Author’s response to reviews

Title: Cellular pharmacodynamic effects of Pycnogenol® in patients with severe osteoarthritis: a randomized controlled pilot study

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Reviewer reports:

Sonja Maria Walzer (Reviewer 1): I would like to thank the authors for the detailed answers and explanations to my comments.

Question: (the authors wrote ....

......We do not think that patients with severe OA would have vast benefits from taking Pycnogenol in the 3 weeks between inclusion and the knee replacement surgery, which was already scheduled. However, as discussed above, we have good reason from results of the clinical studies (cited in references 6-8 in the reference list) that the patients could have less pain and less joint stiffness.

To your opinion and your current findings reported in this manuscript- how would you recommend a Pycnogenol intake to have the optimal long term effects on OA? At which stage should OA patients start?

We would think that it would be highly beneficial for the patients to start Pycnogenol after diagnosis of OA. The earlier the supplement is started the higher the chances that the progression and symptoms of OA are positively influenced. The safety of Pycnogenol is proven and there is no objection against long term use.
Stefan Toegel (Reviewer 2): The authors have adequately responded to most of my comments. I still think that this is an interesting study that qualifies for publication, however, some issues remain to be addressed, as listed below.

*) In their revision, the authors argue that "Due to the very low amount of tissue obtained from all patients and therefore the very low amount of cells and RNA isolated from the tissue, we had decided to check the RNA quantity and purity (A260/A280 ratio) for further processing and not the RNA integrity via agarose gels stained with ethidium bromide". Besides the fact that there are more advanced methods for the quality control of RNA preparations (i.e., Bioanalyzer), which need only tiny amounts of RNA, I wonder about the low RNA yield mentioned by the authors. The Methods section (page 8) clearly states: "The complete residual articular cartilage was removed from the patients' samples". Enzymatic digestion of the entire articular cartilage obtained during total knee replacement surgery should yield several millions of viable chondrocytes. This is also confirmed by the authors stating that "Cell aliquots of approximately 1 x 10^6 cells were shock-frozen in liquid nitrogen and stored at -80 °C. Only cell samples showing > 95 % viability were used for further experiments". Such a cell yield would result in sufficient amounts of RNA to perform any required quality control procedures. Is there a technical problem with RNA isolation procedure in the lab?

There was everything fine both with our chondrocyte as well as with the RNA isolation procedure.

The number of chondrocytes that were isolated from the residual cartilage varied between 2.4 million and 10 Million cells. We would like to again point out that the patients suffered from severe OA, so the chondrocyte yields were low for some patients.

Not all chondrocytes were used for RNA isolation, an aliquot of the chondrocytes was seeded for cell culture experiments. We aimed at measuring secreted mediators. Unfortunately this approach was not successful and is therefore not reported in this manuscript.

Due the limited number of chondrocytes the RNA yields for 9 out of 30 patients were sufficient only for determination of the A260/A280 ratio and subsequent qRT-PCR analysis, for 2 patients (# 101 and # 104) the yield was even insufficient for qRT-PCR analysis.

*) The authors' ambition to follow the MIQE guidelines is truly appreciated! However, I still have concerns regarding the authors' approach with reference gene selection/validation. I agree that the use of 3 reference genes is recommended, but of course all of the selected reference genes must be "stably" expressed to allow normalization. I do not see the usefulness of including an unstable reference gene for normalization of qPCR results, and I do not believe that the MIQE guideline intend to give this recommendation. I also do not see the benefit for the reader to present all calculations with 3 reference genes (including "unstable" SDHA) and then to exclude SDHA for recalculation. This is rather part of assay validation that each lab should perform before publishing the most robust results. The notion that SDHA is "unstable" might qualify for a technical note (maybe in the Methods section or in Supplementary files) but, in my eyes, it is not valid to foreground this aspect in the final paper. In my view, the authors have two options. Either they find a third stable reference gene and present a novel set of recalculated results, or
they simply use the 2 most stable (already identified) reference genes which would also fairly comply with the MIQE requirements. In any case, this will improve the manuscript with regard to clarity.

There is also a second remark related to this issue: The authors should clearly describe their approach for reference gene stability assessment. Which parameter was used to identify "stability", what was the cut-off value? Without such a description, the methodology leaves the impression of arbitrary decisions. Maybe geNorm would be a better option as this tool (besides stability rankings) also provides suggestions on the optimal number of reference genes required for reliable normalization of qPCR data.

We followed the suggestion of the reviewer and omitted SDHA as reference gene, using only the two most stable reference genes. We recalculated the results and present them in the revised manuscript.

As detailed in the manuscript we used the BestKeeper software package which is appropriate for reference gene stability assessment. This software uses the standard deviation as measure for gene expression stability. The cut-off is a standard deviation of +/- 1 Cp value. We added this information to the manuscript.

*) Similarly, when two patients were defined as outliers regarding pre-established criteria, it would be more straightforward to exclude associated data from calculations. At present, the presentation of results (particularly on page 12, line 280-300) is rather confusing.

As mentioned before we do not want to leave the impression that we suppress data or report incomplete or only the most expedient data. With a population of 30 patients the exclusion of a patient (even if fully justified) might induce change in terms of statistical significance. So we prefer reporting both values, with and without exclusion of the respective patient.

We tried to present the results on page 12, lines 280-300, more clearly.

*) The manuscript should include a clear description of the origin of the Pycnogenol capsules (company,…).

We added this information.

*) line 50: remove "at".

We changed this.

*) line 171: Life Technologies (capital letters)

We changed this.

*) "osteoarthritis" is not consistently abbreviated as OA within the manuscript.
We changed this. However, we prefer to spell out the abbreviation at the beginning of each new section (e.g. Introduction, Results etc.) to make the manuscript easier readable for those who start reading a selected part first.