Author’s response to reviews

Title: Ex vivo anti-microbial efficacy of various formaldehyde releasers against antibiotic resistant and antibiotic sensitive microorganisms involved in infectious keratitis.

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Author’s response to reviews:

Dear reviewers, we are grateful for your comments.

Please find below answers for your remarks highlighted in red. In addition, modified text in the manuscript file is highlighted in a different shade of red.

Reviewer #1

1. The title needs to be a little more specific 'Ex vivo anti-microbial efficacy of various formaldehyde releasers of antibiotic resistant and antibiotic sensitive microorganisms'. There no need for abbreviations on the title

Response: The title has been revised as follows (page 1 line 1): “Ex vivo anti-microbial efficacy of various formaldehyde releasers against antibiotic resistant and antibiotic sensitive microorganisms involved in bacterial keratitis”. We agree that the title should be more specific and the above-mentioned version corresponds with the study’s aim and results better. However, we would like to highlight that the selection of the strains studied was based on the most common keratitis pathogens.
2. p values should be present in the result section of your abstract

Response: We have corrected the manuscript accordingly on page 3 line 100 as follows:

Against MRSA, 40 mM SMG at 120 min showed a >95% kill rate, p<0.02. Against VRE, 40mM DAU for 120 min showed a >94% kill rate, p<0.001.

3. The last two sentences of the introduction section are not needed.

Response: The introduction was revised accordingly. Page 5 line 174.

4. Chemical, do not start a sentence with a number 50%.

Mention company name, city and country in parenthesis of all the commercial products you used in your study.

Response: We have corrected the sentence commencing with the number. All the chemical products used in the study are now described as suggested. Revised text can be found on page 5 line 179 as follows:

The bactericidal effect of five different formaldehyde-releasing agents were studied. Key chemicals were as follows: a) sodium hydroxymethylglycinate 50% (SMG) [Suttocide™, Ashland, Columbus OH, USA], b) 2-nitro-1-propanol (NP), c) diazolidinyl urea (DAU) [Sigma Aldrich, Saint Louis, USA], d) 1,3-bis(hydroxymethyl)-5,5-dimethyl-2,4-imidazolidinedione (DMDM) and e) 2-(hydroxymethyl)-2-nitro-1,3-propanediol (NT) [Chemistry Connection LLC, Conway AR, USA]. BBL™ Trypticase™ Soy Broth, BBL™ Trypticase™ Soy Agar, Difco™ Sabouraud Dextrose Broth, Difco D/E Neutralizing Broth [Fisher Scientific, Waltham, MA, USA] were used for bacteria growth. Adult bovine serum albumin (BSA) was bought from Sigma-Aldrich Corp. (St. Louis, MO, USA). All FARs dilutions were made with balanced salt solution, BSS Plus® [Alcon Laboratory Inc, Forth Worth, TX, USA]

5. Bacteria strains: the doctors name should only be in the acknowledgment section.

Response: As suggested, the doctor’s name has been removed from the Methods section Page 6 line 198 and moved to the acknowledgement section Page 12 line 440.

6. In the first paragraph you mention CXL drawbacks considering keratitis treatment, UVA is understandable that may cause toxicity, nevertheless an epi defect on an infected cornea is not a drawback as its anyway present (bacterial infections most likely have epi defects). Furthermore, CXL stiffens mostly the anterior half of the cornea as correctly stated, but you may not
extrapolate this particular information for antimicrobial efficacy of CXL (you are not comparing the same treatment outcomes between two treatment modalities).

Limitations need to be clearly stated in the final paragraph, this is an ex vivo study - in vivo the behavior may be different. Toxicity concerns etc.

Response: We have revised the text to reflect reviewer #1 comments. Please see page 10 line 359 and 364 for omitted text as well as additional text on page 12 line 408 as follows:

Once again, we emphasize that this is an in vitro study and the effects and considerations for in vivo use can be very different.

Reviewer #2

1. Please write the pathogen names in "italic" throughout the manuscript.

Response: We have edited the text according to the above-mentioned remark.

2. Please correct the spelling "nitoalcohols"

Response: This spelling mistake has been corrected on page 5 line 166.

3. Please mention MSSA growth conditions too.

Response: MSSA growth conditions were the same as for MRSA, PA, and VRE. This text has been added to the manuscript in the Methods section on page 6 line 1203.

4. For the graphs, only SMG dosages were plotted or all FARs dosages were plotted? Please correct it.

Response: Thank you for finding this oversight. The text has been corrected on page 7 line 227.

5. Page 7, line 240: Please correct it to "DMDM 100 mM"

Response: the line was accordingly corrected with proper mM abbreviation. See page 7 line 246.

6. Page 8, line 257-259: DAU, DMDM, and SMG all showed some effectiveness with greater effects observed with the longer incubation time of 120 minutes. The mean kill rate was 64% for DAU 20 mM at 60 min and 38% at 120 min. Please explain. Similarly, for PA, the mean kill
rate was 51% for DAU 20 mM at 60 min and 19% at 120 min. For CA, the mean kill rate was 72% for DAU 100 mM at 60 min and 53% at 120 min. For VRE, the mean kill rate was 62% for DMDM 40 mM at 60 min, and at 120 min it was 44%. For PA, the mean kill rate was 69% for DMDM 40 mM at 60 min, and at 120 min it was 16%.

Response: We understand the reviewer’s comment and agree that there were some inconsistencies in the data when comparing rates for the longer incubation time to the shorter time. Additional clarifying text has been added and can be found on page 7 line 247-254.

That being said, greater killing effects were not always observed by extending the exposure time from 60 minutes to 120 minutes for a given concentration. That is, when comparing kill rates between 60 minutes and 120 minutes at the same concentration. The reasons for this inconsistency is unclear, however, explanations include the possibility of polymerization effects occurring as a result of released free formaldehyde (i.e. formaldehyde polymerizing with itself) as well as possible reactions with the FAR products resulting in formation of either the starting material or additional reaction products. An example of this was reported previously by our group. 26

7. Page 8, line 267-268: Similar to MSSA, MRSA growth was inhibited in a dose-dependent manner using DAU, SMG, DMDM and NT.

There was no dose dependent growth inhibition was observed for NT at 120 min.

Response: We agree with the comment and have included the following clarifying text in the revised manuscript on page 8 line 281.

Similar to MSSA, MRSA growth was inhibited in a dose-dependent manner using DAU, SMG, DMDM and NT in 60 min incubation time and for DAU, DMDM and SMG for 120 min incubation time.

8. Table 3: for CA, DMDM 40 mM showed growth kill rate of 42% (p=0.336) at 60 min. But 23% and 37% growth kill rates for 20 mM and 40 mM at 120 min showing p=0.000. Please re-check it.

Response: The reviewer is right. There was a mistake in p-values for DMDM.

Please see the revised Table 3.

9. Please mention the FAR compounds which showed consistent trend (dose vs time dependent) in the manuscript.

Response: Additional text has been included in the Results section of the revised manuscript on page 7-8 line 260-263.
In summary, the FAR/pathogen pairings that showed the most consistent trends (that is, both dose and time dependency) were as follows: SMG against MSSA, MRSA, and PA; DAU against MRSA and VRE; DMDM against MRSA.

10. Figures are not clear. Please make it clearer.

Response: The reproduction quality of figure 1 has been enhanced as requested.

11. This study would be more interesting if, the authors have included more clinical isolates and studied the toxicity of each tested compound using corneal epithelial cell cultures.

Response: The reviewer’s comments are well taken. We used limited resources in this initial screening study. Additional future work will involve testing of clinical isolates as well as toxicity effects, particularly in live animal models.