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Earlier effect of alendronate in mouse metaphyseal versus diaphyseal bone healing

Running title: Alendronate in metaphyseal fractures

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Author contribution statement:

OS helped plan, perform, and evaluate. OS wrote the first draft.

MB helped perform the study and reviewed the manuscript.

PA helped plan and evaluate the study, and write the manuscript.

All authors have read and approved the final submitted manuscript.
Abstract

Healing of injured cancellous bone is characterized by a transient stage of rapid bone formation throughout the traumatized bone volume, often followed by similarly rapid resorption. This is different from the slower diaphyseal healing via an external callus. We therefore hypothesized that antiresorptive treatment might have an earlier positive effects in cancellous bone healing than in diaphyseal fractures. 123 male C57bl6 mice received either an internally stabilized diaphyseal osteotomy of the femur or a screw inserted into the tibial metaphysis. The mice were randomized to daily alendronate injections (200 ug/kg/day), or control injections, and killed for mechanical testing after 14, 21, or 28 days. The hypothesis was tested by a 3-way Anova (time, site, and drug). The ultimate force was increased by bisphosphonate treatment in both models. There was a significant interaction between time, site and drug (p < 0.001) so that the full positive effect of alendronate was evident in the metaphysis at 14 days, but first after 28 days in the diaphysis. While the early effect in the metaphysis might be translated into earlier healing, the late effect in the diaphysis was due to delayed remodeling of the callus, which might have less clinical importance.

Keywords

Fracture, bisphosphonate, metaphysis, cancellous bone, trabecular bone, alendronate
Introduction

Bisphosphonates impair bone remodeling, which is part of the later phases of fracture healing. Therefore, clinicians are unsure of whether to postpone initiation of anti-osteoporosis therapy with bisphosphonates after a fresh fracture. While animal experiments on fracture healing mainly studies diaphyseal fractures, those fractures are less common in the clinic, where metaphyseal fractures dominate. The latter fractures often have the highest impact on quality of life, such as hip fractures and vertebral fractures in the elderly.

The healing of metaphyseal fractures – or rather cancellous bone - has recently attracted increasing interest. These fractures appear to heal by mechanisms that in some regards differ from those of diaphyseal bone [1-3], perhaps because of the relatively large availability of mesenchymal stem cells (MSCs) in the metaphyseal marrow, as compared to the diaphysis.[4]

The animal models that currently dominate in fracture healing research use the diaphysis, where healing involves an external callus and usually a cartilaginous phase. External callus and cartilage seem to be less important in metaphyseal fractures, both in humans and rodents. Apart from the expected responses of traumatized periosteum, metaphyseal healing involves a component of cancellous healing, which is instead characterized by a transient stage of rapid bone formation throughout the traumatized bone marrow compartment, often followed by similarly rapid resorption.[1, 5] This early response can be quantified by screw pull-out testing (Fig 1).[6] Inhibiting the resorptive phase with antiresorptive drugs, such as bisphosphonates, might have the potential to increase the amount of bone remaining in the traumatized metaphysis.
We therefore hypothesized that bisphosphonate treatment will have a stronger positive effect on the metaphyseal type of healing, as compared to diaphyseal healing.

![Diagram of bone healing response at different time points: 1 day, 1 week, 6 weeks.](image)

**Fig 1.** Screw insertion in the proximal metaphysis elicits a bone healing response with new woven bone. After 1 week the new bone serves as a threaded nut, holding the screw and increasing the force required to pull it out [6]. At this time, the model measures the initial fracture healing response. With time the new bone will remodel and adapt to the implant, changing the relevance of the model from fracture healing to implant fixation (6 weeks; lowest image).

**Method**

This study used 123 male C57BL6 mice, 10 weeks old (Janvier, Saint-Berthevin Cedex, France). The mean weight was 24 grams, SD 1 gram. The mice were randomized to receive
either an unstable femoral diaphyseal osteotomy, or bilateral drill holes into the proximal
tibial metaphysis, with a screw inserted into the right drill hole.

