Peer Review File

Article information: https://dx.doi.org/10.21037/cdt-21-364

Reviewer A:

General Comments:

This study in a canine model of myocardial infarction evaluated transthoracic ultrasound-guided intramyocardial delivery of the Ang1 plasmid alone (IM-Ang1) or in a microbubble (IM-Ang1-UTMD) versus intramyocardial delivery of a control microbubble with negative control plasmid (IM-UTMD) or intravenous delivery of Ang1 plasmid in microbubble (IV-Ang-UTMD). Therapies were administered in 42 dogs (10-11 dogs per group) 1 day after coronary microembolization of the LAD after the first diagonal. Ultrasound guidance facilitated percutaneous intramyocardial delivery and verification of delivery. Plasmid delivery was followed by 20 minutes of ultrasound irradiation to improve transfection. They employ FITC labeled Ang1 to track degree of transfection. The goal was to stimulate angiogenesis and improve function post therapy, which was assessed with ultrasound noninvasively. Sudden cardiac death was not different between the groups (27-30%). There were no differences in the complications rate. Contrast echocardiography was used to evaluate tissue perfusion and appeared slightly improved with IM delivery of Ang1 in microbubbles. There was no difference in LV remodeling 1 month post therapy, although the IM-Ang1-UTMD group also demonstrated some mild improvement in global LVEF. The global functional assessment was limited to those animals that survived a full month and there was no assessment of regional function or myocardial strain. The authors should provide information regarding the comparability of baseline LV function post-MI and changes in function for each group. How did they assure that infarct size was comparable between groups? The investigators also evaluated tissues postmortem for angiogenesis and fibrosis. They report decreased fibrosis and increased angiogenesis in the IM-Ang1-UTMD dogs. However, it is not clear how they accounted for analysis of postmortem tissues from animals that died prematurely. The numbers of animals that are depicted in each graph (figures 4-7) should be defined. The work have been strengthened if they performed a more quantitative analysis of myocardial flow, since one of the primary goals was stimulation of myocardial angiogenesis.

Reply to “The authors should provide information regarding the comparability of baseline LV function post-MI and changes in function for each group.”: The reviewer's advice is very good. We added the baseline information of LV function post-MI and changes in function for each group in the parts of “Results” and “Table 2”. One day after MI, there were no differences in the LVEDD, LVESD and LVEF during all 4 groups.

Changes in the text: Page 15, Line 310, 311; Table 2

Reply to “How did they assure that infarct size was comparable between groups”: We think that there were no differences in the size of left ventricular after the MI modeling for the following two reasons: first, the same artery was selected for embolization in all MI dogs; Second, echocardiographic measurements of left ventricular size and systolic function at 1 day after MI showed no statistical difference during the groups.

Changes in the text: Page 15, Line 310, 311; Table 2

Reply to “it is not clear how they accounted for analysis of postmortem tissues from animals that died prematurely”. We used the death of premature dogs immediately occurred within the first week after MI modeling because of the experimental animal ethics principle of 3R--reduction, refinement and

replacement. On the one hand, we are to detect the FITC fluorescence distribution under the frozen section to confirm the success of intramyocardial injection and the amount of injection. These can be explained by the myocardial tissue of the early-dead dog, without the need to sacrifice more dogs. On the other hand, it can also be used to extract protein early and indirectly reflect the expression of target gene Ang1 after intramyocardial injection. The angiogenesis and fibrosis of the myocardial tissue were evaluated one month after MI.

Changes in the text: Page 10, Line 212, 213; Page 11, 214, 215, 216, 217.

Specific Comments:

Methods: The model of microembolization may not mimic the typical pattern of ischemia and reperfusion that is generally observed clinically following MI, where coronary reperfusion is the primary treatment goal. The potential limitations of this model should be discussed. They also need to provide better justification and rationale for use of only two intramyocardial injection sites in the treatment of an LAD infarct.

Reply: The MI model was established by transcatheter coronary embolization. The coronary artery was embolized by the injection of 150~350 μm of gelatin sponge particles, which could be absorbed in about a week. Therefore, although the model did not achieve early coronary reperfusion in time, revascularization can still occur in about a week. We have added a discussion about the limitations of the myocardial infarction model, that is, the time of myocardial ischemia is long, the period of vascular recanalization is relatively late, and it takes about a week for the gelatin sponge particles to be completely absorbed.

