Author’s response to reviews

Title: Chitosan in viscosupplementation: in vivo effect on rabbit subchondral bone

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Author’s response to reviews:

Reviewer reports:

1. Stephan Zeiter (Reviewer #1):

• Major comments

In this review the authors investigate the effect of viscosupplementation with hyaluronic acid (HA) alone or combined with chitosan on the subchondral bone in a rabbit OA model.

The manuscript is well written and enough details for replication are provided or referenced. The findings are interesting and of relevance. However, in order to get published two major points and some minor points need to be addressed.

1.1. The major limitation of this work is that the untreated, negative control group is missing. In the reviewer’s opinion, this group where saline instead of HA is intraarticularly injected, is needed in order to attribute changes observed to the development of the disease or the effect of HA alone or combined with chitosan.
Authors’ response:

Thank you for raising the question about the “negative control group” (Saline-group), which gives us the opportunity to give more power to the results of the study. Indeed, the overall study did include a “saline group” though we initially chose not to present it to simplify the comparisons.

As suggested by the reviewer, we added this group in the revised version of the manuscript. This allows us to answer whether the changes observed are due to the development of the disease or the effect of HA alone or combined with chitosan.

Consequently,

- previous figures have been replaced in the results section by Figure 3 – 4 – 5. Text has been updated accordingly,

- the table summarizing the results has been updated accordingly (line 195),

- the formulation administration in material and method has been updated accordingly,

- throughout the text reference to Saline-group has been added accordingly (highlighted in yellow)

- notably, the following paragraph has been added into the discussion (line 269):

“A significant decrease of C.Th mean value confirming OA induction was observed when the Saline-group was compared to the Contralateral-group, while no significant changes were
observed for all the bone parameters. Contrastingly, no significant decrease of C.Th mean value was observed when the HA and Hybrid-groups were compared to the Contralateral-group suggesting a preserved chondroprotective effect of HA when combined to Cs. Those results are in accordance with other studies [19–21,38,39].”

1.2. Alternatively, historical data from previous publications could be used. The contralateral leg provides information on the comparison

Authors’ response:

Since we added the “Saline-group” as suggested by Dr Zeiter, the use of historical data from previous publications was not inserted in the article. Indeed, there is an important variability regarding study designs in the literature which can affect microarchitectural parameters, i.e. age, weight and breed of the rabbit used.

1.3. Further, the manuscript would be strengthened if histology would be included in the analysis. This could provide additional insight into the pathogenesis of the observed changes.

We kindly invite the readers to refer to our previous article [Kaderli et al. 2015] where histology is discussed for the same animal groups.

Reference:

• Minor comments

1.4. The reviewer would be interested to know why the authors did not do within animal comparison with the contralateral side - this would be statistically more powerful.

Authors’ response:

Following ACLT, gait alterations and joint unloading in the rabbit model have been previously reported. This phenomenon is attributed to lower loads bearing on the ACLT hindlimb, this increases load bearing of the contralateral side (Bray RC et al, 1992). In a treatment evaluation study, as it is the case in the present study, treatment can potentially cause clinical efficacy and an increased weigh bearing capacity on the ACLT knee can then be a secondary effect. The contralateral weight bearing is then reduced.

In our study, there were no differences between the HA-group contralateral samples, Saline-group contralateral samples and Hybrid-group contralateral samples. Thus, normalization was not needed and a “Contralateral-group” with randomly chosen legs from each treated group was created in order to avoid group’s size discrepancies.

The authors wish to add the following information for the reviewer:

In our study, the same results (comparison between groups) were found whether the medial or lateral condyle was investigated. This suggested equilibrated contact-loads on tibial condyles and normal gait (Table 1 p.8).

Reference:

1.5. Age/ weight of the rabbits: please include the range of this parameters

Authors’ response:

Line 93, weight parameter has been detailed accordingly:

3.7±0.18 kg

All rabbits were 5 months old.

1.6. The authors should consider to add data on the housing (single vs group housed etc) and feeding (see also comment below on obstipation).

Authors’ response:

Accordingly to the reviewer question, the following information was added in Animal Model section line 94:

“After 2 weeks in acclimation and quarantine in individual boxes, …”

1.7. Due to the short acting of buprenorphine it is suggested to repeat injection every 8 to 12 hours. Additionally the use of NSAID should be considered.

Authors’ response:

The reviewer’s observation is absolutely right. The rabbits did receive buprenorphine the day of surgery and twice a day (bid) for 4 days. This was corrected in the material and method line 107 as follows:
“The rabbits received subcutaneous injection of 0.01 mg/kg buprenorphine bid for 4 days to avoid pain”.

Following ACLT surgery, meloxicam can be used as NSAID (subcutaneous or IV injection, or per os) even though it is an “off-label” use. In order to fully evaluate the efficacy (including anti-inflammatory properties) of the novel viscosupplements, in our study, it was decided to only use NSAID on the day of surgery. Instead, buprenorphine was used post-surgery and veterinarian supervision was performed to closely monitor and treat any sign of pain.

1.8. What was the rationale for the authors to use metoclopramide and Feligastryl? increased risk of obstipation due to opioid intake?

Authors’ response:

In the rabbit species, the use of opioids, the pain, or the length of an anesthesia can increase the risk of post-surgical obstipation and anorexia. To reduce this risk, Emeprid® (metoclopramide) and Feligastryl® (eserine) were used to favor the intestinal motility, ease the gastric emptying and provide orexigen effects. This is a protocol commonly used in clinical setting for treating rabbits suffering from obstipation/anorexia.

