Author's response to reviews

Title: TOL19-001 reduces inflammation and MMP expression in monolayer cultures of tendon cells

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Author's response to reviews: see over
Dear editor,

Please find attached a revised version of our manuscript. We thank the reviewers for their valuable and very constructive comments, which helped us to improve the quality of the manuscript. As indicated in our point-by-point response, we have incorporated changes as suggested by the reviewers. Thank you very much for giving us the opportunity to resubmit a revised version of the manuscript. We strongly hope that the manuscript will be now suitable for publication.

Yours sincerely,

Catherine Baugé

Point to point response to reviewers

Reviewer 1:

There is no major corrections and need some minor correction

Comments

1. In page no 2, line no 9, check the spelling of developed
   
   We corrected the spelling

2. In page no 2 and 5, line no 26 and 5 respectively, check the spelling of ciprofloxacin
   
   We corrected the spelling

3. It would be better if authors provide the ethical committee number, page no 5 line no 10
   
   We added the ethical committee number (ref #A13-D46-VOL.19)

4. In page no 5, line no 21, give the reference for Trizol method of RNA isolation and WST1 assay
   
   We added information about Trizol extraction (Trizol® Reagent (Life Technologies, #15596-018) (Baugé et al., 2007; Chomczynski, 1993)) and Wst1 assay (Product No 05015944001, Roche Diagnostics, Meylan, France) (Baugé et al., 2015; Mosmann, 1983).

5. Page no 11, line no 19, check the spelling of Spirulina
   
   We corrected the spelling.

6. In reference section some journal names are abbreviated and some are written in full, for example reference no 23, please check it.
   
   We corrected the reference styles, and now used the styles of BMC
Reviewer 2

Summary:

Treatment of tendinopathies provides a clinical challenge, as current treatments with non-steroidal anti-inflammatory drugs (NSAIDs) or corticosteroids often results in reduced tendon specific matrix production and loss of tissue stability. In the presented study TOL19-001 (a combinational drug from spirulina, glucosamine, sulfate, ginseng, selenium, sillicium, iron, vitamin E and zinc) was investigated for its beneficial effects on interleukin-1β and ciprofloxacin stimulated tenocytes in vitro. RT-PCR and ELISA demonstrated TOL19-001 reduced interleukin-1β and ciprofloxacin induced MMPs, PGE2, scleraxis and p65 levels and enhanced collagen I expression.

Comments:

As treatment of tendinopathies often has an unsatisfactory outcome, due to loss of tendon mechanical stability, new treatment approaches are necessary to be investigated. Treatment with TOL19-001 seems interesting, however several points in the presented study need to be clarified and investigated further.

1. As only one in vitro condition, monolayer culture, was used, please change this in the title.

We modified the title. Now, we indicated that experiments were done in monolayer cultures. New title is: “TOL19-001 reduces inflammation and MMP expression in monolayer cultures of tendon cells”

This is also now clearly indicated in the abstract (section “methods”).

2. The abstract should present all results obtained in the study. For example, results on expression of scleraxis, TIMPs, and metabolic activity with Wst1 assay are missing. Overall the abstract is poorly written.

We completed abstract and added information about metabolic activity as well as results about scleraxis and TIMP1.

3. The manuscript should be checked carefully for uniform writing of Interleukin1-β (IL-1β).

IL-1β spelling was checked and corrected.

4. In Materials and Methods the authors should provide an experimental design.

An experimental design is now indicated in the Material and methods section.

5. Authors should demonstrate at first that the cells they isolated from tendon are tenocytes. This can be done for example with LM pictures, IF, IHC staining, western blotting, PCR and/or others to demonstrate (i) tenocytes cellular morphology and (ii) investigation of tenocyte typical gene/protein expression profile such as scleraxis,
collagen I.

As showed in figure 3 by RT-PCR, tendon cells used expressed collagen I as well as scleraxis.

6. Treatment with only 1 concentration of IL-1β is too little.

We agreed with reviewer that treatment with increased concentration of IL1 may give more information than using a single concentration. However, numerous articles studying tendon cells, used similar protocol and used also a single concentration of interleukin-1 (for instance: Dakin et al., J Biol Chem, 2014, 289, 4919-4927, Busch et al., J Biol Chem. 2012, 287(45): 38050–38063; Busch et al., J Biol Chem, 2012 287, 25770-25781, etc). Here, we used a concentration of 1 ng/ml because it is a classical dose used for this aim in papers using monolayer cell culture (Corps et al., Matrix Biology2004, 23(3): 163–169), and our results clearly show that IL-1 at this concentration is able to induce MMPs expression and PGE2 release in monolayer cultures of tendon cells. However, as discussed later, we were not able to detect an induction of NFkB pathways by IL-1. So it is possible that using higher dose of cytokines may permit to observe NFkB activation by IL-1. We add this hypothesis in the discussion section.

