Author’s response to reviews

Title: In vitro antibacterial activity of Bioactive Glass S53P4 on multiresistant pathogens causing osteomyelitis and prosthetic joint infection

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Author’s response to reviews:

Dear Giovanni Battista Orsi

Editor, BMC Infectious Diseases.

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Article Title: In vitro antibacterial activity of Bioactive Glass S53P4 on multiresistant pathogens causing osteomyelitis and prosthetic joint infection

On behalf of my coauthors, I am pleased to resubmit our amended manuscript to BMC Infectious Diseases and would like to extend our gratitude to the reviewers who have helped us improve our manuscript, by providing constructive and useful comments. We have revised our manuscript and have addressed the comments and queries provided by the reviewers below, point by point. Below we inventory each reviewer comment and our response. The revised manuscript has also been submitted with all changes tracked.

Thank you very much for the opportunity to resubmit our revised manuscript.

Kind regards,

Mauro Jose Costa Salles, MD, MSc, PhD
Reviewer comments:

Reviewer #1 (Major Comments for the Author):

Trinconi Cunha et al performed an in vitro study evaluating the efficacy of bioactive glass on bacterial growth. There is few data in literature about the usefulness and efficacy of bioactive glass in the treatment of osteomyelitis, so in my opinion it provides a nice addition to literature. The paper is clear and easy to read, although I would suggest a native speaker to review and improve the grammar. I do have several questions that I think need to be addressed prior to publication (especially the limitations concerning the study design should be more thoroughly discussed), and suggestions to improve the paper:

Authors’ response: We thank the reviewer for these thoughtful comments. The manuscript has been double-checked by an English-speaking native. The study design section was also rewritten in order to better clarify our methodology.

Specific comments

Gentamicin should be written with an i instead of y throughout the entire manuscript.

Authors’ response: We thank the reviewer for pointing out these mistakes. This issue has been addressed and all instances of the word “gentamycin” have been replaced by “gentamicin”.

The Abstract:

There is double info in the material and method section and result section on the type of bacterial strains used.

Authors’ response: We appreciate this query. The abstract was reviewed, and we kept the description in the ‘method’ section and removed the same information in the ‘result’ section.

I would prefer to delete the sentence about statistical analysis and add a sentence about the methods used (culture in broth, and subsequently study the growth on agar plates).
Authors’ response: We thank the reviewer for this suggestion. We added additional information to the abstract regarding the methodology performed in our study.

The 0.5-0.8 mm is the length of the inert glass? Because now it is written in parenthesis behind the bioactive glass.

Authors’ response: This point is well taken. In fact, 0.5 – 0.8 mm is the diameter range of bioactive glass (BAG-S53P4) granules used in the experiment. The Inert glass beads measured 2 mm in diameter. We amended the text to make it clearer.

In General:

It is not clear for me why to speak of oxacillin resistant strains instead of methicillin resistant. Now both terms are used throughout the paper.

Authors’ response: This point is well taken. In Brazil, methicillin is unavailable and therefore, Oxacillin is the standard drug applied for the detection of staphylococcal resistance to β-lactams.

We are aware that Oxacillin and Methicillin are equivalent drugs and MRSA (Methicillin-Resistant S. aureus) is the most widespread terminology. In the revised manuscript, we amended the text to use only Methicillin-Resistant S. aureus (MRSA).

Background:

Just a general comment; in my knowledge very few osteomyelitis cases are described due to CoNS without any osteosynthetic material present.

Authors’ response: We thank the reviewer for this thoughtful comment. In fact, the presence of CoNS as a causative agent of chronic osteomyelitis (OM) may vary according to the epidemiology and type of OM. It may be a common cause of posttraumatic and postoperative OM, especially among those patients with unrecognized or insufficiently treated infection associated with internal fracture fixation device, even after hardware removal. Unfortunately, this is a common epidemiological scenario in many developing countries, including Brazil. Our group identified CoNS as the second most frequent agent (17.7% on tissue cultures and 22.7% on sonication fluid cultures) causing OM associated with osteosynthesis [1]. Additionally, among 67 patients presenting posttraumatic and postoperative osteomyelitis in Germany, 33% were caused by coagulase-negative staphylococci [2]. In patients with diabetic foot osteomyelitis, CoNS is also commonly identified on tissue cultures [3].

In my experience bioactive glass is still present in the bone months or even years after insertion, so theoretically this can act as foreign material as well. Do the authors have any information, or are they aware of studies suggesting otherwise? Can the authors comment on this in the discussion section? In addition, can the authors comment on how long the release of antibiotics from the beads is expected compared to the high pH from the glass?
Authors’ response: We appreciate these queries. Indeed, the ideal bone substitute after debridement of devitalized tissue in osteomyelitis shall be biocompatible, osteoconductive, able to provide mechanical strength and bioabsorbable to avoid additional surgeries for removal. Bioactive glass is still present into the bone for long period of time after insertion, possibly due to its glass composition with high silica content, making it theoretically able to act as foreign material [4]. Heikkila et al., identified by radiography the persistence of granules of Bioglass S53P4 11 years after surgical insertion for tibial plateau fractures [5]. We have added this information into the discussion section.

