Author's response to reviews

Title: In vitro-activity of oily calcium hydroxide suspension on microorganisms as well as on human alveolar osteoblasts and periodontal ligament fibroblasts

Authors:

Anton Sculean (anton.sculean@zmk.unibe.ch)
Sigrun Eick (sigrun.eick@zmk.unibe.ch)
Rick Miron (richard.miron@zmk.unibe.ch)
Tatjana Strugar (tanja.strugar@bluewin.ch)

Version: 2
Date: 2 January 2014

Author's response to reviews: see over
Dear Dr. Klein,

We thank the reviewers for their time and valuable feedback in reviewing our submitted manuscript “In vitro-activity of oily calcium hydroxide suspension on microorganisms as well as on human alveolar osteoblasts and periodontal ligament fibroblasts” and thank you for giving us the possibility to resubmit a revised version.

We went carefully through the reviewers’ comments and have modified the manuscript accordingly. Please find below our detailed response to the reviewers.

We hope that our manuscript now meets yours and the reviewers’ approval.

Yours sincerely on behalf of the authors

Our response to reviewer 1:

Reviewer 1:
“1. While it is justifiable to test a product that is supposed to work on the bone during periodontal surgery on bone cells and PDL fibroblasts, why did the authors assume that there could be any “antimicrobial properties” attributed to the compound used? This does not mean that there may not be any antibacterial activities of the product but the hypothesis is not clear regarding the microbiological testing.”

Our response: As introduced materials used clinically for bone regeneration often lack full investigation of their various roles on different cells and bacteria, one of the aims of this study was to determine if a possible antimicrobial property could be observed for OCHS. Furthermore, the material which was tested here contains a high percentage of calcium hydroxide known to act bactericidal, thus it wasn’t a far stretch to determine if such properties exist. As a response to this reviewer, a specific aim has been specified in the introduction section within the abstract accordingly.

Reviewer 1:
“2. In parallel, why did they test the experiments on co-culturing the bacteria with osteoblasts? What is the relevance and interpretation of these studies and findings?”

Our response: Bacteria and their products interact and interfere with host cells. An infection may negatively influence a possible positive effect of a material used in bone regeneration. We rewrote and restructured the discussion in part to make it clearer.

“Following experimental testing of OCHS with cells from the periodontium, a co-culture system was designed to determine the influence of OCHS on cells exposed to periodontal pathogens simultaneously to simulate a clinical setting…. Therefore, the results from this experiment demonstrate the potential use of OCHS even in the presence of periodontal pathogens without affecting the potential benefit from OCHS.”

Our response to reviewer 2:

Reviewer 2:
“Briefly, this study evaluates the in vitro antimicrobial properties as well as the effect on cell proliferation and mineralization of an oily calcium hydroxide
suspension. The topic is interesting and clinically relevant. However, some issues with the study need to be addressed before it can be considered for publication. The paper is overall well written and the purpose of the study well delineated. However, specifically the methods need major clarifications, as outlined in details below. Discussion also needs to clarify some of the results found in the study.

Please see detailed comments below and let me know if any additional information is needed for this review.”

We would like to thank this reviewer for his valuable comments which will help us improve the manuscript significantly. Major changes have now been brought within the materials and methods section as well as the discussion.

“1- Abstract
- Please provide more details on the periodontopathgens used in the study. What kind of antimicrobial activity and against which species? Please define
- Results: please provide more details here (whats moderate concentration? What is high?) as well as statistics on the results.”

Our response: We now specify the antimicrobial activity in the revised manuscript. Furthermore, addition of the included bacterial strains is now included. The concentrations are now specified and the statistics were added.

“Furthermore potential growth inhibitory activity on microorganisms associated with periodontal disease (e.g. Porphyromonas gingivalis, Tannerella forsythia, Aggregatibacter actinomycetemcomitans) as well…

More than a 2-fold increase in adherent HAO cells was observed at 4 h following application of OCHS when compared to the control group (t-test: p=0.007 for 2.5 mg). Proliferation of HAO cells at 48 h was stimulated by moderate concentrations (2.5 mg; 5 mg) of OCHS (each p<0.001), whereas a high concentration (7.5 mg) of OCHS was inhibitory (p=0.009).”

Reviewer 2:
“2- Introduction:
- Need grammar review throughout the paper. Mistakes found throughout”

Our response: The manuscript has now been revised by a native English speaker. If specific sentences need further modification, please list the troublesome sentences and they will be modified accordingly.

Reviewer 2:
“- Page 5: last paragraph. 3rd line: The study…which study? The present study? Also, watch certain abbreviations: incl.??”

Our response: We followed the reviewer’s suggestion and changed the manuscript accordingly.

“The aim of the present study was two fold; 1) To determine a potential antimicrobial activity of OCHS including its components against bacterial species involved in pathogenesis of periodontitis and 2) to determine the effect on attachment and proliferation of host cells (periodontal ligament fibroblasts and osteoblasts).”

Reviewer 2:
“3- Materials and Methods:
- ‘Determination of antimicrobial efficacy of oily calcium hydroxide suspension’: second line – …bacterial strains…please add: “described below”.”
Our response: We agree with the reviewer’s comment. Now we placed the description of the used microorganisms at the beginning of the section.

Reviewer 2:
“- Checking for purity: how?”
Our response:
This is typically a standard laboratory procedure in all analyses using bacterial cultures. Subcultivations are screened for uniformity of the colonies. We agree that adding this information might be confusing. In order to avoid further confusion, this statement was removed from the manuscript.