*Surgery of the diaphysis of the femur*

An assistant sedated the mice using isoflurane gas (Forene, Abbot Scandinavia, Solna
Sweden). Analgesic and antibiotics were given, 0.1 mg/kg buprenorphine (Temgesic,
Schering-Plough, Brussels, Belgium) and 0.2 mg/kg oxytetracycline (Engemycin, Intervet,
Boxmeer, Holland). The left hind limb was shaved and washed with chlorhexidine. The
mouse was thereafter placed in front of the operator, covered with surgical tape with the left
leg sticking out. This was washed again in chlorhexidine and the surgery performed in a
sterile fashion. A 7 mm skin incision was made on the lateral side of the thigh. The knee-cap
was then luxated medially and a 0.4 mm needle was used to drill a hole into the marrow
cavity. A 0.4 mm needle with the end-part bent into a hook was inserted, pushed as far
proximally as possible, and rotated 180 degrees so as to allow the hook to anchor the needle
into the bone. The needle was then cut flush with the distal condyles. Thereafter a custom-
made scissor was used to cut the bone in the diaphyseal part. Finally a suture was placed to
hold the patella in place, and the skin sutured. The mice were kept on analgesics for 72 hours
post-surgery. The process is described in greater detail elsewhere.[5]

*Surgery of the metaphysis of the tibia*

In terms of analgesia, antibiotics, and pre-surgery handling these mice received the same
treatment as described for the mice receiving a diaphyseal osteotomy, except that the tibia
mice had both their hind limbs shaved. A 5 mm skin incision was made at the antero-medial
side of the tibia, below the knee. Thereafter the operator cut away the musculature covering
the medial side of the proximal metaphysis of the right tibia. A 0.4 mm diameter needle was
used to drill a hole approximately 0.6 mm below the physis into the proximal tibia, and an 0.7
mm diameter custom made screw (M0.7; Rydahl Precision Components, Karlstad, Sweden) made from Ti6A14V grade 2, was screwed into place. The skin was sutured. A similar drill-hole was made in the left leg but without screw insertion, and with a 0.6 mm needle instead of a 0.4 mm needle.

Treatment plan

After surgery all animals were randomized to receive either 200 ug/kg alendronate (Alendronate sodium trihydrate, A4978; Sigma-Aldrich Sweden AB, Stockholm, Sweden) diluted in sterile saline, or only saline, injected subcutaneously 6 times per week.

Within the respective treatment arm, animals were randomized into groups being killed after 14 or 21 days with n = 10 for each combination of time point, drug treatment, and method (Fig 2). A similar experiment with 28 days follow up was added afterwards.

**Fig 2. Experimental lay-out**
Mechanical testing of the diaphyseal model

After harvest, the proximal end of the diaphysis was removed and the intramedullary needle removed. The femur was thereafter put in a moist wrapping and left at 5°C overnight. Within 24 hours of killing the animal, the femur was tested in a mechanical testing machine (100R; DDL Inc, Eden Prarie, MN, USA) set for 3-point bending. The cross head was set to push down on the central part of the callus with a speed of 0.05 mm/s with the ends of the bone resting on supports. Maximum force, stiffness, and energy uptake, was measured.

Mechanical testing of the metaphyseal model

After harvest, the right tibia was separated from the foot. The distal part of the femur was left attached to the tibia. Muscle was removed from the area around the screw. The bone was mounted in the material testing machine mentioned above. The tibia was fixated to the machine using suture threads to either side of the screw and a clamp held on to the screw head and pulled it out at a speed of 0.01 mm/s. Maximum force, stiffness, and energy uptake, was measured.

microCT scans and data preparation

All microCT scans were done on an 1174 SkyScan microCT system (Bruker, MA, USA) using a 0.25 mm Al filter, with 50 kV, 180 degrees scans. Frame averaging of 3, and a rotation step of 0.3 degrees was used. For the 28 day animals a pixel size of 11 um was used, the other time points used 12 um. Post processing was done using dedicated software (Nrecon and CTan, Bruker, MA, USA), ring artifacts and beam hardening were corrected for. Phantoms were used to allow for estimation of bone mineral density (BMD) and tissue mineral density (TMD).

The 28 day femurs were scanned after mechanical testing (due to a temporary malfunction of the microCT). The other time points were scanned prior to mechanical testing, the samples
were kept moist throughout. A VOI was set up comprising of a 2 mm long portion of the central callus, delineated manually. This VOI excluded the old cortex and medullar cavity.

This VOI was analyzed for TV, BV, BV/TV, BMD, and TMD.

The left tibia, which at surgery had received a 0.6 mm drill hole but no screw, was after harvest put into 4% paraformaldehyde. Within 1 week the samples were analyzed in moist condition in the microCT. A cylindrical VOI with a diameter of 0.5 mm and a length of 1 mm was centered over the drill hole, starting from but not including, the cortex. This volume was analyzed for TV, BV, BV/TV, BMD, and TMD.

