Microvascular Research 157 (2025) 104761

Contents lists available at ScienceDirect

Microvascular Research

journal homepage: www.elsevier.com/locate/ymvre

Increased angiopoietin-1 improves nailfold capillary morphology in patients with systemic sclerosis

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ARTICLE INFO

Keywords:
Systemic sclerosis
Raynaud's phenomenon
Heating
Angiopoietin-1
Capillaroscopy

ABSTRACT

Objective: Raynaud's phenomenon is a common symptom of systemic sclerosis. We previously reported that elbow heating increases angiopoietin-1 in the fingertips and alleviates Raynaud's phenomenon. Angiopoietin-1 levels decrease in patients with systemic sclerosis with severe capillary damage. We aimed to conduct a prospective study to confirm whether the increase in angiopoietin-1 caused by heating modifies capillary morphology.

Methods: The left ring fingers of 19 patients with systemic sclerosis were monitored six times at 4-week intervals using capillaroscopy, during which both elbows were heated using disposable heating pads for 8 weeks. Blood samples were collected from the same fingertips four times—before heating, twice during heating, and once after heating—to measure angiopoietin-1.

Results: In six patients, the peak increase in angiopoietin-1 occurred 4 weeks after the start of heating, whereas in seven patients, the peak value was observed 4 weeks after the termination thereof. No change in the density of the front-row capillaries was observed by capillaroscopy. The proportion of hairpin-shaped capillaries increased from 20.2 % during the preheating period to 26.6 % during the heating period ($p = 0.00107$). When a correlation coefficient of 0.6 or higher was set as significant, there was a strong correlation between changes in fingertip angiopoietin-1 levels and changes in the proportion of hairpin-shaped capillaries in six patients.

Conclusion: Increased angiopoietin-1 levels in the fingertip due to elbow heating may improve the peripheral capillary morphology in patients with systemic sclerosis.

1. Introduction

Systemic sclerosis (SSc) is a connective tissue disease with an unknown etiology. Various clinical symptoms have been observed, including skin sclerosis, reflux esophagitis, and lung fibrosis (Denton and Khanna, 2017). Raynaud's phenomenon, in which peripheral blood circulation is impaired by cold stimuli or mental tension, is also a representative symptom of SSc. Apart from intense pain, it causes skin ulceration and sometimes necrosis of the phalanges. Capillaroscopic studies have revealed that the cause of this phenomenon is a decrease in tissue blood flow due to abnormal meandering and branching of the capillaries (Cutolo et al., 2000). As a cause of this capillary change, abnormal angiopoietin-1 (Angpt-1) levels in SSc is one candidate. Angpt-1 is one of the molecules involved in capillary metabolism. This molecule binds to Tie2 receptors on vascular endothelial cells and contributes to the linear extension of capillaries and the stabilization of vascular morphology (Jain, 2003). Angpt-1 is decreased in SSc and is lower in patients with more severe capillary deformation observed by capillaroscopy (Michalska-Jakubus et al., 2019). The decrease in Angpt-1 expression is thought to be involved in the capillary damage observed in SSc. It has been reported that thermal stimulation enhances Angpt-1 production. Li et al. reported that thermal stimulation enhanced Angpt-1 production in co-cultures of human osteoblasts and endothelial cells (Li et al., 2014), and Kuhlenhoelter et al. reported that heating the human body increased the production of Angpt-1 in muscle tissue (Kuhlenhoelter et al., 2016). Furthermore, Chakroborty et al. reported

Abbreviations: Angpt-1, angiopoietin-1; ELISA, Enzyme-linked immunosorbent assay; SSc, Systemic sclerosis; VEGF, Vascular endothelial growth factor.

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https://doi.org/10.1016/j.mvr.2024.104761
Received 5 August 2024; Received in revised form 4 November 2024; Accepted 5 November 2024
Available online 7 November 2024

that dopamine stimulation induces the production of Angpt-1 from capillary pericytes, suggesting a relationship between autonomic nervous system excitation and Angpt-1 production (Chakroborty et al., 2011).

