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ORIGINAL ARTICLE

Exogenous Local Hyperthermia at 41°C Is Effective to Eliminate Mouse Model of Sporotrichosis, Independent of Neutrophil Extracellular Traps Formation

Ya-Lan Wang, Rui-Qun Qi, Jing Lan, Zheng-Xiu Li, Xing-Hua Gao

Department of Dermatology, The First Hospital of China Medical University, Shenyang, China

Background: Local hyperthermia is recommended for the treatment of patients with fixed cutaneous sporotrichosis, though the effectiveness and mechanisms of action remain elusive. While neutrophils represent the main inflammatory cells associated with sporotrichosis lesions, the issue of whether hyperthermia is involved with interactions between neutrophils and *Sporothrix globosa* remains unclear. **Objective:** To evaluate the effect of local hyperthermia on sporotrichosis and determine whether local hyperthermia involves effects of neutrophils against *Sporothrix*. **Methods:** For the *in vivo* study, mice were infected with yeast cells of *S. globosa* followed by treatment with local hyperthermia. *In vitro*, an isolated *Sporothrix* strain was co-cultured with or without neutrophils and subjected under different temperatures. Immunofluorescence was used to assess the formation of neutrophil extracellular trap (NETs) were formed under these different culture conditions and the number of fungi colony forming units were compared. **Results:** Hyperthermia was significantly more effective in clearing the lesions in the mouse model, as compared to sham treatment. Neutrophils failed to exert any fungicidal effects against *S. globosa* in response to hyperthermia. Moreover, NETs were formed after interaction with *S. globosa*, and the percentage of NETs formed was not significantly different at 41°C or 37°C. **Conclusion:** While hyperthermia could serve as an effective therapy for fixed cutaneous sporotrichosis, this ability does not involve the formation of NETs. **(Ann Dermatol 33(1) 37～45, 2021)**

-Keywords-
Extracellular traps, Hyperthermia, Induced, *Sporothrix*, Sporotrichosis

INTRODUCTION

Sporotrichosis is a common mycosis, primarily resulting from infection by *Sporothrix globosa*. This condition is quite prevalent in Northeast China due to contact with reeds or cornstalks[1]. It manifests in a variety of clinical forms, with fixed cutaneous sporotrichosis and lymphocutaneous sporotrichosis representing the most common types[2]. Local hyperthermia is preferred for treating patients with fixed cutaneous sporotrichosis who cannot safely receive other regimens, such as pregnant and nursing women[3].

Despite the recommendations for use, and apparent effectiveness of, local hyperthermia for the treatment of fixed cutaneous sporotrichosis and lymphocutaneous sporotrichosis, no randomized placebo-controlled trials or comparisons with other treatments are available to enable any assessment of the validity and reliability of this procedure. Moreover, as there exist some case reports of spontaneous cures for this condition in the absence of any therapy[2,4], more evidence is required to determine whether or not local hyperthermia is effective.

Pathologically, in patients with sporotrichosis there is a mixed suppurative and granulomatous inflammatory re-

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Corresponding author: Xing-Hua Gao, Department of Dermatology, The First Hospital of China Medical University, 155N Nanjing Street, Heping District, Shenyang 110001, China. Tel: 86-2483282525, Fax: 86-2483282525, E-mail: gaobarry@hotmail.com
ORCID: https://orcid.org/0000-0001-8809-8564

action in the dermis and subcutaneous tissue, which is frequently accompanied by microabscess and fibrosis[5]. Neutrophils represent one of the main infiltrating cells in this condition[6], and that they may form neutrophil extracellular traps (NETs)[7]. NETs are chromatin expulsed from neutrophils decorated with nuclear and cytosolic components[8]. A number of fungi, including *Aspergillus nidulans*, *Candida albicans*, *Candida dubliniensis*, and *Cryptococcus neoformans* were susceptible to NETs[9]. It remains unclear as to whether NETs exert antifungal effects against *S. globosa*. Therefore, given that local hyperthermia has been reported to be effective in treating sporotrichosis[10,11], and there is evidence suggesting a role for neutrophils as described above, the purpose of this report was to assess whether neutrophils play a role in disease recovery of sporotrichosis after hyperthermia.