Regarding the length of time for which Bioglass sustains local pH increase and PMMA beads elutes antibiotics at high local concentration, we argue that it is difficult to be compared. Several factors may affect the elution rate of antibiotics, ranging from type and viscosity of PMMA, the type and concentration of the antibiotic, and the structural characteristics of the beads [6]. Although still a matter of scientific debate and not completely resolved, from PMMA beads there is a burst release of antibiotic during the first 24h, followed by a decreasing secondary elution of over one or two weeks [7]. Meanwhile, studies analyzing the rate and duration of bioactive glass-induced local pH increase are scarce, possibly because of the great variety of powder diameters and lack of standard concentrations. However, studies that have briefly addressed this matter suggest that the pH change promoted by bioactive glass granules lasts for at least 120 hours [8, 9]. Further experiments are necessary to answer this question.

Material and Method:

Where did the authors obtain the 'inert glass?' And how was it processed?

Authors’ response: 2mm inert glass beads were manufactured by Plena Lab, Sao Paulo, SP, Brazil. The beads were sterilized by autoclaving. We have included this information on methods section – p.06, line 144.

'BAG was blended' ; do the authors mean that it was mixed with the broth? Or was the glass crushed so it dissolved with the broth? If the latter is the case, the results can probably not be extrapolated to clinical practice.

Authors’ response: Thank you for pointing this translation error. By ‘blended’, we meant that BAG was mixed with the broth using a vortex mixer, in order to create a suspension. This issue was addressed and the word ‘blended’ was replaced with ‘mixed’.

Only if the pH was above 10 this was considered as suitable for the experiment. How often then did the authors measured a pH that was below this point? This crucial information, and in addition; what pH can be expected to occur in in vivo situations? I think this is an important limitation that needs to be discussed in the discussion section.

Authors’ response: Interestingly, we have not detected pH measures below 10. The bioactive glass + TSB mixtures were incubated for 48 hours before pH measurement and bacterial inoculations. An incubation period of 24-48 hours, along with a suitability pH threshold of 10-11
were applied in previous studies [8,10,11], and we decided to apply the same to keep homogeneous methods and obtain comparable results. Taking that into consideration and analyzing pH curves obtained in previous studies [7,8], we can conclude that the pH level was probably in its peak values during the time of measurements, justifying our lack of unsuitable samples.

Bioactive glass S53P4 has been studied for decades for clinical use mainly in the field of odontology and cranio-maxillofacial defects, with some in vivo studies been published before [12,13]. Only recently, BAG-S53P4 has been successfully used as the adjunctive treatment of chronic osteomyelitis of the long bones, but as far as we know in vivo studies analyzing the antimicrobial activity of BAG S53P4 in osteomyelitis using pH measurement as a surrogate marker are lacking. We speculate that in in vivo situations, one may expect initially lower local pH after implantation of BAG, due to physiological buffer mechanisms and the lack of an incubation period before application. However, in experimental body fluid models, S53P4 may show an increased maximum pH value of 11.65 [14].

In addition to the previous point, I have the same concern with 'testing 3 different concentrations of BAG'. If these optimal conditions are needed to show the benefit of the glass, how can this be expected to occur in patients? This needs to be explained and discussed.

Authors’ response: We thank the reviewer for this thoughtful comment. The optimum bioactive glass concentration was the minimal concentration that we have found that would inhibit bacterial growth. The concentration discovered in this experiment, and then used in the main experiment was 0.8 mg/mL. We argue that such a measurement is much like the minimal inhibitory concentration (MIC) determination we traditionally apply to better guide antibiotic therapy in the microbiology laboratory.

Volume-wise, this quantity was enough to, after vortexing and sedimenting, fill the solution up to about 3mL, leaving 2mL supernatant TSB. Considering that, without any external compression whatsoever, the granules should occupy about 60% of the bone defect, completely filling the defect with BAG, which is the standard use of the substance. We also have added this information into the discussion section.

Please explain on what clinical ground the 3 to 4 CFU are choosen and if this is comparable to the inoculum we can expect in patients with OM that are debrided.

Authors’ response: We thank the reviewer for this query. The 3 to 4 CFU are randomly chosen from isolated dot-shaped colonies and suspended at 0.5 McFarland (1.5 x 108 CFU/mL). Since 500µL were inoculated in the final mixture of 5mL, we obtain 1.5 x 105 CFU/mL. Considering that peri-implanted bacterial reservoirs used in animal prosthetic joint infection models range, in order of magnitude, from 104 to 106 CFU/mL (depending on the subject’s body mass and species) [15-17] the inoculum may be comparable to what we can expect in patients with OM.