Statistics

Statistical analysis was done using SPSS software (SPSS, Version 23, SPSS, Inc., Chicago IL, USA). Data was tested with Shapiro-Wilks test for normality.

We tested the hypothesis that the effect of drug treatment on maximum force would have different timing in the metaphyseal versus the diaphyseal model. This was tested with a 3-way Anova using log-transformed maximum force as the dependent variable and drug treatment, model type, and time point, as independent.

95% confidence intervals and p-values for the effect of alendronate on the different models and time points was estimated using t-statistics, or Hodges-Lehmans estimate if data was not normally distributed. P-values for the non-parametric tests were calculated using Mann-Whitney U-test.

Animal housing and Ethical approvals

Animals were kept up to 4 by 4 in cages with 12:12 light. Water and standard rodent food was provided *ad libitum*. Housing and nesting material was provided. All procedures were
approved by the regional ethics committee (2012 85-12), and reporting complies with the ARRIVE (Animal Research: Reporting of *in vivo* guidelines).

**Results**

*Mechanical testing*

Alendronate did not have negative effects on the healing of any model at any time. On the contrary, the healing in both models was positively influenced by bisphosphonate treatment (Fig 3).

The diaphyseal model did not show any increase in maximum force day 14, but day 21 there was a 52% increase as compared to saline (*p* = 0.01). This increased to 170% by day 28 (*p* < 0.001; Fig 3). Stiffness and energy were much increased by day 28, but less so day 21 (Table 1).

The metaphyseal model showed a markedly faster response to alendronate treatment, with a 130% increase in maximum force compared to saline already day 14 (*p* < 0.001). Thereafter the difference between the groups decreased, because the controls began to catch up with the alendronate group. By day 21, the difference was 69% compared to saline (*p* = 0.001) and day 28 there was no significant difference. Stiffness and energy showed similar patterns.

A 3-way Anova of maximum force as dependent and time, treatment, and model type as independent showed significant interaction between the 3 factors (*p* < 0.001).

*MicroCT results*

In the diaphyseal model, no effect of alendronate on BMD was seen day 14, but there was a 51% increase day 21 (*p* < 0.001) and an 180% increase day 28 (*p* < 0.001); (Table 1, Fig 3, Fig 4). The other parameters showed similar behavior.
In the metaphyseal model, once again, the effect was visible already day 14, when bone mineral density (BMD) was increased by 120%, as compared to saline controls ($p < 0.001$; Table 1, Fig 3, Fig 4). Day 21 and 28 this difference was roughly 200% ($p < 0.001$). The reason was a decrease in the control group, and the absolute value of the alendronate treated group did not change from day 14 to day 28. BV followed the same pattern. Tissue mineral density (TMD) was increased by 25% day 14, 12% day 21, and 20% day 28, as compared to controls ($p = 0.001, 0.004, \text{ and } < 0.001$ respectively).

**Fig 3, BMD and maximum force for the different time points and models.** Note the early and consistent separation between alendronate and saline controls in the metaphysis. This is contrasted by an initial overlap in the diaphysis with a separation occurring over time.
4. MicroCT images showing the samples closest to the mean BMD of each group.
Table 1. Effect of alendronate treatment on mechanical and microCT variables. Group differences and confidence intervals for group differences are expressed as percent of saline.