MATERIALS AND METHODS

Fungal isolates and growth conditions

One isolate strain was harvested from a patient with fixed cutaneous sporotrichosis and identified by nuclear ribosomal internal transcribed spacer region sequencing. The isolated strain was grown on an Emmons' modification of Sabouraud's agar plate. After fourteen days, the hyphae form of fungus was collected by sterile swabs, and transferred to brain heart infusion agar medium at 35°C for fourteen days. The yeast cells were suspended in sterile phosphate buffer saline (PBS), adjusted with hemocytometry and then diluted in RPMI 1640 medium without phenol red (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (BI; Montevideo, Uruguay) and 1% penicillin/streptomycin (BI; Kibbutz Beit Haemek, Israel) or PBS to obtain the required concentrations.

Neutrophil isolation

Blood samples were drawn from 10 healthy donor volunteers upon fully informed consents according to the principles stated in the Declaration of Helsinki and instructions of Medical ethics committee, the First Affiliated Hospital of China Medical University (no. 2019-246-2). Human neutrophils were isolated from venous blood as previously described[12]. Briefly, neutrophils were isolated by density gradient centrifugation (Histopaque 1119 and 1077; Sigma-Aldrich, St. Louis, MO, USA). Purified neutrophils were resuspended in RPMI 1640 medium. Quantification was performed with use of a hemocytometer and viability of polymorphonuclear leukocytes (PMNs) were determined using the trypan blue exclusion test. Only neutrophil suspensions with viabilities greater than 95% were used in these experiments.

NETs visualization

Neutrophil suspensions (0.5 ml) were seeded at a density of 4×10^5 cells/ml in 24-well plates on poly-L-lysine coated coverslips. These suspensions were seeded in either 0.2 ml yeast cell suspensions at a final density of 2×10^5 cells/ml or 0.2 ml phorbol myristate acetate (PMA; MCE, Monmouth Junction, NJ, USA) at a final concentration of 25nM or in 0.2 ml RPMI 1640 medium. One 24-well plate was then placed in a water bath at a temperature of 41°C, while a second 24-well plate was placed in the water bath at a temperature of 37°C. After 30 minutes, two 24-well plates were placed in a cell incubator at 37°C in 5% CO_2 atmosphere for 4 hours. The cells were then fixed and permeabilized, followed by blocking with 1% bull serum albumin (BSA; Solarbio, Beijing, China). Next, cells were incubated with anti-human neutrophil elastase antibody (R&D Systems, Minneapolis, MN, USA), and detected with Alexa Fluor 488 goat anti-mouse (CST, Boston, MA, USA), followed by staining with DAPI. Imaging data were acquired using a Live Cell Station (Bio-Tek, Winooski, VT, USA). The number of NETs and neutrophils were counted and the percentage of NETs formed was calculated. The experiment was replicated five times.

Sporothrix globosa viability assay

To examine possible effects of neutrophils on viability of yeast cells as determined at different temperatures, two 24-well plates were prepared. Yeast cells at a final density of 2×10^5 cells/ml were co-cultured in a water bath at 41°C or 37°C in the presence or absence of neutrophils at an identical final density. After 30 minutes, two plates were placed in a cell incubator at 37°C in 5% CO_2 atmosphere for 6 hours.

To explore the susceptibility of yeast cells to NETs, yeast cells were co-cultured with neutrophils, PMA or NETs which was induced by PMA at 37°C in 5% CO_2 atmosphere for 6 hours.

After 6 hours incubation period, each group was diluted with PBS, plated on Emmons' modification of Sabouraud's agar plates and aliquoted into three petri dishes. Colony forming units (CFU) were counted after fourteen days and the number of CFU was compared among the groups. The experiment was replicated five times.

