Peer Review File

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Reviewer A

First of all, my major concerns regarding this study are the problematic conclusion on the "greatest efficacy of 10% BAS" this is because the non-statistically significance differences between the RL-positive rates across the three groups and the authors did not provide the findings on 15% or 20% BAS. The term "greatest" in the abstract and main text is questionable and not strict.

Reply 1: Thanks for the comments. Our study was designed based on current agents that were mostly applied during practice (air, 5% blood, and 10% blood). Therefore, we didn't include 15% or higher percentage of BAS in our study. We have modified our text for better illustration as advised (see Page 2, line 4; Page 2, line 25; Page 4, line 20, 22-25, and 27-28. References are changed accordingly).

Change in the text (text in red):

… AS without blood, AS with 5% blood (5% BAS), and AS with 10% blood were prepared based on previous studies and observed under microscopy…

… The 10% BAS would be suggested in c-TCD as it addressed larger RLS by increasing the number and stability of microbubbles, and it improves the diagnosis of PFO.

… controversy remains as to the optimal amount of blood, from one drop to 10%, in the AS, and the mechanism for improving c-TCD performance is unclear ….

…and AS with 10% blood (10% BAS), which were common agents applied in clinical practice, …

Second, the title needs to indicate the outcome of this study, positive rates, and the research design of this study, in vitro and in vivo experimental study.

Reply 2: Thanks for the comment. We have changed our title as advised (Page 1, line 3)

Change in text (red)

10% blood-air-saline increases number and stability of microbubbles in detection right-to-left shunt by contrast-enhanced transcranial doppler: an in *vitro* and in *vivo* observational study

Third, the abstract needs to clarify the potential clinical significance of this research focus in the introduction, and the authors should have comments for the clinical implications in the conclusion, not to repeat the main findings again.

Reply 3: Thanks for the comment. We have modified our conclusion as advised. (Page 2, line 25)

Change in text (red):

The 10% BAS would be suggested in c-TCD as it addressed larger RLS by increasing the number and stability of microbubbles

Fourth, in the introduction of the main text, the authors need to use detailed examples to support "controversy remains as to both the optimal amount of blood in the AS and the mechanism for improving c-TCD performance", analyze the reasons for the controversy, have comments on

limitations of prior studies, and explain why the current research methodology is able to address the controversy.

Reply 4: Thanks for the comment. We have added text as advised (Page 4, line 20-25). References are changed accordingly.

Change in text (red):

… however, controversy remains as to the optimal amount of blood, from one drop to 10%, in the AS, and the mechanism for improving c-TCD performance is unclear. Gentile et al showed 1ml blood in AS significantly improved RLS results (8). Differently, Hao and colleagues demonstrated the superiority of adding one drop of blood to AS in detecting RLS (20). No matter how much of blood was added, the mechanism for improving c-TCD performance might come from the enhancement by red cell or more bubbles (9, 12) …

Fifth, in statistics, Chi-square test is not suitable for comparing the rates across the three groups, since the data were one-way ordinal response variable table data. Please consider the Ridit analysis. Please ensure P<0.05 is two-sided.

Reply 5: Thanks for the comment. In our study, we uesd the Crosstabs to analysis the difference between the three groups (table 2). We compare the number but not the rates between the 3 groups. We have modified our text for better illustration. (Page 6, line 20).

Change in text (red):

The categorical variables as RLS classification were compared by Crosstabs with the χ^2 test.

Sixth, in discussion, please discuss the inadequate sample size as the potential reasons for the non-significant differences in the positive rates across the three groups as a major limitation of this study. The other limitation is no data of the 15% Bas or higher. Please also consider to tone down the current conclusion and strictly make it based on the findings.

Reply 6: Thanks for the comments. We have modified text as advised. (Page 9, line 11-15; line 22.)

Change in text (red):

…Firstly, and most importantly, the number of cases included in our study was small, which might lead to the results of no statistically significant differences among three agents. Secondly, we only studied three amounts of blood in AS, which were none, 5%, and 10% based on previous reports and clinical experiences. Other amounts of blood in AS may further address the difference and significance. Thirdly, unlike from previous…

… The use of 10% BAS will improve the diagnosis of PFO…

Reviewer B

1. Abstract

Please defined BAS in the abstract.

Reply 1: Thanks for the comments. We have changed as advised (Page 2, line 1-2)

Change in text (red):

In vitro, AS without blood, agitated saline with 5% blood (5% BAS), and agitated saline with 10% blood (10% BAS) were prepared...

2. Figure 3

Please explain C-TCD in the legend.

Reply: Thanks for the comments. We have changed as advised (Page 17, line 1)

Change in text (red):

Figure 3 c-TCD results for different contrast agents. c-TCD was performed in the same patient using different agents. (A) AS; (B) 5% BAS; (C) 10% BAS. c-TCD, contrast-enhanced transcranial Doppler; AS, agitated saline without blood; .....

3. Table 1

Please explain SD in the table footnote.

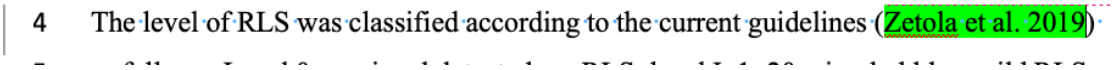
Repl: Thanks for the comments. We have changed as advised (Page 14, line 2)

Change in text (red):

SD, standard deviation; TIA, transient ischemic attack.

4. References/Citations

a) Please add the citation for this study at the end of the sentence.

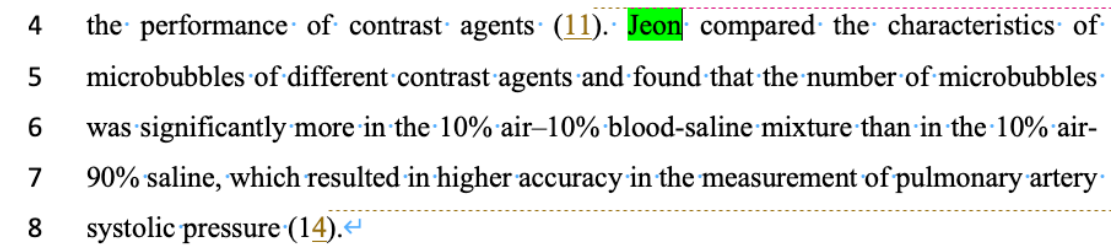


Reply 5a: Thanks for the comments. We have changed as advised (Page 6, line 8)

Change in text (red):

The level of RLS was classified according to the current guidelines (18) as follows...

b) Please check if Jeon is an author of the reference 14, if yes, please revise it as "Jeon et al.".



Reply 5b: Thanks for the comments. We have changed as advised (Page 8, line 10)

Change in text (red):

Jeon et al. compared the characteristics of...