Peer Review File

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All changes made to the manuscript are colored red. Below is a list of reviewers' comments (C), and our corresponding responses.(R)

<Reviewer A Comment>

C1. Mechanism needs to be discussed more. There is a trend toward reduced swelling... is this because of less blood-side extravasation or increased lymph flow? Neither is demonstrated in this study.

R: Thank you for your valuable comment. Because not only there was no studies on the therapeutic efficacy of microcurrent therapy (MT) in lymphedema, but also this study was coducted morphologic (circumference of the forelimb), Immunohistochemistry, and western blot analysis, the exact mechanism was hard to be investigated.

Our results showed that after MT, swollen circumference of the limb was improved and vascular maker was increased. Therefore, as mentioned in the discussion, we speculated that MT could promote vascular function and increase the number of vessles and that could have improved limb swelling. In the future, we will conduct researches to reveal more accurate mechanisms (less blood-side extravasation or increased lymph flow and so on) according to your advice.

C2. Changes to non-lymphedema tissue are not analyzed either. With the minimal effect on swelling, are the tissues changes even biologically-relevant?

R: Thank you for your comment. We designed each opposite leg with no treatement (non-

lymphedema limb) as the naive and compared it to the experimental group. I think there was a lack of clear explantation. Let me describe it more clearly.

2.1 Rat model of forelimb lymphedema

Two weeks after surgery, 12 rats were randomly divided into a group that underwent MT in the lymphedematous forelimb (MT, n = 6) and a sham MT in the lymphedematous forelimb group (sham MT, n = 6). The treated forelimbs were compared with the non-edematous opposite forelimb of each rat (naive).

3.2 Increased vessel labeling in tissue of MT-treated rats

Representative images of the immunohistochemically stained blood vessels ~~and lymphatic vessels~~ are shown in Figure 2A. The vessel density (% area) was significantly higher in the MT group than in the sham MT and in the non-edematous contralateral forelimbs groups (5.33% vs. 3.90% vs. 1.77%) ($p < 0.05$) (Figure 2B).

3.3 Increased expression of VEGR3 and VEFG-C in MT-treated rats

Western blot analysis was performed 14 days after the last MT application to identify the protein expression of VEGFR3 and VEGF-C ~~in the developing lymphatic vessels in the skin~~ in the tissue levels. The expression of VEGFR3 was 2.02-fold higher in the MT group compared to the non-edematous contralateral forelimbs group. Expression in the MT group was significantly higher than that in the other groups ($p = 0.035$) (Figure 4A). VEGF-C expression was 2.27-fold higher in the non-edematous MT group than in the contralateral forelimbs group;

however, the difference between the groups was not statistically significant ($p = 0.051$) (Figure 4B).

Figure Legends

Figure 1. Circumferences of the forelimb at the level of the carpal joint (A) and 2.5cm above the carpal joint (B) in the MT, Sham MT groups, and non-edematous contralateral forelimbs (naive) ~~3 groups~~. The circumference at the carpal joint was significantly decreased in MT group at 14 days after the last MT compared to the sham MT group ($p = 0.021$). MT, microcurrent therapy

Figure 2. Immunohistochemical analysis of CD31 expression in rat forelimbs 14 days after the last microcurrent therapy (MT). (A) Immunohistochemical staining (brown) for CD 31 that underwent MT or sham MT in the lymphedemaous forelimbs and in the non-edematous contralateral forelimbs (naive) ~~in the 3 groups of rat forelimbs~~ (x200). (B) Quantification of the mean area (%) of CD 31 stained vessels in each group. Percentage of CD31- positive area was measured by ImageJ software. The statistical evidence ($p < 0.05$) was analyzed by Kruskal Wallis's test followed by Dunn's procedure. CD31, cluster of differentiation 31; MT, microcurrent therapy

