Author's response to reviews

Title: Effects of BIS076 in a model of osteoarthritis induced by anterior cruciate ligament transection in ovariectomised rats

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Author's response to reviews: see over
EDITORIAL REQUIREMENTS:
"1. Please reformat the title page at the front of your manuscript file. It should contain, at minimum, the names, institutions, countries and email addresses of all authors, and the full postal address of the submitting author."

The title page has been reformatted, as required.

REFEREE 1
Major Compulsory Revisions:
"1) Fig.1 Histologic analyses. The examples of histologic sections shown in Figure 1 are of poor quality. If these are representative of the whole group, then reliable histologic scoring would be challenging. Furthermore using HE stained sections only would prohibit scoring of aggrecan loss which is a component of the OARSI scoring scheme. Toluidine blue or Safranin O-fast green stained sections would be more suited for this purpose"

Images in Fig. 1 (hematoxylin/eosin staining) have replaced by other examples of higher quality. In addition, as we also performed the safranin O staining, we have added the new Fig. 2 to show some examples.

"2) P6 last sentence: the composition of the porcine cartilage extract is listed as 40-65% type II collagen and 15-25% glycosaminoglycans, mainly chondroitin sulphate). How variable is the content of the porcine cartilage extract composition and was only one batch used in this preparation." Only one batch was used in this study (PP-1). For the sake of clarity, we have included the exact composition of the only BIS076 batch used in this study (page 7, first sentence). The procedure was validated by other batches:

<table>
<thead>
<tr>
<th></th>
<th>Batch PP-1</th>
<th>Batch PP-2</th>
<th>Batch PP-3</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typer II Collagen</td>
<td>55.5% on dry basis</td>
<td>57.3 % odb</td>
<td>54.9 % odb</td>
<td>55.9 % odb</td>
</tr>
<tr>
<td>Chondroitin sulphate</td>
<td>21.3% odb</td>
<td>18.3 % odb</td>
<td>19.7% odb</td>
<td>19.8 % odb</td>
</tr>
</tbody>
</table>

"3) P7, 1st paragraph: clarification of the experimental groups is required. It is assumed that all rats underwent ovariectomy and then the groups were split into sham surgery and ACLT surgery groups and these then allocated to treatment groups. A sham surgery group + treatment are not included, which would have allowed assessment of BIS076 on the tissue and cytokine expression independent of OA."

All rats did not undergo ovariectomy and were then split into ACLT and sham surgery groups: Sham group animals underwent simulated ovariectomy (week 0) followed by simulated ACLT (week 2). In page 6, lines 17-18 it was indicated that "Another group of animals (Sham) underwent sham operations". To better describe the experimental groups we have replaced it by:
"Another group of animals (Sham) underwent sham ovariectomy at week 0 followed by sham ACLT at week 2."
And we have added in page 6, lines 23-24: "Rats subjected to ovariectomy+ACLT were divided into three groups: Control and two treatment groups (T1 and T2)."

On the other hand, the inclusion of more animal control groups is restricted by the ethical committee of our institution and it would require a new application and approval after the confirmation of the interest of BIS076 effects. Therefore, this is a point for future studies.

"4) P10, 2nd paragraph, Fig. 3 and Table 2. MicroCT studies.
- It is not clear, what the area for bone structure analysis was for the epiphysis? Did this include the subchondral bone (below cartilage but above the growth plate) and the primary spongiosa (immediately below the growth plate as shown in Fig 3. If this is the case, then an analysis of just the subchondral bone above the growth plate would be more informative."

This observation is right. We agree that analysis of subchondral bone alone is very important in OA and obviously in human studies. Nevertheless, in this experimental model we have effects in several bone compartments related to the response to ACLT and ovariectomy and previous experiments indicated more reproducible results when the whole area was selected for analysis.

"- The authors claim that these is a similar tendency for bone loss in the epiphysis as observed for the metaphysis upon treatment. This is not correct. There was no difference (or trend) in any of the parameters in the epiphysis."

In page 10, last paragraph, the order of some sentences have been changed to clear up that this commentary refers to sham and control groups only (as the changes in the different parameters comparing sham and control groups followed a similar profile in metaphysis and epiphysis) and not to treatment groups:

"μCT analysis indicated loss of bone mass and structural alterations in control rats compared with sham-operated animals (Table 2 and Figure 3). Main bone changes were observed in metaphysis, with significant reductions in bone volume fraction (BV/TV), bone surface density (BS/TV), trabecular number (Tb.N) and volumetric bone mineral density (vBMD) whereas trabecular factor (Tb.Pf) significantly increased. A similar tendency was observed in the parameters measured in epiphysis although these changes did not reach statistical significance. Interestingly, BIS076 counteracted all bone alterations shown in control animals in the metaphysis only, with statistically significant effects at the higher dose (T2)."

5) It is a little surprising that the changes in the metaphyseal bone are much greater then those observed in the epiphysal bone both in terms of bone lost between ACLT and sham surgery groups and the ACLT control and treatment groups. Could the authors speculate why this may be the case. Furthermore treatment had a stronger affect on the metaphyseal bone compared to the epiphyseal bone. Can the authors explain the differences in the different compartments of the bone?