Recently, we have reported that continuous heating of the elbow with disposable heating pads alleviates Raynaud's phenomenon and increases Angpt-1 expression in the fingertips (Shima et al., 2022, Watanabe et al., 2023). Although Angpt-1 is a capillary stabilizing factor, it is still unclear whether increasing Angpt-1 in the fingertips improves capillary morphology. Because heat stimulation causes vasodilation, it is highly likely that wearing a heating pad alleviated Raynaud's phenomenon, but if increased Angpt-1 repairs meandering capillaries, continuous heating may make Raynaud's phenomenon less likely to occur. We, therefore, prospectively observed capillary morphology for six months and established an elbow heating period during the observation to examine whether heating would improve capillary morphology with increased Angpt-1 levels.

2. Material and methods

2.1. Study procedure

This was a single-arm open-label prospective study. The study protocol was approved by the Institutional Review Board of Osaka University Hospital (2021Oct26, #20178–2) and was published in the University Hospital Medical Information Network, titled "Prospective capillaroscopic observation of AngioGenesis and New Induction vessels by warming in systemic sclerosis [AGNI study]" (UMIN 000041821). The study was initiated after receiving permission from the hospital director and was conducted in accordance with the principles of the Declaration of Helsinki.

Entry criteria were as follows: 1) patients aged 20–90 years; 2) patients who met the 2013 classification criteria for SSc: an American College of Rheumatology/European League against Rheumatism (van den Hoogen et al., 2013); 3) patients attending Osaka University Hospital as outpatients; and 4) patients who gave their free written consent after fully understanding the study. The exclusion criteria were as follows: 1) patients with skin abnormalities other than systemic sclerosis at the site where the disposable heating pads were worn; 2) patients with chronic diseases associated with fever or complicated by infection; 3) patients with diabetes mellitus; 4) patients who could not voluntarily remove and insert the device; 5) patients with peripheral neuropathy in the upper limb; 6) patients with shunt vessels in the upper limb for hemodialysis; and 7) patients deemed inappropriate by the principal investigator. Written informed consent was obtained after written and verbal explanations. The study procedure is illustrated in Fig. 1.

Six capillaroscopy tests were performed every four weeks. Blood samples were collected from the phalanges using a blood glucose lancet at four visits (visits 2, 3, 4, and 5). Disposable heating pads were placed on both upper arms near the elbow joints for 8 weeks between visits 2 and 4.

2.2. Capillaroscopy test

The capillaroscope used in this study was a TOKU-Capillaro (TOKU, Tokyo, Japan), and the capillaries in the nailfold were photographed using fixed-point observation. Matching the observation finger with the blood sampling finger and frequently performing fixed-point observations on eight fingers requires patience from the participants; we, therefore, decided to follow only the ring finger on the non-dominant hand.

Nailfold capillaries were photographed at low magnification (×40) to avoid arbitrary selection of the field of view, and the characteristic blood vessels that served as landmarks were selected. The same examiner photographed the same field of view as much as possible in six tests, using the target vessel as a landmark. The number of capillaries in the front row was counted by the number of blood vessels observed within a 3.2-mm width, which was the visual field limit of the equipment used. In the ninety-degree method, a capillary loop is considered a front-row loop if the angle between the apex of the capillary and the apex of its two adjacent capillaries is >90°, which is used to determine whether the capillary is in the front row or not (Etehad Tavakol et al., 2015). Capillaries were counted by a single assessor other than the imager, and hairpin-shaped capillaries were selected according to Smith's classification (Smith et al., 2016).

2.3. Angiopoietin-1 measurement

Fingertip blood was sampled using a blood glucose lancet. In total, 20 μL of fingertip blood was diluted into 180 μL of phosphate-buffered saline and centrifuged at 1200 rpm for 5 min to obtain the supernatant, which was stored at −80 °C. Fingertip Angpt-1 concentration was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine® ELISA Human Angiopoietin-1 Immunoassay, R&D Systems, Minneapolis, MN).