Figure 3. Immunohistochemical analysis of fibrotic tissue in the skin of rat forelimbs 14 days after the last microcurrent therapy (MT). (A) Masson's trichrome staining (blue) for fibrotic tissue that underwent MT or sham MT in the lymphedematous forelimbs and in the non-edematous contralateral forelimbs (naive) ~~in the 3 groups of rat forelimbs~~ (200x). (B) Quantification of the extent of fibrotic tissue area (%) in each group. The percentage of trichrome stained area was measured using ImageJ software. The mean stained area in the MT

group was significantly larger than that in sham MT and naïve groups. The statistical evidence ($p < 0.05$) was analyzed by Kruskal Wallis's test followed by the Dunn's procedure. MT, microcurrent therapy

C3. For the CD31 analysis, cannot really say that this is angiogenesis and lymphangiogenesis as dermal lymphatics label weakly for CD31.

R: Thank you for your valuable comment. As your comment, CD 31 mainly represent endothelial cells in the blood vessels. In order to clarify the study, we decided to omit the word 'lymphangiogenesis' in our article. Also, we revised the limitation as below. We will plan to study more specific immunohistochemical staining for LYVE-1 or Podoplanin in the future studies.

1. Discussion

The present study has several limitations. First, the sample size was insufficient for obtaining statistically significant results. Second, the study was conducted over a relatively short period, and further studies are needed to assess the long-term effects of MT. Third, additional studies are needed to evaluate the effects of MT at different frequencies, intensities, and durations. Fourth, we did not evaluate lymphatic circulaition using lymphography/lymphoscintigraphy according to the changes of forelimb circumference as well as immunohistochemical results. Fifth, in future studies, the quantification of lymphangiogenesis using lymphatic endothelial cell specific markers as podoplanin, LYVE-1 or Prox-1 will be needed.

C4. R3 or VC protein levels are not specific to 'developing lymphatics in the skin' as stated;

R3 is potentially expressed in angiogenic blood endothelium. Just say 'tissue levels'.

R: Thank you for your valuable comment. It was revised as below.

3.3 ~~Expression of VEGR3 and VEFG-C~~ Increased expression of VEGR3 and VEFG-C in MT-treated rats

Western blot analysis was performed 14 days after the last MT application to identify the protein expression of VEGFR3 and VEGF-C ~~in the developing lymphatic vessels in the skin~~ in the tissue levels.

C5. The method of "measuring the % of trichome stained area" makes no sense

R: Thank you for your valuable comment. As trichrome dye indicated the presence of collagen fibers in the tissue, we used ImageJ software to observe the degree of trichrome dying. Using ImageJ software, we evaluated the percentage of blue staining in the whole area with reference to previous studies. We revised it more clearly. The reference is mentioned below.

2.4 Immunohistochemical analysis

Quantification of the area covered by vessel ~~and the extent of fibrotic tissue were~~ was determined by measuring the percentage of CD31- positive area. ~~and by measuring the percentage of trichrome stained area.~~ The percentage of blue staining in each image for Masson's trichrome stain was evaluated by colour deconvolution technique, with the dye indicating the presence of collagen fibers in the tissue. (13)

Reference

12. Caetano GF, Fronza M, Leite MN, Gomes A, Frade MA. Comparison of collagen content in skin wounds evaluated by biochemical assay and by computer-aided histomorphometric analysis. Pharm Biol. 2016;54(11):2555-9.

C6. Minor:

1) Headings in section 3 should be the results, for example, 3.2 "Increased vessel labeling in tissue of MT-treated rats"

3.1 Decreased circumference of the forelimb in MT-treated rats

3.2 Increased vessel labeling in tissue of MT-treated rats

3.3 Increased expression of VEGR3 and VEFG-C in MT-treated rats

2) In the results, statements should directly reference which panel (Reference Figure 1A, etc. in the results)

3) In the Highlight Box need to spell out MT

4) Figure 1 is lacking the A and B labels

5) I would prefer data be graphed with individual data points or, minimally, use SD and SEM is not statistically informative.

R: Thank you for your valuable comment. The manuscript and figure were revised as your comments. We revised figures as boxplots.