We only used two intramyocardial injection sites because of the special curved shape of the canine chest anatomy and the limitation of the puncture site. Observation from the right chest shows that the right heart is in front of the image. The right chest myocardial puncture has a greater risk of complications, and the left chest puncture is relatively safer. When in the supine position, the heart moves significantly back, resulting in a deeper position. On the one hand, it affects the ultrasound observation. On the other hand, it is not suitable for intramyocardial injection through the subcostal region. So, we can only choose the left chest injection, ultrasound positioning at the level of the papillary parasternal short-axis view of the left ventricle anterior wall.

Changes in the text: Page 19, Line 401, 402, 403, 404, 405, 406, 410, 411; Page 20, Line 412, 413, 414, 415, 416, 417.

Methods: How did they assure that postmortem tissue that was evaluated microscopically and with immunohistochemistry represented those regions that received intramyocardial therapy? Greater detail needs to be provided regarding the method of tissue sampling and tissue analysis.

Reply: Due to the limitations of the anatomical position and the myocardial puncture site, we can only use the 2-point injection method, both of which choose the myocardial tissue at the level of the papillary muscle in the middle of the left ventricle anterior wall. Therefore, we only take the myocardial tissue in the middle of left ventricle anterior wall to ensure that it is consistent with the ultrasound-guided puncture site.

In addition, we injected the target gene labelling with FITC. We can observe under the microscope whether there is a green fluorescent at the injection area, but this green fluorescent display was only displayed within 1 week. Therefore, we only choose to observe green fluorescence in the early frozen section, and cannot continue to observe for one month.

From the general specimen color and texture, it can be seen that the myocardium in the infarcted area is white and harder, which is obviously different from the normal myocardial tissue, which is red

and soft. Therefore, we can combine the anatomical location of the ultrasound puncture site and the appearance of the specimen to ensure the accuracy of myocardial tissue extraction.

Changes in the text: Page 7, Line 145, 146, 147; Page 8, Line 148; Page 11, Line 233, 234, 235; Page 12, Line 236, 237, 238, 239, 240, 241. Page 19, Line 410, 411; Page 20, Line 412, 413, 414, 415, 416, 417.

Methods, page 6: They need to provide greater detail regarding how they quantified myocardial perfusion with contrast echocardiography. How were images analyzed and ROIs drawn to assure reproducible estimation of tissue perfusion? It would have been stronger if they had performed dynamic imaging to evaluate intramyocardial blood volume and/or flow using previously well-established methods.

Reply: We added the detailed procedure about contrast echocardiography process and the quantifying. For example: MCE was performed with the angiographic mode to evaluate myocardial microvascular perfusion before MI, 1 day after MI and 1 month after treatment. After obtaining an adequate acoustic window with suitable depth and gain, the SonoVue contrast agent was infused intravenously. When the contrast agent was filled with plateaued in the myocardium, a flash with high (1.40) mechanical index impulse was given to destroy the contrast agent microbubbles. Then, a low (0.12) mechanical index of MCE was switched on, the myocardial contrast replenishment was visualized and evaluated. The dynamic images were acquired at the parasternal short-axis view of LV mid-papillary muscle level for up to 15 cardiac cycles after the flash for offline evaluation.

For qualitative analysis of myocardial perfusion, the region of interest was placed in the middle of each myocardial segment by using Qlab software quantitatively analyzing the reperfusion curve of real-time acoustic radiography. The mean value of three times was taken for the parameters in each region. Plots of time-contrast intensity were constructed and fitted to an exponential function, $y = A(1-e^{-\beta t})$. The plateau of signal intensity (A) and the slope of maximum signal intensity rise (β) were measured, and the product of A × β was computed. Then the mean value of A, β, and A×β in each segment was calculated. A, β, and A×β represent myocardial blood volume, myocardial blood flow velocity, and myocardial blood flow, respectively.

Changes in the text: Page 9, Line 184, 185, 186, 187, 188, 189, 190, 191; Page 10, Line 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 208.

Reviewer B:

1. The paper is interesting and showed the feasibility and efficacy of intramyocardial injection of ultrasound-targeted microbubble destruction mediated gene therapy. A few major issues:

1. There is no justification using angiogenin-1 gene in this study. Why not just use a eGFP plasmid?

Reply: We used the Western blot to detect the relative expression level of angiogenin-1 gene in the myocardial tissue at the injection site, as shown in the results. As following "The Western blot results showed that the relative expression of Ang1 protein was significantly higher in the IM-Ang1-UTMD group (54.1%±5.7%) and the IM-Ang1 group (45.9%±5.8%) than in the IV-Ang1-UTMD group (34.2%±3.9%) and the IM-UTMD group (8.5%±1.1%), with significant differences".