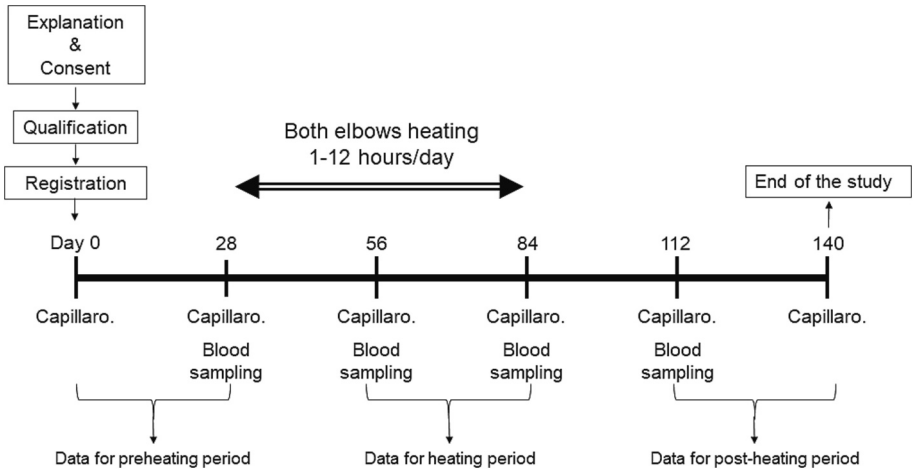

Fig. 1. Study procedure
The participants visited our hospital six times every 28 days and underwent capillaroscopy. Two capillaroscopy tests before the start of elbow heating were used as the preheating period data. Similarly, the tests during and at the end of heating were defined as the heating period data, and the tests at 4 and 8 weeks after the end of heating were defined as the post-heating period data. Capillaro, capillaroscopy test.

2.4. Disposable heating pad

The disposable heating pad used in this study was 7 × 9 cm in size, contained iron powder that generates heat through oxidation, and was designed to ensure that the skin temperature in the area of application did not exceed a maximum of 42 °C. This exothermic temperature was set to avoid thermal injury based on data from Moritz et al. (Moritz and Henriques, 1947). Heating pads were inserted into a pair of special holders and attached to the proximal sides of both elbow joints. The heating pad can be heated for 6 h, and up to two pairs of pads can be used per day. Thus, participants could receive heating for a total of up to 12 h daily, with usage set between 1 and 12 h at their discretion to avoid burns. For safety, the participants were instructed to use the heating pads only during the daytime and not at night or while sleeping, to avoid burns.

2.5. Statistical analysis

To avoid a contingent effect on the capillaroscopy test, for example, weather, the tests were performed twice with a 4-week interval for each period. The average of the two tests before heating was used as the "preheating period" value, the average of the two tests during the heating period was used as the "heating period" value, and the average of the two tests after the heating period was used as the "post-heating" value. To evaluate the effect of concomitant drugs on the extent of the increase in Angpt-1, Student's *t*-tests were performed between two groups: users and non-users. When observing the changing of capillaroscopy data, it is necessary to use "one-way analysis of variance (ANOVA)" to compare the three groups of "preheating period," "heating period," and "post-heating period." However, because we have already reported that heating stimulation shows a carry-over effect (Shima et al., 2022), a one-way ANOVA does not properly evaluate the difference. Therefore, we used a paired *t*-test to examine the effect of heating by comparing the two groups of "preheating period" and "heating period." Correlation coefficients between the concentration of Angpt-1 in the fingertips and the number of hairpin-shaped capillaries were obtained using Pearson's function. Bell Curve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan) was used for these calculations.

3. Results

3.1. Patients

A total of 20 entries were obtained. One patient was hospitalized with meningitis before the start of the heating intervention and was excluded from the study. The remaining 19 cases constituted the population for the analysis group, although two cases were missed in the last sixth capillaroscopy test owing to patient inconvenience. The characteristics of the 19 patients are shown in Table 1.