Animals

Female BALB/c mice (8～10 weeks of age; body weight, 19～20 g) were purchased from Liaoning Changsheng Biotechnology Co. Ltd (Benxi, Liaoning, China). All treatments and maintenance of the mice were strictly performed in

accordance with approved institutional animal care and maintenance protocols. Protocols were approved by Animal Care and Use Committee, China Medical University (no. 2019170).

Skin infection and antifungal treatments

A 1×10^8 CFU/ml suspension of yeast cells in sterile PBS was prepared for infection injection of the mice. The right dorsal skin area was disinfected with 75% alcohol, followed by a 0.1 ml injection of either yeast cell suspensions or PBS. After one week, some of these mice were euthanized for skin removal, while the remaining mice were randomly divided into a hyperthermia or control group. Mice of hyperthermia group were anesthetized with isoflurane and subjected to a daily heating regimen with thermal bags for 30 minutes with temperatures ranging from 40°C～41°C. Mice within the control group were similarly anesthetized but received no heating treatments. Changes in appearance of rashes were observed and recorded weekly after treatment. Mice were euthanized for skin removal after one or three weeks of treatment.

Histology and immunohistochemistry of mouse tissue sections

Skin samples were fixed in 4% paraformaldehyde solution overnight and embedded in paraffin. For histology, tissue sections (3 μm) were mounted on glass slides. The tissue was stained with Periodic-Acid Schiff (PAS) reagent, counterstained with hematoxylin and then examined for pathological changes and fungal burden within the skin. Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA) was used to calculate the pixel area of the selected area in the abscess cavity. The number of yeast cells in the selected area was counted and the number of yeast cells per pixel area was calculated. To confirm the formation of NETs in abscess cavity, tissue sections on poly-l-lysine-coated glass slides were incubated with rat anti-Neutrophil Elastase antibody (Abcam, Cambridge, UK) overnight at 4°C. The EliVision Super Kit (Maixin, Fuzhou, China) was used for immunostaining. Images were acquired with cellSens Standard (OLYMPUS, Tokyo, Japan) and analyzed with Image-Pro Plus 6.0 (Media Cybernetics).

Statistics

Data were analyzed with use of SPSS 22 (IBM Corp., Armonk, NY, USA) and plotted by GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Supplementary Table 1 and Table 1 were generated with use of Office 2016 and data were presented as mean±standard deviation.

Results of differences in rash widths during treatment were analyzed using a repeated measures analysis of variance. Fisher's exact test was used to evaluate differences in cure rates between the two groups. The number of yeast cells/per pixel area of the two groups, as determined at the end of the third week of treatment, failed to show a normal distribution and was therefore analyzed using the nonparametric Mann–Whitney test, with data presented as median. The number of yeast cells/per pixel area of two groups in the first week was normally distributed and analyzed with use of independent-samples t-test, but data were also presented as median. Independent-samples t-test was used to evaluate the viability of *S. globosa* after different treatment *in vitro* and the difference in the percentage of NETs formed *in vitro*. A $p<0.05$ was required for results to be considered as statistically significant.

Table 1. Cure frequencies (cure rates) within mice of the hyperthermia and control groups after cessation of treatment

Time (wk)	Hyperthermia group	Control group
1	11 (73.3)	5 (33.3)
2	14 (93.3)	6 (40.0)
3	15 (100)	8 (53.3)
4	15 (100)	11 (73.3)

Values are presented as number (%). There were fifteen mice in the hyperthermia group, fifteen mice in the control group.

RESULTS

Morphology of mycelia and yeast

One isolate of *S. globosa* was obtained from a patient's fixed sporotrichosis lesion. After 5 days of inoculation at 25°C within modified Sabouraud's agar medium, brown colonies with wrinkled and folded surfaces were present (Supplementary Fig. 1A). When transferred to the brain heart infusion agar and cultured for 7 days at 35°C, creamy white-to-tan yeast colonies were observed (Supplementary Fig. 1B).