<table>
<thead>
<tr>
<th>Location</th>
<th>Time</th>
<th>Quantity</th>
<th>Alendronate (SD)</th>
<th>Saline (SD)</th>
<th>Diff, %</th>
<th>P</th>
<th>% CI low</th>
<th>% CI high</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 14</strong></td>
<td>Max force, N</td>
<td>8.5 (5.1)</td>
<td>6.0 (1.7)</td>
<td>40</td>
<td>0.18</td>
<td>-19</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>Max force, N</td>
<td>11 (5.4)</td>
<td>6.3 (1.4)</td>
<td>52</td>
<td>0.01</td>
<td>18</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stiffness, Mpa</td>
<td>4.9 (1.8)</td>
<td>3.6 (0.78)</td>
<td>38</td>
<td>0.05</td>
<td>1</td>
<td>75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy, Nm</td>
<td>5.7 (4.8)</td>
<td>3.6 (1.1)</td>
<td>59</td>
<td>0.22</td>
<td>-39</td>
<td>160</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD, g/cm³ CaHA</td>
<td>0.17 (0.079)</td>
<td>0.15 (0.033)</td>
<td>4</td>
<td>0.71</td>
<td>-19</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/TV, %</td>
<td>33 (16)</td>
<td>29 (9.8)</td>
<td>1</td>
<td>0.82</td>
<td>-30</td>
<td>41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV, mm³</td>
<td>6.8 (1.3)</td>
<td>6.0 (2.3)</td>
<td>10</td>
<td>0.55</td>
<td>-19</td>
<td>41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TV, mm³</td>
<td>23 (6.3)</td>
<td>20 (4.2)</td>
<td>16</td>
<td>0.12</td>
<td>-8</td>
<td>36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMD, g/cm³ CaHA</td>
<td>0.38 (0.040)</td>
<td>0.34 (0.022)</td>
<td>8</td>
<td>0.01</td>
<td>3</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Day 21</strong></td>
<td>Max force, N</td>
<td>14 (6.7)</td>
<td>10 (3.0)</td>
<td>39</td>
<td>0.11</td>
<td>-10</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>Stiffness, Mpa</td>
<td>4.1 (2.8)</td>
<td>2.0 (1.4)</td>
<td>100</td>
<td>0.01</td>
<td>44</td>
<td>220</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy, Nm</td>
<td>0.41 (0.068)</td>
<td>0.27 (0.034)</td>
<td>51</td>
<td>0.000</td>
<td>31</td>
<td>71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD, g/cm³ CaHA</td>
<td>76 (9.1)</td>
<td>56 (5.5)</td>
<td>35</td>
<td>0.000</td>
<td>22</td>
<td>48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/TV, %</td>
<td>16 (3.7)</td>
<td>9.6 (0.90)</td>
<td>66</td>
<td>0.000</td>
<td>38</td>
<td>95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TV, mm³</td>
<td>21 (4.4)</td>
<td>17 (2.4)</td>
<td>22</td>
<td>0.032</td>
<td>2</td>
<td>42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMD, g/cm³ CaHA</td>
<td>0.51 (0.045)</td>
<td>0.42 (0.030)</td>
<td>22</td>
<td>0.000</td>
<td>13</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Day 28</strong></td>
<td>Max force, N</td>
<td>21 (8.6)</td>
<td>7.7 (3.2)</td>
<td>170</td>
<td>0.000</td>
<td>99</td>
<td>260</td>
<td></td>
</tr>
<tr>
<td>Stiffness, Mpa</td>
<td>42 (22)</td>
<td>12 (8.3)</td>
<td>270</td>
<td>0.000</td>
<td>120</td>
<td>450</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy, Nm</td>
<td>4.9 (2.2)</td>
<td>3.0 (1.9)</td>
<td>62</td>
<td>0.05</td>
<td>0</td>
<td>120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD, g/cm³ CaHA</td>
<td>0.38 (0.042)</td>
<td>0.14 (0.032)</td>
<td>180</td>
<td>0.000</td>
<td>170</td>
<td>210</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/TV, %</td>
<td>85 (4.4)</td>
<td>34 (7.2)</td>
<td>150</td>
<td>0.000</td>
<td>140</td>
<td>170</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TV, mm³</td>
<td>14 (3.0)</td>
<td>3.4 (0.91)</td>
<td>320</td>
<td>0.000</td>
<td>260</td>
<td>380</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMD, g/cm³ CaHA</td>
