Author’s response to reviews

Title: In-vitro sonothrombolysis using thick-shelled polymer microbubbles - A comparison with thin-shelled microbubbles

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Author’s response to reviews:

Dear Dr Rosa Sicari,

Thank you very much for reviewing our manuscript. Efforts have been made to comply with the issues raised by the reviewers and we have prepared a new, revised version of the manuscript that you will find enclosed. We hope that the manuscript in its present form will satisfy the reviewers, and we would be most grateful if you would reconsider the revised manuscript for publication in Cardiovascular Ultrasound.

The specific comments to the reviewers and the change addressed by the authors follow below. All changes made in the manuscript are highlighted in yellow.

Reviewer's comments:

1) Phantom properties: authors cited another publication (ref. 23) about mechanical properties of the phantom. However, the process described by Fromageau et al. is quite different respect to the process of the present paper. Accordingly, it is not clear how authors evaluated properties of the phantom sed for experiments. Please clarify and comment.

The reference #23 with its the associated text has been removed, and the following text explaining assessment of attenuation coefficient and speed of sound for the phantom material has been added to the method section:

“Phantom characteristics was assessed by estimation of attenuation coefficient and speed of sound. For the estimation of attenuation coefficient, a flat sample (thickness 10mm) of the phantom material was positioned in deionized water on a metal reflector. A single element transducer, delivering ultrasound at frequency of 2.25MHz, was positioned opposite to the reflector and perpendicularly to the sample. The ultrasound was delivered using an ultrasonic pulser-receiver (Olympus, USA). The frequency spectrum was recorded using an oscilloscope (Tektronix, USA). The frequency spectrum was computed firstly with only the metal reflector...
without the sample and, secondly, with the metal reflector and the sample. The two spectra were
than used for computing the attenuation coefficient knowing the distance of the transducer from
the sample (10cm) and the ultrasound velocity in water (1500m/s). Assuming a linear
dependence between the attenuation coefficient and the frequency, the final attenuation
coefficient obtained was 0.5 dBcm-1MHz-1. The speed of sound of the phantom was assessed
using the same equipment as for the attenuation coefficient estimation. The speed of sound was
estimated to 1510m/s and was calculated by dividing the thickness of the phantom with the time
difference between the echos from the front side and back side of the sample.”

2) Line 125: information about the material of the pore mesh should be added. In particular, it is
not clear if pore mesh can interact with ultrasound beam thus having a protective (or deleterious)
addictive effect on blood clots. Please comment and change the text accordingly. Finally, a
picture of the pore mesh should be added with and without the blood clot.

We fully agree with the reviewer that the lack of information about the pore mesh raise questions
of potential impact. Now, more details about the size and the material of the pore mesh has been
added. With this information it should be clear to the reader that the impact of the pore mesh
could be considered as negligible. Note that the mesh is hardly visible in the ultrasound image
(figure 3), and its presence generates no artefacts. Due to corona virus, we are not allowed to be
at the university, which makes it impossible for the time being to prepare new blood clots for a
picture. Perhaps a picture can be added later as a supplement, or maybe it is less important now
when the new information has been added?

The following text has been added to the method section:
Methods, In-vitro set-up and phantom construction: “A 15µm thick filter of nylon with 40µm
pores was prepared by removing the polypropylene frame from a cell strainer (Falcon Franklin
Lakes, USA). The vessel phantom was composed of two halves, which were connected after
positioning the 40µm pore mesh between them (Figure 2).”

3) Phantom: authors would like to mimic vessel properties. However, in a clinical scenario of
thrombolysis, the vessel wall constitutes only a limited amount of tissue that should be crossed
by ultrasound, while fat and muscle tissues are the most. Accordingly, the ideal phantom is a
fat/muscle mimicking one.

We agree with the reviewer. However, as mentioned in manuscript we had some problem with
leakage in the experimental set-up. When the pressure rose due to the clot obstruction, a higher
force was applied on the vessel walls, which bent leading to a change in the overall shape of the
phantom and allowing the leakage of the solution. We tested different phantom materials and the
softer tissue/fat-like phantom (3% (w/w) agar, 4% (w/w) graphite powder) had more pronounced
leakage. We also tested a stiffer silicone rubber phantom, but this phantom had an attenuation
coefficient of 3.2dBcm-1MHz-1 which was high above attenuation coefficient of soft tissue
thereby reducing the ultrasound intensity in the clot region too much. The chosen phantom
material was the best compromise between stiffness and acoustic properties of the tested
materials, but as the reviewer pointed out not fully realistic.
4) Probe distance: the distance between the probe and phantom should be 15 mm instead of 10 mm in the case of high frequency probe according to figure 2. Please clarify. Furthermore, both distances seem to be unrealistic respect to clinical cases where the occluded vessel is deeper in the body, thus limiting the clinical usefulness.

5) Blood flow velocity is constant and quite far from the physiological blood flow velocity wave. This could be a limitation of the in vitro setup.

6) Lines 151-154: discarding of pressure measurements according to observations of leakage could introduce bias in measurements. This should be recognized as another limitation of the study.

9) Limitations in pulse customization of the Verasonics system were already recognized. However, the difference between protocol 1 and protocols 2-4 makes difficult to compare results. Please comment and strongly highlight this point in the limitations section.