A lot of GN were gentamicin resistant according to Table S2. This probably explains the reduced efficacy of beads in GN-infections. What do the authors think / propose is the explanation of the reduced efficacy of bioactive glass in these GN cases?
Authors’ response: This point is well taken. To our knowledge, few previous in vitro studies analyzed the bactericidal activity of BAG S53P4 on multiresistant Gram-negative bacteria (MDR-GNB), and to date no resistance to the action of BAG-S53P4 has been identified [17-19]. Moreover, Bioglass seems not to induce bacterial resistance, even during a prolonged in vitro exposure of BAG to GNB18. In our experiment, BAG start killing Gram-negative bacilli slower than Gram-positive. We therefore, may only speculate for possible reasons to reduced efficacy of BAG S53P4 upon MDR-GNB. P. aeruginosa and K. pneumoniae are able to easily express a large variety of genes, which provides an overwhelming osmotolerance, counteracting part of the antibacterial mechanism of S53P4 [20-22]. Besides, the pH of natural habitats of P. aeruginosa range from 4.5 – 9.5, very close to the pH 10 threshold we set for bioactive glass suitability. We have added this information into the discussion section.

Results:

Maybe I don't fully understand the concept, but please explain why, in the Figures, the CFU in the inert glass group is constant, and does not increase in time.

Authors’ response: After 24 hours of plating, the bacteria covered the whole surface of control group plates since the first count (t = 0h), sometimes presenting slight decreases during different measurements (probably human error during plating). Since there were slightly more than 100 CFU/cm2, the formula used to estimate the number of CFU is = (Petri dish area)/(bacterial dilution), which is 58cm2/(1,5 x 105 CFU/mL), resulting in approximately 3.8 x 106 CFU in each full plate.

Unfortunately, 3.8 x 106 is the largest CFU count possible for us to obtain through this method. Therefore, we plotted this as the maximum quantity in our figures, approximating the log to 6.

Based on the questions above, I think the discussion section should be improved, addressing several limitations of the study design (in relation to clinical practice). It would also be nice to add one section about the results that have been published about the bioactive glass in clinical studies.

Authors’ response: We thank the reviewer for these thoughtful comments. We have modified the manuscript in order to better answer the reviewer’s questions, including addressing the suggested topics on the discussion.

Reviewer #2 (Major Comments for the Author):

This article is interesting, as local bone cements or bone substitutes with antimicrobial activity is an emerging option in orthopedic septic surgery. Authors studied the antibacterial activity of a bioactive glass and antibiotic-loaded PMMA. They concluded that the bioactive glass presented similar antibacterial properties as antibiotic-loaded PMMA. The experiments seem to be well conducted. However, some clarifications are needed. Indeed authors used reference strains and clinical methicillin-resistant staphylococci and Gram-Negative bacilli, including carbapenemase
enterobacteria and P. aeruginosa. I suppose that the reference strains are susceptible to gentamicin, but not clinical isolates. Is it right? It may considerably influence the results. What was the gentamicin MIC of clinical isolates? It is of importance, as high local gentamicin concentration can also have an antibacterial activity even in gentamicin-resistant strains. Finally no differences in the bactericidal activity on enterobacteria and P. Aeruginosa (the latter is usually less susceptible to gentamicin)? Please clarify these points.

Authors’ response: We thank the reviewer for these thoughtful comments.

Indeed, the clinical isolates used are resistant to gentamicin, while the ATCC strains are not. Even though they have different resistance profiles, the ATCC strains exhibited time-kill curves similar to those of the clinical isolates, so even if they affected the results, their influence probably wouldn’t be significant.

Unfortunately, for these strains we did not performed MIC measurement, instead we used a qualitative single disc diffusion tests. Eventually, no difference was observed between P. aeruginosa and K. pneumoniae time-kill curves. Probably this was due the high local gentamicin concentrations.

Specific comments

Methicillin-resistant instead of oxacillin-resistant (so MRSA and not ORSA, etc.)

This point is well taken. In Brazil, methicillin is unavailable and therefore, Oxacillin is the standard drug applied for the detection of staphylococcal resistance to β-lactams. We are aware that Oxacillin and Methicillin are equivalent drugs and MRSA (Methicillin-Resistant S. aureus) is the most widespread terminology. In the revised manuscript, we amended the text to apply only Methicillin-Resistant S. aureus (MRSA).

Gentamicin is sometimes wrote with a i and sometimes with a y (please always use a I)

Authors’ response: We thank the reviewer for pointing out this mistake. This problem has been addressed and all instances of the word “gentamycin” have been replaced with “gentamicin”.

References