Animal infection

All mice in the two experimental groups survived and showed no significant weight loss. After injection of the yeast cell suspension, a nodule gradually developed at the injection site, while no rashes were seen in mice injected with PBS. Within mice receiving the yeast cell suspension rash width decreased from 0.75±0.14 cm to 0.32±0.19 cm in response to 4 weeks of hyperthermia treatment. Corresponding changes in rash width within the control group were 0.67±0.15 cm to 0.40±0.16 cm (Supplementary Table 1, Fig. 1). When calculating the value of differences in the rash width (Difference=rash width before treatment–rash

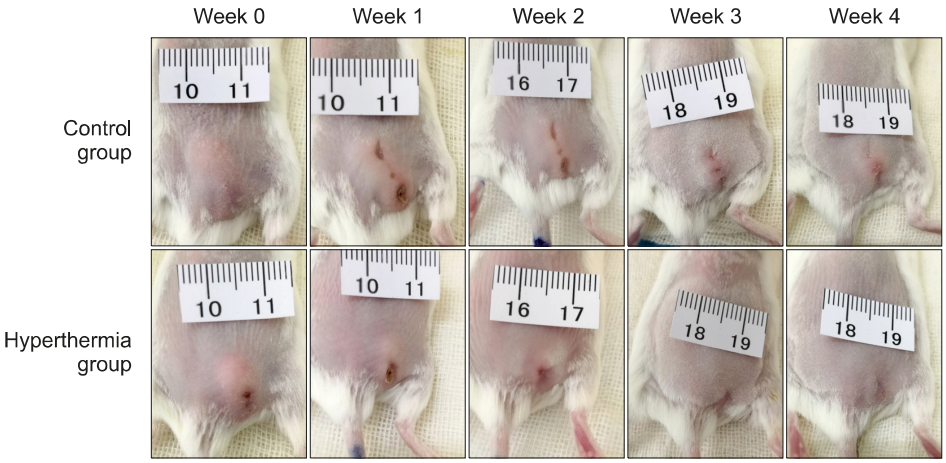

Fig. 1. Photos of infected mouse models in response to hyperthermia.

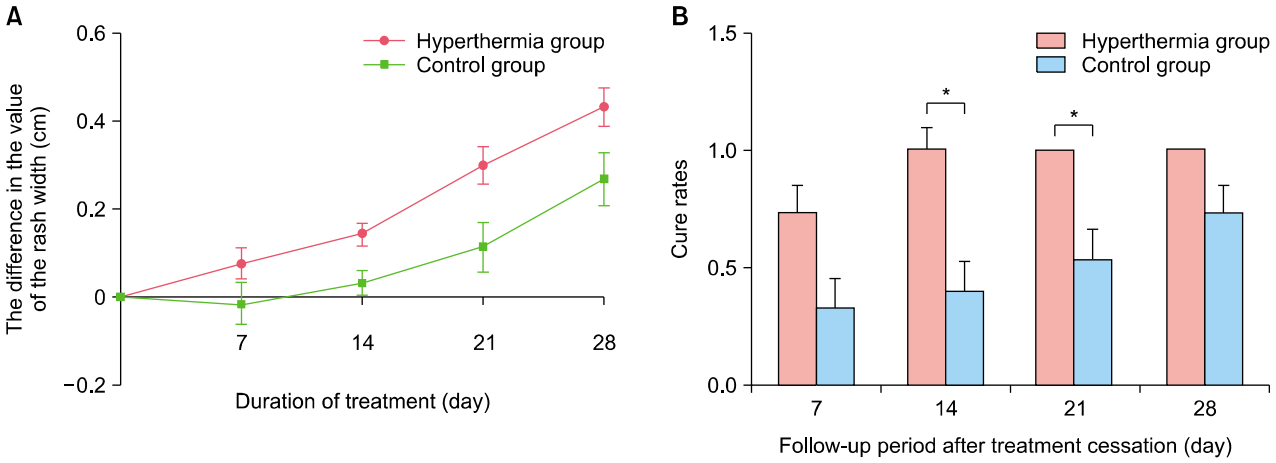

Fig. 2. Changes in the rash appearance in response to different treatments. Data were presented as means±standard error of the mean. (A) Rash width changes during treatment in both hyperthermia and control group of mice expressed as the difference in the value of the rash width (rash width before treatment−rash width during treatment). Significant differences in rash width were obtained as a function of treatment times in both the hyperthermia and the control groups. F (2.257, 63.200)=72.206, p=0.000. Differences in the value of rash width were significantly different at each time point during the treatment period within the hyperthermia versus control group. F (1,28)=9.583, p=0.004. (B) Recovery rate of rashes in the different treatment groups during the follow-up period after treatment cessation (*p<0.05).

