reader to be familiar with their first study. Although this is partly legitimate, each manuscript has to stand on its own. Therefore, the authors need to be more specific about the surgical method of implantation and screw fixation. Also the method of anaesthesia and analgesia of the rats should be given to allow the reader to assess whether animal welfare was maintained during the study. The description of how the authors performed the statistical analysis is missing, although they report in the results sections their P-values. Please, give short description.

The methods section has been rewritten. The surgical methods are described more rigorously, the method of anesthesia and analgesia given as well as the statistical methods.

**Conclusions:**
Again, the authors should focus on their own study with bone grafts and discuss these results.

See above

A critical comparison of results of the current with their previous study is missing.

This is discussed in the first section of the discussion which has been partly rewritten and added accordingly. The results show that the drug can be administered less often using the more potent zoledronate than the previously used alendronate.

Also missing is a critical view on graft resorption in controls in a chamber without mechanical load. Would another animal model, where grafts would have been subjected to mechanical load, have had another outcome?

Excellent point and the answer is probably yes. The ingrowing tissue in the unloaded chamber is stress-shielded and the resorptive drive high and therefore the effect of an antiresorptive drug larger than in a less stress-shielded model. We have used alendronate in a loaded chamber model and the remodeling bone then collapses to a larger extent in the non-bisphosphonate-treated specimens. (Tagil M, Astrand J, Westman L, Aspenberg P Alendronate prevents collapse in mechanically loaded osteochondral grafts: a bone chamber study in rats. Acta Orthop Scand. 2004 Dec;75(6):756-61) A sententence commenting this is added (Conclusions, line 5).

They mix their discussion and conclusions with other orthopedic diseases that they have not studied in this experiments and therefore should exclude.
We do not, see above. We are thoroughly dividing what we conclude and what we discuss.

**Specific comments:**
Page 3, first paragraph: Background:
Line 1: The first sentence about osteonecrosis is out of context. Focus on the grafts.

See above

Line 4: ……osteonecrosis occurs …..

See above

Line 6: Was it really necrotic bone or bone grafts?

Given the right conditions, osteoclasts does not seem to distinguish between the two.

Page 3, second paragraph: Methods
Line 3: The rats were killed, and their tibiae were harvested.

That is correct.

Page 3, third paragraph: Results
Line 1: …control specimens in the titanium chambers were almost totally resorbed …..

That is correct (as shown in Fig 2 and 3 and in the table )

Page 4, first paragraph: Conclusions
Line 4: In our model an increased net formation of new bone was found in the grafts which…..

That is correct but note that we speculate that this probably is because more newly formed bone is still there when we harvest.

Page 5, first paragraph: Background
Delete entire paragraph and use second paragraph for introduction about the effect of bisphosphonates.

See above. This introduction is essential to the whole study as one crucial point in necrotic bone remodelling, be it due to osteonecrosis, dead bone in fracture healing, (or even bone malignancies) all rely on the action of osteoclasts. This action will occur first during revascularization.

Page 5, second paragraph:
Line 7: Did they really show that bisphosphonates reduce the resorption of necrotic bone in the true pathological sense, or just mineralized bone? Tumor metastases may contain necrotic bone, but usually there are also viable cells and living bone parts. Osteoporosis has also nothing to do with necrotic bone.

“The true pathological sense” of the resorption of necrotic bone is an interesting philosophical point which we could expand on but beyond the scope of most studies on bone metabolism. Michael Rogers works mainly with in vitro studies searching for the
mechanisms of how the bisphosphonates decrease the osteoclastic activity. The reference is an overview of his pioneering work. The sentence with tumor metastases and osteoporosis just reflects the clinical indications of the bisphosphonates today. A reference can be added but we do not think this is necessary.

Page 6, paragraph 2:
Use this paragraph as introduction about bisphosphonates. Instead add a paragraph about the implantation of pretreated allografts including immunological aspects. Take reference to the first study with alendronate.

See above

Page 7, paragraph 1:
Specify the surgical implantation enough that the reader can follow the technique. Give anaesthetic and analgetic regimen.

This has been added in the text

Page 7, paragraph 2: Grafts
Line 1: How were the donor animals killed?

Overdose of pentobarbital. Added

Line 4: Were the bone grafts harvested under constant irrigation while using the cutter?

No. If the comment concerns heat necrosis, the hole cutter was not motorized and grafts were taken by hand power (added in the text). We believe the grafts were necrotic due to devascularization and freezing, not heating.

Page 7, paragraph 3: Surgical procedure
Line 3: What was the size and diameter of the hole?

The size of the burr making the hole is 2.7 mm, which has been added in the text. The inner diameter (2 mm) and the length 7 mm is also given in the text

Line 4: Give screw size

See above

Line 5: Was the chamber now placed subcortically or subcutaneously?