This is not surprising if we must take into account the characteristics of the experimental model used. Morphological changes in rat tibia after ovariectomy have been widely described. It is known that trabecular bone at the metaphysis of the rat tibia deteriorates to a greater extent than at the epiphysis (e.g. Sheng et al. 2007, Acta Radiol 48, 131, Brounwers et al. J Orthopedic Res
This is in fact the case in our model, as we have observed in many different experiments.

"6) p11 first paragraph and Figure 5.
- Analysis of serum markers of cartilage degradation and bone cell activity. The authors state in the text that there is an enhanced level of CTX-II in the sera between sham and control, however in Fig 5 this is not evident. Furthermore, although the authors are showing SD, many of the statistical significance is not convincing. Perhaps the authors could plot their data as a box and whisker plot or with individual points within the groups to show the spread of data."

The difference in CTX-II between Sham and Control groups is significant (ANOVA followed by Bonferroni's test), t=3.294, ** (GraphPad Prism 5).

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Control</th>
<th>T1</th>
<th>T2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>5.86</td>
<td>12.19</td>
<td>13.45</td>
<td>5.30</td>
</tr>
<tr>
<td>SD</td>
<td>3.77</td>
<td>5.24</td>
<td>5.14</td>
<td>4.55</td>
</tr>
<tr>
<td>n</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>15</td>
</tr>
</tbody>
</table>

Following the reviewer’s suggestion, Figure 5 (now Fig. 6) is presented now as box and whisker plot. Figure 4 (now Fig. 5) is also presented as box and whisker plot. In addition, the statistical analysis has been checked.

"- The apparent reduction in TRAP5B levels in the ACLT+control group compared to the Sham group is a little surprising given that there is a significant bone loss within the metaphysis. Can the investigators explain why this is. Have they done osteoclast counts within the histologic sections? - Serum CTX-1 levels would have provided a better measure of bone resorption in addition to TRAP5b activity and RANKL/OPG.="

This reduction is known to occur after ovariectomy in rats. Depending on the time of measure, although osteoclasts are active and bone resorption is present in certain areas (as we have shown by microCT) the total number of osteoclasts and serum TRAP5b can decrease. We agree that assessment of several biomarkers is necessary to follow bone turnover. It is also known that ovariectomy-related bone loss in rats is a RANKL-dependent event which can be efficiently blocked by OPG (e.g. Omsinski et al. J Bone Mineral Res 2008, 23, 672), and for this reason the ratio RANKL/OPG was determined in these experiments. Other biomarkers can be used, we agree on the interest of CTX-1 which will considered for future experiments as well as histological osteoclast count.

7) The authors do not provide any discussion on how BISO76 may be eliciting its effects. This should be included in the discussion.

We have added in this revised version a last paragraph in the discussion section and new references (40-44) supporting our views.

Minor Essential Revisions
"1) P8 microCT methods paragraph. A clear description of how the region of interest for each anatomical location where bone microarchitecture was assessed should be provided. Details of the software used for microCT analyses (including versions) and thresholds used for analysis should also be listed."

Details of microCT method have been added to materials and methods, as suggested.

"2) In the background section of the abstract it is stated that “some of the available treatments are dietary supplements providing natural components that can help to preserve structural integrity of the joint”. To this reviewer’s knowledge, this statement is largely incorrect, at least in the treatment of human OA."

We agree that demonstrated effects on structural integrity in human OA are doubtful in many cases. For the sake of accuracy, the last statement has been deleted.

-REFEREE 2
"The authors have analyzed the effects of a new formulation of natural porcine cartilage extracts (BIS076) in a model of osteoarthritis induced by ACLT in ovariectomized rat. The main criticism of this study is relative to the experimental design that is not clear in different points: number of treated animals/group/treatment, description of the time points analyzed for histopathology, μCT, inflammatory mediators, serum biomarkers. Moreover, since they selected a model that is a mix of two pathologies (osteoporosis and osteoarthritis) to dissect the real effects observed on the different compartments (cartilage, bone) they should consider also a group of only ovariectomized rats and a group of only ACLT treated rats."

In the first version, the number of animals per group was indicated in page 6, line 14, and also in the legends to figure. The treatments appeared in pages 6-7. The time point for histopathology and all determinations was the end of the experiment (week 12). All these points have been clear up in the revised version of the manuscript, with all changes marked in red. Concerning this model, in preliminary experiments during the setup phase we compared it with ovariectomised rats and ACLT rats in order to know the characteristics and limitations of this experimental procedure as a model related to alterations of cartilage and bone. We selected it for this study as a suitable model to assess drug effects on cartilage and bone using the possible lowest number of animals.

"Minor point: 1. The background has not a clear sequence of the arguments treated in the paragraphs."

The background is just an introduction to understand the rationale for this study. It comments from general aspects of OA and bone loss to the need of treatments and the experimental model used.