7. Results, page 7, line 23: Authors state that IL-1β did not activate NF-kB. However this has been demonstrated in several reports (Mobasheri A, and Shakibaei M. Histol Histopathol. 2013, Busch F. J Biol Chem. 2012, Buhrmann C. J Biol Chem. 2011). This result, as it stands opposite to other studies, should have been investigated in more detail.

We are aware that this result is surprising. That’s why we discuss this point in discussion section. We do not assert that IL-1 does not induce NFkB activation. We only write that we was not able to observe it, and give different hypothesis to explain that. It may be not clear in the first version of the manuscript. So, we now try to make it more apparent. We added more information about concentration of IL-1 and incubation time in the description of results, and discussed more this observation in the discussion section.

“Surprisingly, we did not observe an increased expression of p65 mRNA after IL-1β treatment. However, we could not exclude that, in tenocytes, IL-1β induces NFκB activation through a post-transcriptional regulation. Indeed, NF-κB is present in the cytoplasm in its resting stage as a heterotrimer complex consisting of two subunits and an additional inhibitory subunit, IκBα [46]. During the activation process, the inhibitory subunit IκBα is phosphorylated at Ser-32 and Ser-36 residues by IKK kinase (IκBα kinase) and is subsequently degraded. Once released, subunits of activated NF-κB translocate to the nucleus and mediate transcription of various inflammatory and catabolic gene products [47, 48]. Thus, it is likely that IL-1β induces NF-κB activation in tenocytes [43], whereas it does not upregulate p65 mRNA expression, and consequently by a different mechanism than CIP.”

We now add that this could also due to an inadequate dose of IL-1b or time of treatments.

8. Results, page 8, line 14-15: Authors speak about ultrastructure of the cells. They should show this in the manuscript!

When we performed experiments, we did not take pictures (our camera was out of order). So we cannot document more this information Consequently, we suppress the sentence in the paragraph.
9. Authors should present in more detail subcellular mechanisms that are targeted by TOL19-001 treatment. For example, if TOL19-001 counteracts IL-1# induced effects, this has to be demonstrated by showing were TOL19-001 interferes with IL-1# inflammatory and degradative pathway! Possible effect on NF-kB-activation/signaling has to be investigated in more detail!

10. To identify more precisely were TOL19-001 may interact with NF-kB pathway inhibitors of different members of this pathway should be employed.

As indicated in discussion, NFkB is a major mediator of IL-1 pathways and inflammation. However, other signaling pathways, namely MAPK, can also be activated by IL-1 and have also a major role in its function. So, we totally agreed with the reviewer that this pathway deserves to be studied more in details. That’s why we think that this putative relationship between IL-1, NFkB and TOL19-001 should be studied in an extensive way (using different concentrations of IL-1 and TOL19-001 and different time incubation), which would required a specific study.

11. Overall, it is too little to only present RT-PCR and ELISA results. Results have to be made more plausible by employing additional techniques such as western blotting and IF.

We do not understand this remark. Indeed, RT-PCR and ELISA are often used to document gene expression. This two techniques are totally complementary since one evaluates gene expression at mRNA level, and the second at protein level. These both techniques are very specific and give a good information about gene expression. In addition, quantitative (even semi-quantitative) analyses are very difficult by western-blot and IF.

12. How does TOL19-001 influence the ultrastructure of the cells and the tenocytes matrix? As the ultimate goal is to obtain mechanically more stable tendon tissue this question is important.

This question is, indeed, very pertinent. But it is difficult to respond. Numerous and long experiments will be required. So it may be the topic of another study.

13. Figure 5 and 6: It is not clear from the manuscript if the tenocytes were treated with TOL19-001 or only the spirulina extract. As TOL19-001 contains additionally several components assumingly this would make a difference!

As indicated in the legend figures, and now also in the material and methods section (experimental design), the tendon cells are always treated with TOL19-001, and never with spirulina alone. This point is well discussed in the “discussion” section.

14. Discussion, page 9, line 17: Throughout the paper the authors talk about using two different models, and here they speak of two different conditions. This should be changed everywhere in the paper into using 1 in vitro condition (monolayer culture!) and different treatments (IL-1#, Ciprofloxacin, TOL19-001).

Effectively, we used only monolayer culture of tendon cells. However, two treatments were compared : IL-1 versus CIP. These both treatment are used to mimic some processes involved in tenopathies. That’s why we used the term of two models, whereas
both of them were did in monolayer culture.
We tried to make it less confusing and now clearly indicated when we used the term of models that both of them were did with monolayer culture of tendon cells. We also replace model term by treatment when it was confusing.

15. Discussion, page 10, line 1-11: Belongs to Introduction
We suppressed the paragraph.

16. Discussion, page 10, line 14: The authors use a monolayer, not a 3D model! They cannot speak about this being the “best model to study tendinopathy”!
We modify the text. And indicated that IL1 may be a better model than CIP.

17. Discussion, page 11, line 26: Again, this is not an adequate in vitro model to study tendinopathies.
We modified the text, and now, we clearly indicated that we used monolayer culture of tendon cells.