<Reviewer B Comment>

C1. If authors attempt to clarify the therapeutic effect for skin fibrosis of chronic lymphedema, it takes over 12 weeks. Forty days is too short to evaluate skin fibrosis of lymphedema. These facts had been already reported in previous report. Therefore, this model did not appropriate to evaluate therapeutic effect for skin fibrosis of chronic lymphedema.

R: Thank you for your valuable comment. The course of lymphedema in rat is quite different from that of humans. According to our study, limb edema was rapidly noticeable from the third day of post-ALND surgery, and reached maximum swelling around 10 to 14 days of post-ALND surgery. It has also been reported that in the rat, limb swelling has improved within several weeks following ALND unlike humans. Around at 50 days (ref. 1) to at 66 days (ref. 2) post-ALND surgery, no detectable swelling was found in rat model of forelimb lymphedema. Of course that is not the meaning of the lymphangiogenesis. Therefore, the timing of fibrosis seems to be difference from that of humnas. Fibro-fatty tissue deposition and greater skin thickness was found even before 4 weeks after lymphedema surgery in previous animal studies (ref. 3) We coducted our reserach considering the overall secondary lymphadenoma course in rat mentioned above.

However, as your advice, we will try to create a more meaniningful model of chronic lymphedema in rat how to worsen fibrosis in limb and how to sustain tissue swlling in limb.

ref.1 Laura L. Lynch, Uziel Mendez, Anna B. Waller, Amani A. Gillette, Roger J. Guillory, II, and Jeremy Goldman. Fibrosis worsens chronic lymphedema in rodent tissues

ref.2 Uziel Mendez, Emily M. Stroup, Laura L. Lynch, Anna B. Waller, and Jeremy Goldman. A chronic and latent lymphatic insufficiency follows recovery from acutelymphedema in the rat foreleg

ref.3 In Gul Kim, Ji Youl Lee, David S. Lee, Jeong Yi Kwon, Ji Hye Hwang. Extracorporeal Shock Wave Therapy Combined with Vascular Endothelial Growth Factor-C Hydrogel for Lymphangiogenesis

C2. In Fig.2, immunohistochemical staining for CD31 was performed. However, CD31 mainly represent endothelial cells in blood vessels, not lymphatic endothelial cells. Authors should perform immunohistochemical staining for podoplanin, LYVE-1 or Prox-1 to identify lymphatic endothelial cells..

R: Thank you for your valuable comment. As your comment, CD 31 mainly represent endothelial cells in blood vessls. In order to clarify the stduy, we decided to omit the word 'lymphangiogenesis' in our article. Also, we revised the limitation as below. We will also plan to study more specific immunohistochemical staining for LYVE-1 or Podoplanin in future studies as your advice.

2. Discussion

The present study has several limitations. First, the sample size was insufficient for obtaining statistically significant results. Second, the study was conducted over a relatively short period, and further studies are needed to assess the long-term effects of MT. Third, additional studies are needed to evaluate the effects of MT at different frequencies, intensities, and durations. Fourth, we did not evaluate lymphatic circulaition using lymphgraphy/lymphoscintigraphy

according to the changes of forelimb circumference as well as immunohistochemical results. Fifth, in future studies, the quantification of lymphangiogenesis using lymphatic endothelial cell specific markers as podoplanin, LYVE-1 or Prox-1 will be needed.

C3. In Fig.2b and 3b, Kruskal Wallis's test was performed to analyze these data. Kruskal Wallis's test was typically used for median. These figures should be represented in boxplot..

R: Thank you for your valuable comment. We revised the figures as you mentioned.

C4. Authors should show lymphatic circulation using lymphography and increase of lymphatic vessels histologically.

R: Thank you for your valuable comment. I also agree that to observe lymphatic flow, it is good to check the lymphograph. But it was impossible because of the absence of animal experiment equipment. We revised the limitation section as below.