width during treatment), these differences were found to significantly increase over this 4-week period of the experiment within both groups (p<0.05; Fig. 2A). In addition, these differences in the value of rash width were significantly different at each time point during the treatment period within the hyperthermia versus control group (p<0.05; Fig. 2A), mice in the hyperthermia group had a greater reduction in rash width than those in the control group. Similarly, cure rates (the number of mice with complete clearance of lesions by the number of mice) increased in both groups during the follow-up period after treatment cessation, with the hyperthermia group showing overall more rapid cure rates. Cure rates in the control versus hyperthermia groups were 33.3% vs. 73.3% (p>0.05), 40% vs. 93.3% (p<0.05), 53.3% vs. 100% (p<0.05), respectively, at the end of the first, second, and third week after treatment cessation. At the end of the fourth week, all mice in the hyperthermia group remained cured as compared with 73.3% in the control group (p>0.05; Table 1, Fig. 2B).

Number of fungi from infection sites

Yeast cells were limited to the abscess cavity in all mice of both groups. The median of yeast cell count/per pixel area was 31.94×10^{-6} yeast cells per pixel area in the abscess cavity before treatment (Fig. 3A) and this number decreased over the duration of the experiment (Fig. 3F). However, no statistically significant differences were present between the control and hyperthermia groups (p>0.05; Fig. 3F).