Reviewer comment 4, 5, 6 and 9 address limitations of the study. We have added much more information in the limitations section of the manuscript to comply with the issues raised by the reviewer. The text in the new limitation section in the discussion is seen below:

“The experimental set-up had some limitations. There were problems with leakages which affected the number and quality of pressure measurements, making them less reliable as compared with the clot mass computations. Also, the disregarding of pressure measurements based on visual assessment can potentially introduce bias in the results. Furthermore, the Verasonics system had some limitations in the coding and implementation of different combinations of ultrasound parameters due to the fact that the entire system had power limitations and hardware constraints that did not allow realization of continuous long pulses. In the present study, the 5/10ms ultrasound pulse was composed by several shorter pulses with small idle times in between. Using the L12-5 transducer, the longest pulse achievable with 11.25MHz had a duration of 88.18µs, while the L7-4 transducer transmitting at 4.09MHz enabled pulses with maximal duration of 240µs. Thus, in order to obtain a longer pulse, several of these short pulses were combined. The idle time in between was the lowest achievable and smaller values were not possible due to power limitations. Moreover, each pulse had a duty cycle equal to 37%. The different characteristics between the two transducers used in the study limit direct comparison between protocol 1 and protocol 2-4.

The experimental set-up in-vitro presented several aspects similar to the physiological situation of occluded coronary arteries. The vessel lumen was 4mm in size, comparable to the dimension of the left anterior descending coronary artery. Moreover, the 40µm pore mesh reproduced the microcirculation and the flow velocity was 2mm/s, similar to the real situation. On the contrary, the distance between the clot region and the ultrasound transducer was not comparable to the real distance between the probe and the coronary arteries. Moreover, in a clinical situation the presence of the ribs and other body structures can attenuate and scatter the ultrasound waves which limits any usage of linear transducers. Another limitation of the in-vitro set-up was the continuous flow delivery, which is not present in the real situation where the flow is pulsatile.
However, the continuous flow limited to formation of air bubbles in the set-up, thereby eliminating random peaks in the pressure patterns”

Further comments on comment #4)
Thank you for noticing this error in Figure 2. We have changed the figure according to the correct distance between prob and phantom (blood clot) of 10mm (20mm divided by 2).

Further comments on comment #5)
According to these three studies the coronary blood flow (range 1.05 ml/s-4.81 ml/s) is in accordance with the flow used in the present study.

Coronary Artery Flow Velocity Is Related To Lumen Area and Regional Left Ventricular Mass

Measurement of the blood flow rate and velocity in coronary artery stenosis using intracoronary frequency domain optical coherence tomography: Validation against fractional flow reserve.
Haroon Zafar, Faisal Sharif, Martin J. Leahy. IJC Heart &amp; Vasculature. 2014;5:68-71


7) Lines 179-182: saline with microbubbles at 2x106 MBs/ml was injected. However, this condition seems to be very different respect to the clinical scenario where a small amount of solution with 107-108 MBs/ml were injected in the circulation thus having a limited amount of microbubbles where the clot was formed. Please comment.

In the paper of Borrelli MJ et al. Influences of microbubble diameter and ultrasonic parameters on in vitro sonothrombolysis efficacy. J Vasc Interv Radiol. 2012;23(12):1677-84 e1., they stated that the optimal concentration of microbubbles for sonothrombolysis using MBs with diameters of 1µm and 3µm was 5.4*10^8 MBs/ml and 1.1*10^8 MBs/ml respectively. They also stated that the complex MB-MB interaction influences the relationship between the MB diameter and the MB concentration.

When considering a clinical situation with contrast administration for sonothrombolysis, we believe that infusion would be preferable to bolus injection in order to have a constant flow of MB to the clot area. When considering the European guidelines for contrast echocardiography, they recommend 0.8 mL/min of SonoVue (concentration of 1-5x10^8) when using constant infusion. This value is lower than the one used in this study, however opacification of the phantom vessel was comparable to the clinical situation based on visual assessment and the fact that no shadowing artefacts were detectable. Future studies need to further evaluate the optimal concentration of MBs taking also MB properties and ultrasound output parameters into account as well as the patient safety perspective.

The following text as been added in the discussion section
“To obtain efficient sonothrombolysis, also optimization of MB concentration needs to be considered in future applications. A previous study showed maximal sonothrombolysis at MB concentrations >108 MB/ml and that the concentration is dependent on MB diameter (15). The concentration of MBs used in this study (2x10^6 MB/ml) was lower than the above-mentioned study, and potentially a higher concentration could have yielded a more pronounced effect on the obtained results. On the other hand, this concentration was higher than the recommendations for clinical contrast administration (28). Even though contrast administration is associated with low incidence of adverse events (28), the patient safety perspective must be taken into account when sonothrombolysis is introduced into clinical practice”

8) Figure 3: scale and focus position should be added.

A new figure with scale and focus position has been added.

10) Change in pressure over time was compared only by visual inspection. However, a more quantitative approach with an appropriate statistical test should be preferred.

We do not fully agree with the reviewer. We believe that the low number of pressure measurements per protocol (n=3) make the results more suitable for visual presentation in comparison to statistical analysis. Please, reconsider this comment.

11) Figure 4: mean pressure measurements over time exhibit quite variable variations around the means in the 5 protocols. Please comment.

As mentioned in the discussion, we believe that clots debris might have remained entrapped within the mesh. Since we do not have no method analyzing the material entrapped in the mesh, we do not know its impact on the pressure measurements and how it differs between different measurement sessions.

We have also seen that the set-up is sensitive to air bubbles and even though the infusion pump significantly reduced the problem with air bubbles, air bubbles might occasionally occur during the experiments.

The following text has been added in the discussion section (the air bubble problem is discussed in the new limitation section):

“Moreover, for in-vitro application similar to the present study, the efficiency of the clot lysis should be computed considering also the clot debris stuck in pore mesh. By developing a method for analyzing the material entrapped in the mesh, it would be possible to fully understand if the obtained pressure values with its relatively high variability could be explained by the fact that that clot debris occasionally was bigger in size than the mesh pores.”