The information was added in the Postoperative care paragraph line 109 as follows:

“..., 0.5 mg/kg Emeprid® (metoclopramide) for 3 days, 15 mg/kg Borgal® bid for 9 days and 1 cp/day Feligastryl® (eserine) for 3 days to reduce the risk of obstipation.”

1.9. Since ROI were defined by the operator -was he/she blinded for the assessment?
Authors’ response:

The operator was blinded during the whole ROI definition process. This was detailed in the Microarchitectural parameter measurements paragraph line 141 as follows:

“Image treatment and parameters measurements were performed by a single operator blinded for the assessment …”

2. Teja Guda (Reviewer #2):

The authors conducted an interesting study to develop and evaluate the subchondral bone loss associated with ACL transection by viscosupplementation using either HA or the HA+Cs. The study is well characterized and the details allow easy future reproduction. Some concerns were noted in review:

• Major comments

2.1. Why were non-parametric tests (Kruskal-Wallis and the MWW rank test) used instead of parametric tests? What was the power of the tests? Was this because of the data not being normally distributed, or because of the groups having non-uniform standard deviations.

Authors’ response:

Levene and Shapiro tests revealed homogeneous variance across the groups and normal distribution among each group, which would enable the use of parametric tests such as ANOVA
and then Student test as post hoc. However due to the limited size of the experimental groups (n=6 to n=8) confidence about normal distribution cannot be guaranteed thus non-parametric tests were preferred. Unfortunately, to our knowledge, there is no easy and regular procedure to determine the power of non-parametric tests.

2.2. Why were only 4 non-operated knees (per group) used as contralateral controls? The normalization of the treated groups to the contralateral groups would enable the inter-animal variation to be removed and hence increase the power as well as potentially enable the use of parametric tests. Was the contralateral group data normally distributed - more crucially, was there ANY statistical significance between the HA-group contralateral samples compared to the Hybrid group contralateral samples? More crucially, how were the 4 samples per group chosen (or 2 samples excluded)? I would recommend including all 6 animals in each group.

Authors’ response:

Following ACLT, gait alterations and joint unloading in the rabbit model have been previously reported. This phenomenon is attributed to lower loads bearing on the ACLT hindlimb, this increases load bearing of the contralateral side (Bray RC et al, 1992). In a treatment evaluation study, as is the case in the present study, treatment can potentially cause clinical efficacy and an increased weigh bearing capacity on the ACLT knee can then be a secondary effect. The contralateral weight bearing is then reduced.

In our study, there were no differences between the HA-group contralateral samples, Saline-group contralateral samples and Hybrid-group contralateral samples. Thus, normalization was not needed and a “Contralateral-group” with randomly chosen legs from each treated group was created in order to avoid group’s size discrepancies.

The authors wish to add the following information for the reviewer:

In our study, the same results (comparison between groups) were found whether the medial or lateral condyle was investigated. This suggested equilibrated contact-loads on tibial condyles and normal gait (Table 1 p.8).
Reference:


2.3. The progression of OA is relative and the validity of the rabbit model in terms of cartilage and subchondral healing in the rabbit has been debated widely in literature. While the results of this study are interesting, a major drawback is that only one time point is used. How are the authors confident that this is a progressive deterioration?

Authors’ response:

The aim of this study was to investigate the effect of two HA-based viscosupplements in early OA and in particular the action of Chitosan added to hyaluronic acid. Thus, only one time point was chosen to enable the comparisons. OA progressive characteristic has been demonstrated in different studies thanks to multiple time points, [Yoshioka et al., Vignon et al., and Fischenich et al.].

OA management by experts’ recommendation should takes place at the early phase of the disease [Henrotin Y. et al., 2015]. After careful analysis of the literature, 4 and 8 weeks post-surgery time points were shown to induce macroscopic and microscopic femoral bone alteration considered as early OA signs [Batiste DL et al., 2004, Florea et al. 2015]. Since OA lesions are usually more severe on femoral than on the tibial condyles in the ACLT rabbit model [Wang et al., 2007 – Batiste DL et al., 2004] , a 6 weeks post-surgery time point was considered optimum to investigate early OA tissue changes on tibia.

References:


• Minor comments

2.4. Acronyms "DMOAD" and "SYSADOA" are introduced in the introduction, but never used in the manuscript. Introducing the terminology is necessary; the acronyms themselves are unnecessary and should be removed from the introduction.

Authors’ response:

“DMOAD” and “SYSADOA” acronyms have been removed from the introduction
2.5. The introduction should end with a one sentence purpose - such as what would be indicated if the hypothesis did proved true ("indicating that the supplementation showed OA progression in the preclinical model", etc)

Authors’ response:

Accordingly to the reviewer opinion, the following sentence has been added in the end of the introduction line 86:

“Such an early change on the subchondral bone might be symptomatic of an OA disease modification.”

2.6. The sentence "In order to go one step further in the evaluation of the effect of Cs on subchondral bone, the impact of bone microarchitectural parameters on the mechanical compliance will be evaluated as well as the relationship of this last on the cartilage integrity." Is not appropriate for the conclusion - maybe better at the end of the discussion section.

Authors’ response:

The Conclusion and the last paragraph of the discussion were switched (line 308 and 316).

2.7. Please edit language for sentence phrasing to facilitate easier readability.

Authors’ response:

The whole article has been reviewed by Dr. Joseph Hamm, native English speaker, and modified accordingly before resubmission. The acknowledgement paragraph has been updated (line 347).