Reviewer 2:
“- “defined inoculum”: please provide details here – how much? What concentrations?”
Our response: The following information has been added to the manuscript. “After subcultivation of bacterial strains, a defined inoculum (McFarland 0.5) was added in a ratio of 1 : 9 to broth containing the test substances.”

Reviewer 2:
“- “defined concentrations” – please define how much?”
Our response: We deleted the term “defined concentrations”. The concentrations are given in the sentence immediately thereafter.

Reviewer 2:
“- Describe the way tissue was harvested for culture
Our response:
We added a few more information and a few references. “Human bone chips were cultured from an explant model as previously described [25.26]. Following collagenase digestion, HAO cells were plated in T-75 flasks containing cell cultivation medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% of fetal bovine serum (FBS, Invitrogen). PDL cells were harvested from the middle third portion of tooth and placed in T-25 cell culture flasks till cell confluency [27].”

Reviewer 2:
“- please describe how many replicas were used for each experiment. Authors describe 6 per group except for the antimicrobial assays, how many there? 1U, 2U… not clear what those represented? Authors describe ‘1 U represented 2.5 mg of total material’ what about the others?”
Our response: Each one U was 2.5 mg. But we followed the reviewer’s suggestion and added the information for 2 U and 3 U. “1 U represented 2.5 mg of total material (meaning 2 U is equivalent to 5 mg and 3 U to 7.5 mg).”

Reviewer 2:
“- In addition, calcium hydroxide in aqueous solution was used in this experiment, why wasn’t this used also in the antimicrobial experiment?
Our response: As mentioned in the materials and methods section as well as in the results section, the antimicrobial activity of calcium hydroxide as well as of oleum pedum was tested by using micro-broth dilution technique. This technique per definition uses diluted substances (in solution or suspension). We added the used technique to the heading of table 1. .
Reviewer 2:
“- Statistics also not clear… t test was used to test which samples? Anova was used to test what? Please be more specific.”
Our response: The revised manuscript now specifies the statistical tests that were used for which experiments as follows:
“More than two independent groups were compared by one way ANOVA followed by Post Hoc LSD analysis for comparison with the controls (activity of OCHS and its components on HAO cells and PDL fibroblasts). Statistical analysis was made using Student’s t-test for two independent samples (effect of OCHS on interaction HAO cells’ and PDL fibroblasts’ interaction with bacteria).”

Reviewer 2:
“4 Results
- Second paragraph of the results is describing methods. Please move methods into methods section.
Our response: We followed the reviewer’s suggestion.

Reviewer 2:
“- Table 1” why was the components of OCHS tested but not the actual substance here?”
Our response: As mentioned in one sentence being a repetition of method, it is explained why not MIC values could be provided for OCHS in a total. OCHS was insoluble. Following that we could not make a determination of MIC values by using broth dilution technique.

Reviewer 2:
“- Fig 2: the results indicate that this mineralization was quantified. Methods does how these 10 sections were chosen and analyzed?”
Our response: We specified the selection of the fields and the analysis as follows in the materials and methods.
“Fields were selected equally distributed from the whole slide. A counting grid was used and each subfield (50 µm × 50 µm) with positive staining for calcium noduli was counted in relation to the total numbers of subfields; the mean was used as a single value for analysis.”

Reviewer 2:
“- Page 11 second paragraph described methods again. Move these methods into appropriate section.”
Our response: We followed the reviewer’s suggestion.

Reviewer 2:
“5 Discussion
- Authors mention: ‘attachment of bacteria and the bacteria themselves may serve as a receptor for HAO cells’ please provide reference for that statement. The authors go on to explain the evidence of bacteria, such as Aa, inducing osteoblast cell death and others such as the red complex ones, also arresting ostoblastic proliferation but they fail to explain the reasons why there was more attachment of osteoblasts with Aa and no inhibition of attachment with red complex bacteria. These results are confusing and need better explanation.”
Our response: We agree; these results were surprising and are difficult to explain. We restructured and shortened this part of the discussion. We added one reference showing a stimulating effect on osteoblasts when A. actinomycetemcomitans LPS was used in low concentrations.

“Following experimental testing of OCHS with cells from the periodontium, a coculture system was designed to determine the influence of OCHS on cells exposed to periodontal pathogens simultaneously to simulate a clinical setting. Exposure of HAO cells and PDL fibroblasts to periodontopathogenic bacteria did not negatively influence the promoting effect of OCHS on attachment and proliferation of these cells. Therefore, the results from this experiment demonstrate the potential use of OCHS even in the presence of periodontal pathogens without affecting the potential benefit from OCHS. Surprisingly, the addition of bacteria enhanced the number of attached HAO cells. This result should be taken with caution and might be associated with the in vitro culture conditions. The effect was especially pronounced when A. actinomycetemcomitans was used. The cytotoxic potential of periodontopathogens is well known. Gingipains are responsible for the majority of the proteolytic activity of P. gingivalis [37] and are able to inhibit proliferation of osteoblasts by causing early G1 arrest in cell cycle [38]. A capsular-like polysaccharide antigen of A. actinomycetemcomitans induces apoptotic cell-death in osteoblastic cells [39]. But recently it was shown that a low concentration of A. actinomycetemcomitans LPS in contrast to high concentrations was able to increase bone sialoprotein gene transcription [40]. A topic of future research might be the interfering and interaction of periodontopathogens with host cells and regenerative materials.”