Peer Review File

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Reviewer A

In this study, the author assess the therapeutic effects of long-pulsed ultrasound (US) enhanced microbubble (MB) mediated recombinant tissue-type plasminogen activator (rt-PA) thrombolysis in a rat model of platelet-rich thrombi. The authors reported that long-pulsed US enhanced MB mediated rt-PA thrombolysis offered a powerful approach for the treatment of platelet-rich thrombi. This research has potential application prospects for further understanding the mechanisms and treatments of arterial thrombotic disease.

But I still have the following questions:

1. In the high duty cycle US+MB group, the high duty-cycle US condition + pure MB did not show the effect of dissolving thrombi. Thus, it is suggested to add the relavent disscusion to explain this phenomenon.

Reply: Thank you very much for your insightful comment. We added the explanation in the discussion section. The reason may be the thrombi in our study were full of platelets and quite rigid, high duty-cycle US of acoustic pressure of 0.6MPa mainly caused MB undergo stable cavitation thereby unable to dissolve the thrombi completely. We have modified in the discussion section as advised (see Page 9, line 203-207).

Changes in the text: In our study, pure administration of rt-PA didn't cause patency of the occluded artery, probably because the platelet-rich thrombi were quite solid. In another group, high duty-cycle US (0.6 MPa) with MB typically generates stable cavitation events (Ultrasound Med Biol 2012;38:1589-98) , therefore the radiation forces from microstreaming are unable to completely dissolve the rigid thrombi.

2. In this study, there were only 6 rats been devided in each group. Does the sample size meets the statistical requirements?

Reply: We appreciate the reviewer for bringing up this issue. The sample size was estimated based on a previous study during the corresponding author's stay in Cardiovascular Institute of University of Pittsburgh. In that experiment, the sample size was three. Thrombi (mainly consisted of red blood cells) were prepared by adding 10% $CaCl_2$ solution to venous porcine blood, and the thrombolytic effect was tested in an *in vitro* open loop system. 0.23MPa ultrasound with pulse interval of 1.5 second was used. A very long pulse length of 600,000us (duty cycle 40%) and 5,000us pulse-length (duty cycle 0.3%) US were compared (see the figure below). The thrombolytic rate of pure rt-PA group was 46.64±3.15%, similar to 5,000us US + rt-PA + MB group ($P>0.05$). And 600,000us US + rt-PA + MB group showed the best thrombolytic rate (98.58±2.22%, $P<0.01$vs rt-PA group). There were 3 runs in each group and the results reached a statistical significance. So we used 6 rats in this study. Thank you very much

again for all your valuable comments.

Reviewer B

The manuscript presented an animal study to investigate the effect of ultrasound + MB +rt-PA for the treatment of stroke. While the design and the performance of the study seem robust, I wound not recommend the publication of this study given concerns particularly in innovation, as well as outcome evaluation.

The main concern is in the innovation. FUS in combination with ME and rt-PA to achieve thrombolysis has been extensively studied. Here is one example, "Investigative Radiology 46(3):p 202-207, March 2011". Note that this is just one example, there are many others out there. That being said, I cannot fine new science or engineering in the current study.

While the design of the study seems ok, the outcome evaluation needs further clarification. Color Doppler is used to evaluate the flow, but only "recanalization" and "nonrecanalization" were used. How are these related to flow speed should be quantified. Also, what is the lowest speed color doppler can measure? The outcome evaluation needs to be quantified.

Reply: Thank you for bringing this issue to our attention. Currently the ultrasound used in clinic is usually short cycle(≤5μs). Recently long-pulsed US enhanced MB mediated rt-PA thrombolysis has been investigated as a useful reperfusion therapy for thrombotic vascular obstruction, probably from more sustained cavitation effects (J Am Coll Cardiol 2016;67:2506-15; Theranostics 2017;7:3527-38). The US in the rabbit experiment of the listed literature was traditional pulsed US, different from that 1000μs pulse-length in our study. Several recent references have been added in the introduction section (reference 21, JACC Cardiovasc Imaging 2020;13:641-51. & reference 23, Ultrasound Med Biol 2023;49:152-64) and the novelty of this study was better demonstrated in the discussion section (see Page9, line 186-187). The thrombi in the our model was full of fresh platelets with dense structure, resistant to pure rt-PA. The synergistic effects of long-tone-burst US, MB and rt-PA showed successful thrombolysis, especially under high duty cycle acoustic condition. The disability and mortality rates remain very high in acute myocardial infarction and stroke, although rt-PA and percutaneous intervention techniques have been widely used. Therefore we try to find a new approach for the treatment for arterial thrombosis.