3.2. Angpt-1 measurement

Fig. 2 shows the progress of the 14 cases in which Angpt-1 was measurable in all four blood samples collected. Most patients presented with an increase in Angpt-1 concentration compared to the baseline (day 28). In six cases, an elevated peak was observed on day 56 (4 weeks after the start of heating). In seven cases, the peak appeared on day 112 (4 weeks after the end of heating). To determine the differences between the early and late elevation groups, we compared age, disease duration, auto-antibodies, disease subtype, and clinical symptoms. Age (53.2 ± 11.4 vs 58.6 ± 11.1), disease duration (14.6 ± 8.9 vs 11.7 ± 7.6), or modified Rodnan skin score (2.8 ± 1.3 vs 3 ± 2.3) did not show any significant differences between the two groups. Although the auto-antibodies did not show any deviation in either group, the positivity rate for digital ulcers was higher in the early group (50 %) than in the late group (14 %). The effects of the drugs used were examined, but no statistically significant difference was observed in terms of increase in Angpt-1 concentration from the baseline on day 56 (4 weeks after the start of heating) between users and non-users of glucocorticoids (prednisolone and methylprednisolone) (4.93 ± 4.59 ng/mL vs. 2.06 ± 3.42

Table 1
Characteristics of the participants.

Age (*years*)	55.9 ± 11.7
Disease history (*years*)	13.3 ± 7.6
Sex (*numbers*)	
Male	3
Female	16
Disease subtype (*numbers*)	
dcSSc	5
lcSSc	14
mRSS at entry	4 ± 3.6
SSc related symptoms (*numbers*)	
Digital ulcer	4
Pulmonary fibrosis	8
Pulmonary hypertension	0
Reflux esophagitis	13
Comorbidities (*numbers*)	
Hypercholesterolemia	5
Hypertension	3
Sjöegren syndrome	2
Medications (*numbers*)	
Glucocorticoids user	6
Immunosuppressants user	3
mycophenolate mofetil	1
azathioprine	1
mizoribine	1
Prostacyclin analogues user	6
beraprost	5
limaprost alfadex	1
Ca channel antagonists user	3
PDE5 inhibitors user	0
ETR antagonists user	3

dcSSc, diffuse cutaneous systemic sclerosis; lcSSc, limited cutaneous systemic sclerosis; Ab, antibody; PDE5, Phosphodiesterase-5; ETR, Endothelin receptor.

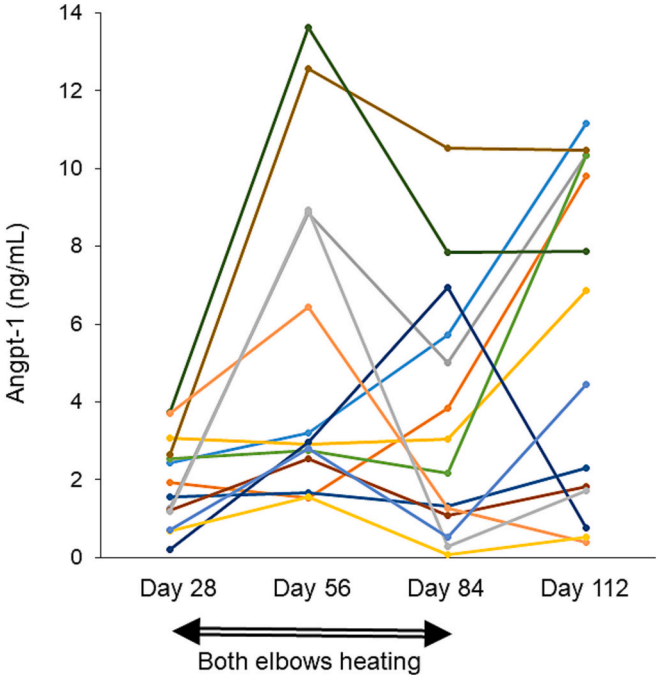

Fig. 2. Increase in angiopoietin-1 levels following the initiation of heating both elbows
Blood samples were collected from the tips of the left ring fingers. Angpt-1, angiopoietin-1.