Another reason we did not use EGFP was the concern that the FITC labeled target gene would be confused with the green fluorescence of EGFP.

2. Sham animal group shall be included.

Reply: We used the IM-UTMD group as the sham group with transthoracic ultrasound-guided percutaneous IM injection of 1.0 ml of the negative control plasmid with UTMD.

Changes in the text: Page 2, Line 32, 33, 34, 35, 36, 37; Page 8, Line 163.

3. Figure 3: it is unclear the green fluorescence was background or FITC, thus shall group animal shall

be included.

Reply: We took samples from different parts of the myocardial tissue of all 4 groups and made frozen sections. Only the injection area showed obvious green fluorescence, while other areas did not show green fluorescence. This suggests that green should be FITC fluorescence.

4. Figure 4B: sample size shall be provided.

Reply: We have replenished the number of samples for each group. N=3 in each group, each experiment was repeated three times.

Changes in the text: Page 25, Line 542, 543.

5. Figure 5: authors shall indicate in the pic where were the filling defects. Representative images of myocardial defects in each animal group shall be presented before, 1 h, and 1 month after MI.

Reply: This suggestion is very good. In the figure, we marked the area of the filling defect with a yellow arrow, and supplemented the ultrasound myocardial perfusion contrast images of each group at each stage.

Changes in the text: we provide a new figure 5.

6. Figure 6: author shall show lower magnification of scar area.

Reply: We have partially replaced the pictures, and now the pictures are lower magnification than before.

Changes in the text: we provide a new figure 6.

7. Figure 6 and 7: the scale bars are the length in the pics and represent 50 um. However, they are from different magnification. This is impossible.

Reply: We are very sorry that we have not noticed such important details before. We have partially replaced the pictures, and now the pictures are all the same magnification in size.

Changes in the text: we provide a new figure 6 and 7.

8. Figure 7: vessel density at border zone of infarct shall be presented also.

Reply: We may not clearly describe it in the methodological part. The density of new blood vessels we observe is the border zone around infarcted myocardium. Most of the infarct area has been fibrotic, with very few new blood vessels.

Changes in the text: Page 12, Line 252; Page 26, Line 560.

9. What's the point of including Figure 8?

Reply: We first wanted to know whether the ultrastructure of myocardial tissue under the projection electron microscope was different in each group after treatment, but finally found that there was no obvious difference that can be observed under the electron microscopy, so only the infarct zone, border zone and normal myocardial tissue were displayed for understanding the electron microscopy images.

Minor issues:

1. Abbreviations shall appear after full spell.

2. Language shall improved. Name a few:

a. Line 54: with UTMD-mediated Ang1 plasmid therapy is a safe and effective method for MI.

b. Line 129: suspension containing approximately 2.0×108 microbubbles/ml was formation.

c. Line 218: Masson’s staining and Sirius Red’s staining for collagen fiber

d. Line 531: C, local myocardium echo enhanced after the intramyocardial injection.

Reply: Thank you for such a detailed suggestion, all minor issues have been revised. We revised the language as required. In order to better meet the requirements, we asked professional editing experts to make modifications for us. We chose the “Premium Editing Service” on www.aje.com. The paper was edited for grammar, phrasing, and punctuation.

Changes in the text: Page 1, Line 3, 4; Page 2, Line 25, 41, 42; Page 3, Line 54, 55; Page 6, Line 124, 125; Page 11, Line 233; Page 12, Line 243, 248; Page 16, Line 326; Page 26, Line 551, 557.

Reviewer C

1. Please draw a flowchart/timeline diagram to illustrate how the study designs were carried out, including the animal model, essential conditions, sample size, allocation, blinding, intervention and measurement at each stage.

Please see excellent examples from NC3Rs: https://eda.nc3rs.org.uk/

Reply: This suggestion is very good. We have added the experimental flow diagram in the manuscript, as shown in Figure 1 and the figure legend.

Changes in the text: Page 6, Line 104; Page 26 Line 532, 533, 534, 535, 536.

2. We suggest the authors registry the study at the Animal Study Registry and provide the registry ID in the manuscript. Please see details at: https://www.animalstudyregistry.org/asr_web/index.action

Reply: This suggestion is very good. In accordance with the principle of ethical approval, we had applied to the ethics committee and obtained approval and registration before carrying out the experiment. The ID was listed in the manuscript.