	Pulse duration	Group	
2016	20 μs	Thomas R Porter	J Am Coll Cardiol
2017	5000 μs	Flordeliza S Villanueva	Theranostics
2017	20 μs	Thomas R Porter	Invest Radiol

2020	30 μs	Jonathan R Lindner	JACC Cardiovasc Imaging
2023	5000 μs	Flordeliza S Villanueva	Ultrasound Med Biol

Besides recanalization rate, arterial recanalization grade was assessed by color Doppler with the lowest measure speed of 1mm/s. Data were presented in supplementary material 1, Table S2**.** Thanks for the reviewer, and we will make efforts to provide more insights for the mechanisms how long-tone-burst US mediates thrombolysis effects in the future.

Changes in the text: In other research, 30-μs pulse-length US was used to augment limb perfusion in patients with peripheral artery disease (21,22). In a rodent model of microvascular obstruction, longer pulses (5000 μs) caused greater microbubble cavitation followed by a more rapid microvascular flow restoration than did a 10-μs pulse US (23)… To our knowledge, this is the first study to show long-pulsed ultrasound mediated successful thrombolysis for platelet-rich thrombus in a totally occluded arterial model.

Reviewer C

Sonothrombolysis is important in treatment of thrombosis. The reported in-vivo study is interesting and some results are impressive. However, the manuscript can be enhanced by considering the following comments:

1. introduction: the state-of-the-art of sonothrombolysis can be covered better by introducing both extracorporeal and intravascular sonothrombolysis techniques; the short pulse and long pulse studies may be introduced with more details such as what are the pulse lengths people have reported, and why this work will just report 1000 us?

P2L51: rephrase this sentence: "Ultrasound (US) enhanced microbubble 51
(MB) therapy has investigated as a therapeutic technique for the ..."

P2L56: "... (7-9)and without..." a space should be inserted between ")" and "a"; there are numerous such typos in this manuscript.

P3L69: "...the initial lyutic rate...", what is "lyutic rate"?

Reply: Thank you very much for your valuable comments. The different short pulse and long pulse studies are reviewed and added in the introduction section (see Page 3, line 61 to Page 4, line 73). Most of the studies at present focus on 10 μs, 20 μs, 30 μs pulse lengths, meanwhile 5000 μs is drawing great attention. So we chose 1000 μs pulse length to investigate.

The sentence on P2L51 has been rephrased (see Page 3, line 55).

A space has been inserted between “...(7-9)” and “and without..." (see Page 3, line 58).

We also worked hard to rectify other typos in our manuscript.

We are sorry for the error. We have revised to "lytic rate"(see Page 4, line 82).

2.Method: the experiment paramegters were not clearly stated, even with the

supplement materials. For example, the definition of high duty cycle and low duty cycle is not clear, rationale on 1000 us , pressure range, etc. can not be found.
P3L90: rephrase the sentence "Make...."
P4L104: figure citation (Fig. S1) may be added next to the sentence " A piece of paper ..."
P4L124: "Mpa" to "MPa"
P4L125: rephrase this sentence : "In 124
addition to pulse interval of 3s, 10ms (duty cycle 10%) was also tested to observe the impact of duty cycle."
P4L127-129: rephrase the sentence "Make sure..."
Reply: Thank you for your constructive suggestions. In our study, ultrasound was delivered with two different kinds of duty cycles: 0.03% (pulse interval 3s) and 10% (pulse interval 10ms). We have revised in the text to make it more clear (see Page 6, line 133-134). Because carotid arteries of the rats are superficial and there is less attenuation for the application of ultrasound (compared to the heart), 0.6MPa acoustic pressure was used. The grammar mistakes pointed out are revised as following.
Changes in the text:
Page 5, line 99-100: To ensure the MBs arrived at the thrombi, visualization was performed with a …
Page 6, line 111-113: …and plastic sheeting to form thrombus (Figure S1 in Supplementary Materials).
Page 6, line 129-130: …0.6MPa (peak negative pressure) was used
Page 6, line 133-134: Subsequently, 1 MHz US was delivered with two different duty cycles: 0.03% (pulse interval 3s) and 10% (pulse interval 10ms).
Page 7, line 136-137: We made sure that the thrombi were entirely covered by therapeutic US before we gently moved the probe up and down.

3. Results:
P6L195: "please add more details about "... a little MBs were used, ..."
We appreciate the reviewer for pointing this out. We have revised as advised (see Page 9, line 195). Thank you very much again for your time and comments.