ng/mL, $p = 0.201$). Similarly, no statistically significant difference was observed with regards to increase in Angpt-1 concentration from the initial value on day 56 between users and non-users of prostacyclin analogs (beraprost and limaprost alphadex) (4.69 ± 5.17 ng/mL vs. 2.14 ± 3.26 ng/mL, $p = 0.259$). The effects of immunosuppressants, phosphodiesterase-5 inhibitors, and endothelin receptor antagonists were also of concern; however, there were only three users of each drug, making statistical comparison impossible.

3.3. Capillaroscopic images

The changes in the number of front-row capillaries in each period are listed in Table 2. There were 13 cases that showed an increase in the heating period compared with the preheating period, and six patients showed a decrease in the heating period compared with the preheating period. In some cases, the density increased during the heating period, but there were no statistically significant differences among the three periods.

Among the front-row capillaries, the average percentage of hairpin-shaped capillaries was 20.2 % during the preheating period, increasing to 26.6 % during the heating period (Fig. 3). During the post-heating period, the average percentage of hairpin-shaped capillaries in the front-row was 25.7 %. A significant difference was observed between the preheating and heating periods ($p = 0.0107$).

The correlation coefficients between the concentration of fingertip Angpt-1 and the rate of hairpin-shaped capillary formation are shown in Fig. 4. Assuming that a coefficient of 0.6 or higher is significant, it was determined that there is a correlation between changes in the rate of hairpin-shaped capillaries and changes in fingertip Angpt-1 concentration in six patients when examining the 17 patients, excluding the two participants who had missing data more than twice. A typical example of a fixed-point observation is shown in Fig. 5. The meandering capillaries observed in the ovals (A) faded out in the picture on days 56 (B) and 84 (C), and the second row of capillaries grew and replaced the front-row capillaries with a hairpin shape (C, D). The hairpin-capillary rate and fingertip Angpt-1 concentration in all cases are shown in Supplementary Fig. 1. In many cases, there appeared to be an association between the hairpin-capillary ratio and the concentration of Angpt-1 in the phalanges, whereas in others, such as case 20, there was no association between the two variables.

3.4. Adverse events

All adverse events observed in this study are listed in Supplementary Table S1. The events related to heating were skin redness (one case), burns (three cases), and itching (one case). Two patients with burns were treated with topical medications, and both recovered.

Table 2
Numbers of front-row capillaries.

No.	Preheating period	Heating period	Post-heating period
1	14.5	**17**	15.5
2	**14.5**	13	13.5
3	15	**19**	16.5
4	16.5	19.5	**22**
5	10	**11**	**11**
6	12	**15**	11.5
7	**18**	17.5	12.5
8	16.5	**18**	18.5
9	11	**14.5**	13
10	10	**12.5**	8
11	16.5	17.5	**18**
12	**8.5**	5.5	7
13	**19.5**	19	17.5
14	16.5	19	**20**
15	**23.5**	17	18
16	16.5	**17**	**17**
17	**13.5**	8.5	11
18	18	**19.5**	19
19	13.5	**14.5**	14

Bold numbers indicate the maximum values for each participant.