The ingrowth openings in the bottom of the chamber are situated subcortically but the other end protrudes above the cortical surface and can be palpated through the skin. (i.e. subcutaneously, see fig 1) Text has been changed to clarify.

Page 7, paragraph 4: Injections
Line 1. When were the injections started, directly after implantation or later?

Day 4,11,18,25 and 32. Clarified in the text

When was the last injection before sacrifice?
Day 32

Page 8, paragraph 2:
Line 4: What was the magnification and how were the power fields chosen, such as parallel or at the front of the original graft?

The magnification was 40x and the fields parallel to the grafts (added in the text)

Line 6: Give number of official animal permission in brackets.

The Animal ethics committee file number has been added.

Page 9, paragraph 1:
Line 1: In all other specimens ......

?????

Line 3: Were all grafts (controls and treated) replaced “halfway through the grafts”? Or were there differences between groups? One would assume if the histologic measurements are considered.

For exact data see table (=bone ingrowth distance). No, there were no significant differences regarding bone ingrowth distance between the groups. This is one of the major conclusions of the study that they differ in resorption but not in ingrowth distance of new bone into the graft (but the amount of retained new bone). The table has been changed to clarify.

Line 4: Did the bone deposit directly onto the mineralized matrix of the control grafts, or was there fibrous or marrow tissue in between remodeled grafts and old matrix?

Fig 2 is representative of the histological appearance. New bone was forming distally to the previously formed incorporating rests of nonresorbed graft bone which then was surrounded by new-formed bone.

Page 10, paragraph 1:
Line 2: It is not correct that the authors showed in this study that the zoledronate is as effective in preventing bone resorption as alendronate. They only demonstrated that in grafts treated with zoledronate more bone was present compared to untreated controls. The authors can discuss their previous results with alendronate in the same animal model, but not include the previous results in the current study. Re-write the first paragraph.

We disagree with the referee’s conclusion. In both studies the bisphosphonates reduces the resorption compared to untreated controls. We do not state a quantitative difference just a qualitative.

Line 4: Discuss sc versus iv. Injection separately, if this point is really based on different results with iv Injections in other studies. Otherwise leave it away.

It is not confirmed in our model but it might be relevant. David Little has shown a difference in release between iv and sc in a scintimetric study in rats at the Bisphosphonate meeting in Davos 2004 (unpublished, abstract in Bone) I delete it if you want me to but it is a weak part of the study I think should be mentioned.
Page 10, paragraph 2:
Line 9: “Osteoclasts are necessary for bone formation by the osteoblasts”. This statement is not correct. The relationship between osteoclasts and osteoblasts is different and more complex. Osteoclasts are dependent on cell signals released by osteoblasts to get recruited and activated (IL-1, IL-6, PGE2 and possibly IL-11). Their activity is again inhibited through the mediator NO, and through the soluble substances released by the bone matrix after osteoclasts resorption.

Although the referee’s description is more accurate our description is still correct but maybe too simplified. We believe the reader knows that the coupling between osteoclasts and osteoblasts is more complex than we write.

Line 10-16: Here the authors refer to osteoporosis and fracture healing including microfractures. Again, the authors have not proven any of these statements and cannot confuse both, fractures and osteoporosis with graft resorption.

See above. Since the same cells mediate the responses, the mechanisms are likely to be the same.

Page 11, paragraph 1:
Line 7: …be discussed, such as that in contrast to cortical bone……..

Relevant. Remodeling of trabecular bone is different to cortical bone. Mechanisms the same but the timing of the catabolism and anabolism differ.

Line 17: leave necrotic away.

The word necrotic is taken away

Page 11, paragraph 2:
At the end the authors should add something about long-term remodeling of the grafts under the influence of bisphophonates?

This is thoroughly discussed in Discussion, second paragraph, line 5 and forwards, mainly in the context of fracture healing though.

Figure & figure legend:
Fig.1: It would be desirable if it were indicated where and how the graft chamber is fixed in relation to the tibia.

An additional figure is added to clarify.

Fig.2 and 3: Make clear where the old graft and the newly deposited bone is in relation to the picture in both, the legend and the figures.

That is marked in the figures 2 and 3 and explained in the legends. In the 20x magnification the graft bone (G) is best seen in the non remodeled area whereas the new formed bone (NB) is best seen in the border zone of new forming bone. Below, in the remodeled zone (M) practically everything is resorbed in the non-treated specimens whereas in the treated a mixture of new formed and old graft bone is seen. We think this is clear to see in the magnifications (figs 2B and 3B)
Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)
*Please specify which term?*

Discretionary Revisions (which the author can choose to ignore)

**What next?:** Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions

**Level of interest:** An article whose findings are important to those with closely related research

Best regards
Magnus Tägil