<td>17 (3.6)</td>
<td>10 (1.4)</td>
<td>68</td>
<td>0.000</td>
<td>43</td>
<td>93</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Day 42</strong></td>
<td>Max force, N</td>
<td>12 (3.5)</td>
<td>5.2 (1.5)</td>
<td>130</td>
<td>0.000</td>
<td>80</td>
<td>180</td>
<td></td>
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<tr>
<td>Stiffness, Mpa</td>
<td>3.9 (1.2)</td>
<td>1.9 (0.28)</td>
<td>100</td>
<td>0.001</td>
<td>56</td>
<td>150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy, Nm</td>
<td>13 (6.1)</td>
<td>4.4 (1.7)</td>
<td>210</td>
<td>0.001</td>
<td>110</td>
<td>310</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD, g/cm³ CaHA</td>
<td>0.45 (0.060)</td>
<td>0.20 (0.056)</td>
<td>120</td>
<td>0.000</td>
<td>98</td>
<td>150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/TV, %</td>
<td>76 (12)</td>
<td>21 (9.3)</td>
<td>260</td>
<td>0.000</td>
<td>210</td>
<td>310</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TV, mm³</td>
<td>0.14 (0.021)</td>
<td>0.039 (0.017)</td>
<td>260</td>
<td>0.000</td>
<td>210</td>
<td>310</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMD, g/cm³ CaHA</td>
<td>0.44 (0.037)</td>
<td>0.34 (0.027)</td>
<td>29</td>
<td>0.000</td>
<td>26</td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Day 21</strong></td>
<td>Max force, N</td>
<td>12 (3.1)</td>
<td>7.1 (2.5)</td>
<td>69</td>
<td>0.001</td>
<td>32</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>Stiffness, Mpa</td>
<td>3.9 (1.1)</td>
<td>2.6 (0.72)</td>
<td>52</td>
<td>0.004</td>
<td>19</td>
<td>86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy, Nm</td>
<td>13 (4.9)</td>
<td>6.3 (2.7)</td>
<td>110</td>
<td>0.002</td>
<td>46</td>
<td>160</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD, g/cm³ CaHA</td>
<td>0.44 (0.072)</td>
<td>0.15 (0.039)</td>
<td>200</td>
<td>0.000</td>
<td>160</td>
<td>230</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/TV, %</td>
<td>73 (14)</td>
<td>12 (7.2)</td>
<td>520</td>
<td>0.000</td>
<td>430</td>
<td>610</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TV, mm³</td>
<td>0.14 (0.027)</td>
<td>0.022 (0.013)</td>
<td>520</td>
<td>0.000</td>
<td>430</td>
<td>610</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMD, g/cm³ CaHA</td>
<td>0.55 (0.030)</td>
<td>0.49 (0.047)</td>
<td>12</td>
<td>0.004</td>
<td>4</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Day 28</strong></td>
<td>Max force, N</td>
<td>11 (3.6)</td>
<td>9.0 (2.4)</td>
<td>20</td>
<td>0.20</td>
<td>-11</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Stiffness, Mpa</td>
<td>3.7 (1.0)</td>
<td>3.3 (0.81)</td>
<td>12</td>
<td>0.35</td>
<td>-14</td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy, Nm</td>
<td>10 (4.7)</td>
<td>7.7 (2.6)</td>
<td>34</td>
<td>0.13</td>
<td>-12</td>
<td>80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD, g/cm³ CaHA</td>
<td>0.43 (0.088)</td>
<td>0.14 (0.028)</td>
<td>200</td>
<td>0.000</td>
<td>180</td>
<td>240</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/TV, %</td>
<td>78 (20)</td>
<td>11 (5.1)</td>
<td>620</td>
<td>0.000</td>
<td>570</td>
<td>750</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TV, mm³</td>
<td>0.15 (0.038)</td>
<td>0.021 (0.010)</td>
<td>630</td>
<td>0.000</td>
<td>570</td>
<td>750</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMD, g/cm³ CaHA</td>
<td>0.51 (0.025)</td>
<td>0.42 (0.039)</td>
<td>20</td>
<td>0.000</td>
<td>12</td>
<td>27</td>
<td></td>
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mean or median. P values are added for convenience (as a descriptive), but do not reflect primary hypothesis testing.