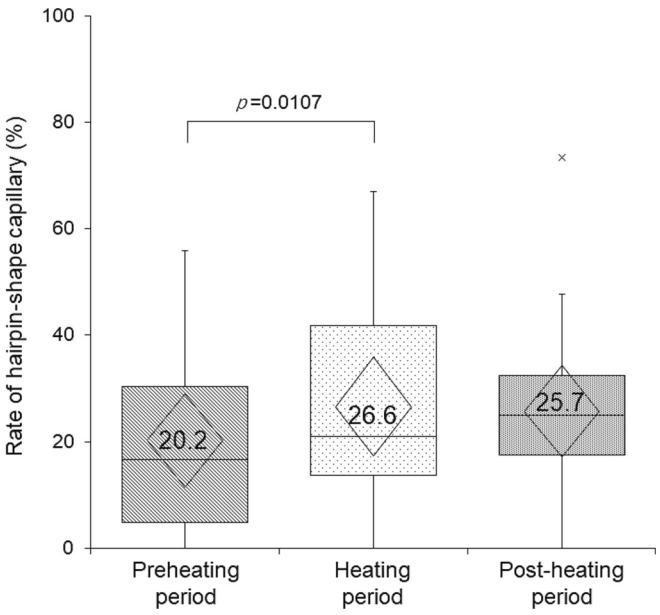

Fig. 3. The rate of hairpin-shaped capillaries
The vertical bars indicate the maximum and minimum values and the horizontal bars in the boxes indicate medians. The numbers in the boxes represent the average values for each period.

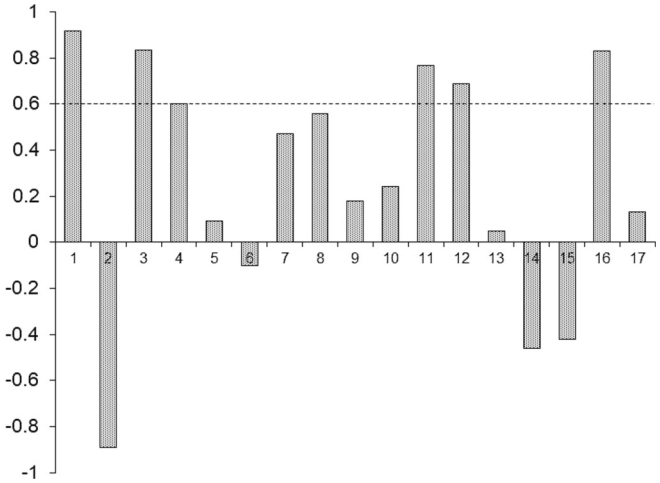

Fig. 4. Correlation coefficients between the concentration of angiopoietin-1 in the fingertip and the rate of hairpin-shaped capillaries
A total of 17 patients were included, and two patients, with two or more missing measurements, were excluded. The vertical axis indicates the correlation coefficients between the angiopoietin-1 data at four time points (days 28, 56, 84, and 112) and the rate of hairpin-shaped capillaries observed at the four time points.

4. Discussion

Raynaud's phenomenon is a common symptom in patients with SSc and is difficult to treat. The effects of calcium channel antagonists, particularly nifedipine, have been extensively investigated as a drug treatment for this phenomenon. However, according to a classic report by Corbin et al., nifedipine administration results in flushing in 52 % of patients, acroparesthesia in 35 %, and headache in 26 % (Corbin et al.,