The present study has several limitations. First, the sample size was insufficient for obtaining statistically significant results. Second, the study was conducted over a relatively short period, and further studies are needed to assess the long-term effects of MT. Third, additional studies are needed to evaluate the effects of MT at different frequencies, intensities, and durations. Fourth, we did not evaluate lymphatic circulaition using lymphography/lymphoscintigraphy according to the changes of forelimb circumference as well as immunohistochemical results. Fifth, in future studies, the quantification of lymphangiogenesis using lymphatic endothelial cell specific markers as podoplanin, LYVE-1 or Prox-1 will be needed.

<Reviewer C Comment>

C1. The number of CD31-positive vessels was significantly higher in the MT group than in the sham MT group. However, CD31 is not a special immunostaining for lymphatic vessels. How about LYVE-1 or Podoplanin?

R: Thank you for your valuable comment. As your comment, CD 31 mainly represent endothelial cells in blood vessls. In order to clarify the stduy, we decided to omit the word 'lymphangiogenesis' in our article. Also, we revised the limitation as below. We will also plan to study more specific immunohistochemical staining for LYVE-1 or Podoplanin in future studies as your advice.

3. Discussion

The present study has several limitations. First, the sample size was insufficient for obtaining statistically significant results. Second, the study was conducted over a relatively short period, and further studies are needed to assess the long-term effects of MT. Third, additional studies are needed to evaluate the effects of MT at different frequencies, intensities, and durations. Fourth, we did not evaluate lymphatic circulaition using lymphography/lymphoscintigraphy according to the changes of forelimb circumference as well as immunohistochemical results. Fifth, in future studies, the quantification of lymphangiogenesis using lymphatic endothelial cell specific markers as podoplanin, LYVE-1 or Prox-1 will be needed.

C2. Why did you evaluate at 2 weeks after the surgery? In general, rats have the ability to regenerate lymphatic vessels naturally. I believe spontaneous lymphatic regeneration may occur at 2 weeks.

R: Thank you for your valuable comment. As you said, the course of lymphedema in rat is quite different from that of humans. Some studies showed that at 3 days after electrocauterization of the lymphatic vessles, the dermal depth of the footpad reached its maximum swelling (ref 1). According to our previous study, limb edema was rapidly noticeable from the third day of post-ALND surgery, and reached maximum swelling around 10 to 14 days of post-ALND surgery (data not shown). That is the reason that application of MT started at two weeks after ALND.

It was also known that around at 50 days (ref. 2) to at 66 days (ref. 3) post-ALND surgery, no detectable swelling was found in rat model of forelimb lymphedema. Of course that is not the meaning of the lymphangiogenesis. Our experiment was conducted for a total of 41 days. we also think there would be natural improvement of swelling, but we don't think there would be much impact on the results of this study.

ref.1 Ji Hye Hwang, In Gul Kim, Ji Young Lee, Shuyu Piao, David S. Lee, Tae Seung Lee, Jeong Chan Ra,Ji Youl Lee. Therapeutic lymphangiogenesis using stem cell and VEGF-C hydrogel

ref.2 Laura L. Lynch, Uziel Mendez, Anna B. Waller, Amani A. Gillette, Roger J. Guillory, II, and Jeremy Goldman. Fibrosis worsens chronic lymphedema in rodent tissues

ref.3 Uziel Mendez, Emily M. Stroup, Laura L. Lynch, Anna B. Waller, and Jeremy Goldman. A chronic and latent lymphatic insufficiency follows recovery from acutelymphedema in the rat foreleg

C3. Same question as #2. To prevent spontaneous regeneration of lymphatic vessels. we irradiate to the lesion. Why did not you perfome X-ray irradiation?

R: Thank you for your valuable comment. Like similar to chronic lymphedema course in humans, we also think about the way worsening fibrosis in limb and the way sustaining tissue swlling in limb in rat model of forelimb lymphedema. As the incidence of secondary lymphedema worsens when radiation therapy is involved and/or fibrotic scarring incurred at the site of surgery, acually we are planning of induction of fibrotic scarring at the site for surgery as well as ALND in experiemtal animal model. In that way, we could perform x-ray irradiation, although we're planning to use chemical substances rather than x-ray irradiation in our experiemtal animal model. Thank you for your good advice.