*FIGURE 3 HERE*

*FIGURE 4 HERE*

*TABLE 1 HERE*

192 **Samples lost**

1 animal in the diaphyseal day 21 control group, and 2 in the day 28 control group were excluded prior to mechanical and microCT testing, due to stabilizing pin protruding distally.

196 **Discussion**

Alendronate increased the ultimate force both in the diaphyseal midshaft osteotomy and in the metaphyseal screw insertion trauma model. The effect was more immediate in the metaphyseal model. The findings were confirmed by microCT, using a metaphyseal empty drill hole as comparison to the diaphyseal fracture gap.

The current literature on shaft fracture healing shows that the main reservation against the use of bisphosphonates in fracture healing is dampened remodeling. This effect may be visible on radiographs, but seems usually not to translate into a negative effect on mechanical strength, possibly with exception for direct healing. Instead the main effect of bisphosphonates on fracture healing is the retention of bone.

In contrast to shaft fractures, cancellous bone healing in the metaphyses gives rise to fast bone formation in the marrow, both in humans and rodents, with woven bone arising from cell condensations in the marrow [3] as early as within the first week. [1, 5] This is probably
made possible in part by the relatively high number of local MSCs in the marrow [4] and entail a different healing process compared to diaphyseal healing, which is dominated by periosteal or endochondral bone formation. The metaphyseal bone formation after trauma is also strictly confined in space; it does not extend outside the traumatized area, as demonstrated by Charnley more than 60 years ago.[7] This is confirmed by our microCT and previous histology findings on mouse metaphyseal drill holes.[1, 5]

Stability influences both metaphyseal and diaphyseal fracture healing.[8] Stability, however, might mean two different things: either a situation leading to cyclic tissue deformation, or a risk of redislocation. Cyclic tissue deformation can influence cell behavior via mechanotransduction. This can be studied in for example unstable rodent diaphyseal models. However, iterated or cyclic deformation seldom occurs in metaphyseal fractures in the clinical situation, even though they may be unstable in the sense that there is a risk of redislocation. The metaphyseal model we use in our experiments is rather stable and might be considered to reflect the relative absence of cyclic deformation that can be expected in metaphyseal fractures in the clinical situation.

The use of two different surgical models in the same statistical analysis requires some explanation. ANOVA is based on differences between groups. However, when using log transformed data, it instead analyzes ratios, since log(x) – log(y) = log(x/y). This means that the hypothesis tested concerns the ratio between bisphosphonate and control group in one surgical model in relation to a similar ratio for the other model. This makes the analysis unaffected by differences in mean values between the two surgical models.

Earlier studies using diaphyseal models have demonstrated that bisphosphonate injections cause a decrease in callus remodeling. Despite this, mechanical strength is typically increased [9-18] or not affected [19, 20], probably because the treatment increases mineral content and
volume of the callus. Time till union seems mostly unaffected. More frequent injections (daily or weekly) have a more marked effect on remodeling than single or less frequent injections.[13, 18] One study contrasted the effect of a bolus injection of zoledronate administered 1 day, 1 week, or 2 weeks after fracture. The result was a stronger inhibitory effect on remodeling with the latest injection, as measured by material properties week 12. The authors suggest that the earlier injections did not affect the remodeling of bone not yet formed at the time of injection. However at neither 6 nor 12 weeks post fracture did the difference in material properties translate into a difference in terms of ultimate load.[13]

However, bisphosphonates have been reported to lead to a reduced bending strength after 6 weeks in rats.[21] Interestingly this model used direct healing of perfectly reduced fractures. Direct healing depends on osteonal growth across the fracture gap, or in rats on bone formation inside the gap. A narrow gap might need widening by osteoclastic resorption to make space for vessels and bone forming units, which could explain the negative effect of bisphosphonates.[22] For the same reason, this inhibitory effect of bisphosphonates has also been described in models for stress fracture.[23, 24]

Although understudied, there are a number of metaphyseal bone healing models in the literature. There is, however, a problem with definitions: from a biological perspective, we are interested in healing mechanisms in cancellous bone, but the metaphysis includes cortical bone contributing to its mechanical properties. In this study, we tried to regard cancellous bone healing as a separate phenomenon. Different metaphyseal models have different relevance for the cancellous part of the healing process. To be relevant for cancellous bone healing, a model should not heal by external callus. Examples are proximal tibial osteotomies in mice [25] and rats [26], fixated with external plates, and a longitudinal osteotomy in rabbits.[3] These models are stable enough to mostly avoid an external cartilaginous callus and in the rats allow measurement of bending stiffness.[26] In comparison, screw pull-out
models might have less variability, and are quite sensitive to bone anabolic or antiresorptive
treatment.[27-31] Partial osteotomies allow for the study of metaphyseal fracture healing
under variable conditions and can add valuable information.[32, 33] A limitation is that the
approach does not allow for mechanical evaluation.

Currently there is no single metaphyseal bone healing model available that can be said to be
without limitations. This is true also of the model used in this study; it can be debated
whether a screw inserted into a metaphysis is a good model for bone regeneration. We have
attempted to validate its relevance.[6] When a hole is drilled into the metaphysis, a fracture
healing response is triggered, leading to new bone formation. This new-formed bone will
form a “threaded nut” that resists the pull-out force. The force will therefore be a measure of
the amount and quality of the new bone (Fig 1).[27, 30] Osseointegration has little effect on
the screw pull out force.[6] Taken together, pull out force seems to be a good readout for
metaphyseal fracture healing, at least in the early phase.

The drug dosage was chosen for maximal effect, and would correspond to almost complete
osteoclast inhibition in humans. This is a limitation with respect to the clinical relevance of
our findings.

**Conclusion**

The effects of bisphosphonate in the metaphysis appeared much earlier. This supports the
idea of different healing mechanisms at the two sites. The early effect could be a crucial
benefit when treating fracture or implant patients, as early fixation is very important in these
cases.

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