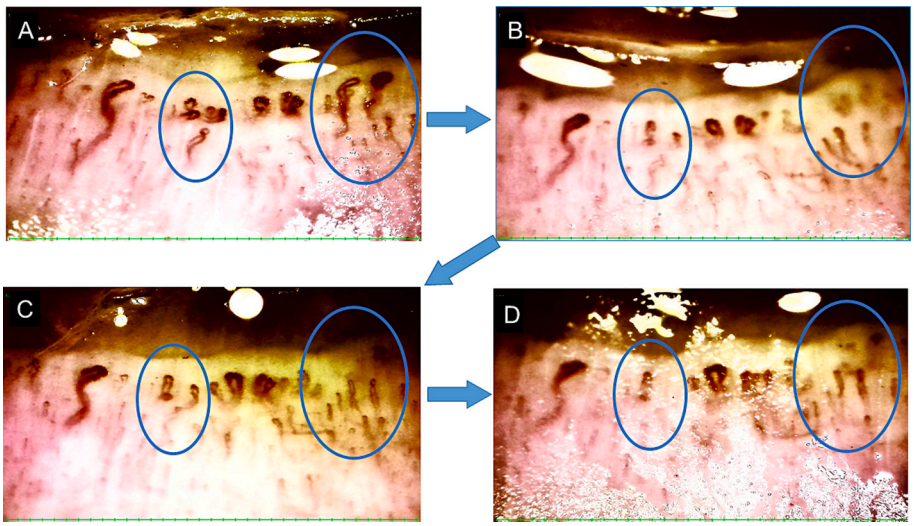

Fig. 5. Typical images of capillary changes
A: Preheating period (day 0). B: Heating for 1 month (day 56). C: Heating for 2 months (day 84). D: Post-heating period (day 112).

1986); side effects occurred in over 60 % of cases, making long-term adherence to nifedipine challenging. The effects of prostanoids, especially iloprost, on Raynaud's phenomenon have also been investigated; however, Pakozdi et al. reported that iloprost inhalation causes headaches or nausea in 40 % of cases and dizziness and flushing in 20 % (Pakozdi et al., 2008). Phosphodiesterase-5 inhibitors, particularly sildenafil, are also candidates for the treatment of RP; however, in studies using sildenafil, discontinuation due to headaches, facial flushing, and nausea have been reported, as well as other drugs with vasodilatory effects (Fries et al., 2005; Costa et al., 2024). Therefore, managing drug treatment for Raynaud's phenomenon requires patience.

We have previously reported that heating the elbow instead of drugs can alleviate Raynaud's phenomenon, and we found that this also increases Angpt-1 in the fingertips (Shima et al., 2022). The results of the present study confirmed that heating increased the concentration of Angpt-1 in the fingertips. Some cases showed a rapid increase in Angpt-1 with heating, whereas others showed increases after 2 months, although the cause of the difference between the two groups is unknown. The group in which Angpt-1 levels increased early after the start of heating had a higher incidence of fingertip ulcers. When the elbows of patients with SSc are heated, the alleviation of Raynaud's phenomenon is more pronounced in those with greater capillaroscopy impairment (Shima et al., 2024), which may be related to the early increase in Angpt-1 observed in patients with positive digital ulcers. In some cases, Angpt-1 peaks are observed during final blood sampling. It is unclear to what extent this increase in Angpt-1 persists in the present study because no fingertip blood samples were taken after four blood tests to minimize the number of samples taken from the fingertip. Although the concentration of Angpt-1 at the fingertip was expected to decrease at some point after the termination of heating, the present results show that this effect persists for some time after the termination of heating. Most of the patients received some drugs. The possibility that the drugs used affected the results cannot be excluded. The effect of immunosuppressants on Angpt-1 may also be considered, but the number of users was small, making it impossible to conduct a comparative study.

The rate of hairpin-shaped capillaries increased in this study, which we considered to be an effect of the intervention. Although skin lesions were not examined in this study, it has been reported that the degree of capillary damage observed by capillaroscopy is related to the degree of skin lesions (Ruaro et al., 2019); therefore, it is important to reduce capillary damage. However, in some cases, fluctuations in Angpt-1 concentration did not correspond to the ratio of hairpin capillaries. In addition to Angpt-1, various other factors influence capillary metabolism. For example, vascular endothelial growth factor (VEGF) has been reported to be elevated in diffuse cutaneous SSc (Distler et al., 2002). Endostatin, an anti-VEGF factor, has also been reported to be elevated in patients with SSc who have widespread skin sclerosis (Hebbar et al., 2000). Abnormal values of angiopoietin-2 (Angpt-2), an Angpt-1 antagonist, have similarly been reported (Michalska-Jakubus et al., 2011). Capillary metabolic factors other than Angpt-1 are likely involved in the hairpin-capillary ratio.

This study has some limitations. First, capillaroscopy was only performed on the left ring finger. The gold standard of capillaroscopy is the observation of eight fingers on both hands. Dinsdale et al. reported that the sensitivity against the diagnostic criteria was 74.6 % for the eight-finger observation and 46.6 % for the left ring finger single observation (Dinsdale et al., 2019). However, the purpose of this study was not to detect abnormal blood vessels but to observe fixed-point abnormal blood vessels that had already been detected. Observation of the Angpt-1 effect on the meandering capillary in SSc requires matching the observation finger with the blood collection finger, but it is not realistic to conduct blood collection from eight fingertips. Second, we focused on Angpt-1 in this study, and there were no data on other factors involved in capillary metabolism. As mentioned earlier, Angpt-2 binds to the Tie2 receptor and antagonizes Angpt-1. An increase or decrease in Angpt-2 expression may have a significant effect on the function of Angpt-1. As the amount of blood collected from the fingertip was very small, it was difficult to measure multiple factors in the same sample. Although the sample volume was very small, it is expected that this problem can be solved using a panel measuring device. Third, only hairpin-shaped capillaries were selected for capillaroscopy tests. In this study, only capillaries with a hairpin loop morphology were selected to examine the function of Angpt-1 as an anti-tortuous factor; however, according to Smith et al. (2016), tortuous shapes (afferent and efferent limbs bend but do not cross), one-crossing shapes, and two-crossing shapes are normal variations. Therefore, capillaroscopy may have led to overestimation in the present study. However, since the hairpin shape is thought to best represent the physiological action of Angpt-1, and it remains clear whether the distortion of the tortuous or crossing capillaries is influenced by Angpt-1 or Angpt-2, only hairpin capillaries were selected for this study. Fourth, while capillaroscopy allowed us to observe that heat stimulation altered capillary morphology, we could not entirely eliminate observer subjectivity in determining these morphological changes. Soluble form suppression of tumorigenicity-2 (sST2), soluble form receptor of interleukin-33 (Pellicano et al., 2022), asymmetric dimethylarginine (ADMA), an endogenous nitric oxide

inhibitor (Pagkopoulou et al., 2023), and Uric acid (UA) (Pagkopoulou et al., 2021) have been reported as markers of capillary involvements. Further research is needed to determine whether these markers are associated with changes in Angpt-1 concentration at the fingertip. The fifth limitation concerns the sample size. Since this study was conducted at a single facility, the sample size was rather small. However, because it was designed as a pre- and post-heating comparison study, rather than a parallel group comparison, it was expected that overall trends could be observed even with a smaller group. However, considering the presence of various confounding factors such as concomitant medications, a larger-scale study is warranted.

5. Conclusion

Wearing a heating pad on the elbow does not cover the hand; therefore, it does not interfere with daily activities. It is also possible to maintain the pad warm on a daily basis, which is expected to alleviate and prevent Raynaud's phenomenon. If repeated heating improves capillary morphology, as demonstrated in this study, it is expected that not only will Raynaud's phenomenon be alleviated while the pad is worn, but its occurrence will also become less likely over time.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mvr.2024.104761.

CRediT authorship contribution statement

Yoshihito Shima: Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation, Conceptualization. **Akane Watanabe:** Investigation, Formal analysis. **Nobuto Inoue:** Resources. **Tetsuya Maruyama:** Resources. **Eiji Kunitomo:** Supervision. **Yuji Matsushima:** Project administration, Funding acquisition. **Atsushi Kumanogoh:** Project administration.

Funding

This research was supported by the joint research of Osaka University Graduate School of Medicine and Kobayashi Pharmaceutical Co., Ltd.

Declaration of competing interest

Research expenses and labor costs for Y.S. and A.W. were provided by Kobayashi Pharmaceutical Co., Ltd. Additionally, N.I., T.M., E.K., and Y.M. received support from Kobayashi Pharmaceutical Co., Ltd. A.K. declares no conflicts of interest regarding this paper.

Acknowledgments

We thank Mrs. Akiko Taguchi and Mrs. Mika Yoneo for their assistance and Dr. Ikuko Ueda for the patient recruitment. We also thank Editage (www.editage.jp) for English language editing.

Data availability

The data that has been used is confidential.

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