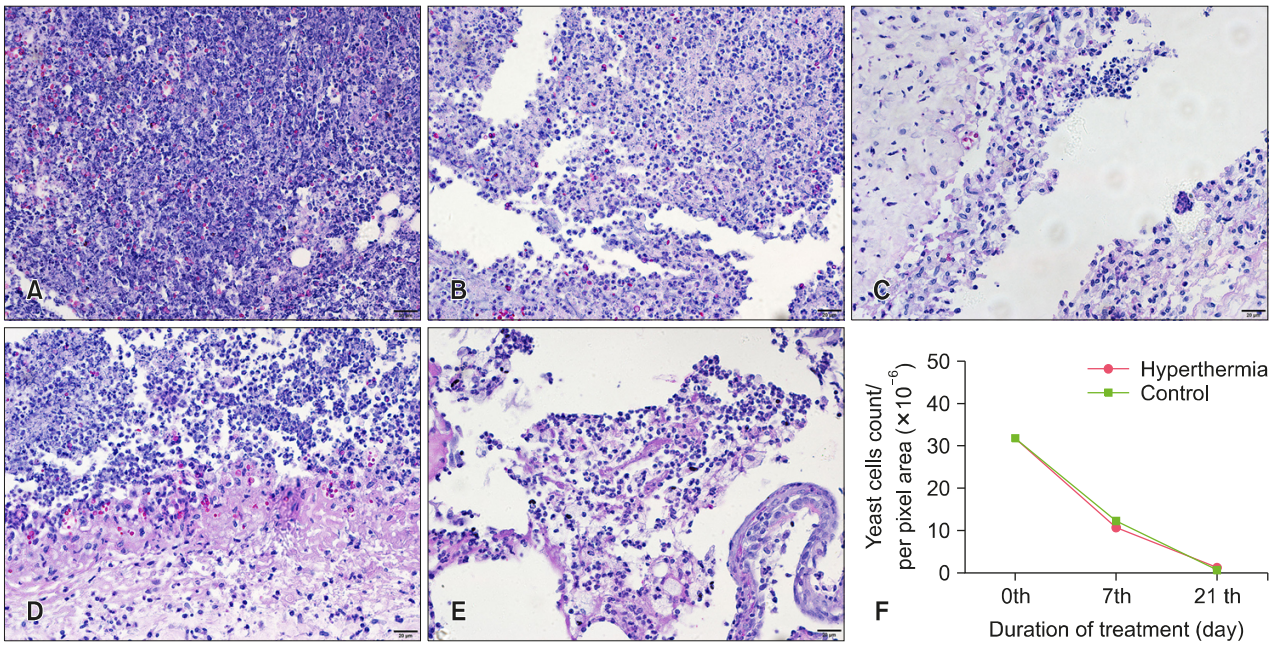

Fig. 3. Changes in number of yeast cells within tissue as a function of treatment/duration. (A∼E) Histopathological analysis in mice stained with Periodic-Acid Schiff (PAS). Round and oval red yest cells can be seen in the lesions stained with PAS. Original magnification: 400× (scale bar=20 μm). (A∼C) Pathological sections of the control group: (A) before treatment, (B) after one week, and (C) after three weeks. (D, E) Pathological sections of the hyperthermia group: (D) after one week and (E) after three weeks of treatment. (F) Number of yeast cells/per pixel area ($\times 10^{-6}$) within abscess cavity. An independent-samples t-test was used to compare the number of yeast cells between the two groups after one week of treatment ($t=-0.768$, $p=0.454$). The nonparametric Mann-Whitney U-test was used to compare the number of yeast cells between the two groups after three weeks of treatment ($Z=-0.180$, $p=0.857$). There were nine mice in each group. Values are presented as median.

The median of yeast cells count/per pixel area in the control versus hyperthermia groups were 12.35×10^{-6} (Fig. 3B) vs. 10.8×10^{-6} (Fig. 3D) after a week of treatment and 0.78×10^{-6} (Fig. 3C) vs. 1.25×10^{-6} (Fig. 3E) after three weeks of treatment. After three weeks of treatment, neutrophils, macrophages, lymphocytes were observed in the lesions of a third of the mice in both the hyperthermia and control group following PAS staining, but no fungus was found in the lesions.

Formation of NETs

Histopathologically, we examined elastase, a critical cationic protein in neutrophils to confirm the web-like structures were NETs. A large number of neutrophils infiltrated the subdermis and formed abscess cavities in both the hyperthermia and control groups. The formation of NETs, which appeared as web-like structures of extracellular DNA (blue) and neutrophil elastase (rust) co-localizations in these structures, was similar within abscess cavities of mice in both the hyperthermia (Fig. 4A, B) and control (Fig. 4C, D) groups. After co-culture with yeast cells, the web-like structures observed were similar to those induced by PMA (Fig. 4E), but the percentage of NETs formed was not significantly different after co-culture with yeast cells at 41°C (mean±standard error of the mean [SEM]: 16.29%±2.806%; Fig. 4F) or 37°C (mean±SEM, 15.55%±1.952%; Fig. 4G).

Effect of neutrophils and temperature on *Sporothrix globosa* viability

Yeast cells were co-cultured with or without neutrophils at 41°C for 30 minutes, then transferred to 37°C and continued in culture for 6 hours. No statistically significant differences in CFU were obtained between the two groups ($p>0.05$; Fig. 5A). Moreover, when yeast cells were co-cultured with neutrophils at 41°C versus 37°C for 30 minutes, followed by transfer to 37°C and continued in culture for 6 hours, no differences in CFU were found between the two groups ($p>0.05$; Fig. 5B). Nor were significant differences present in CFU between the two groups, after yeast cells were cultured at 41°C versus 37°C for 6.5 hours ($p>0.05$; Fig. 5C). We also compared the number of yeast cells cultured at 37°C for 6.5 hour in the presence or absence of neutrophils, and found no significant differences between these two groups ($p>0.05$; Fig. 5D). When examining the effects of neutrophils or NETs induced by PMA on yeast cell viability, no significant differences were

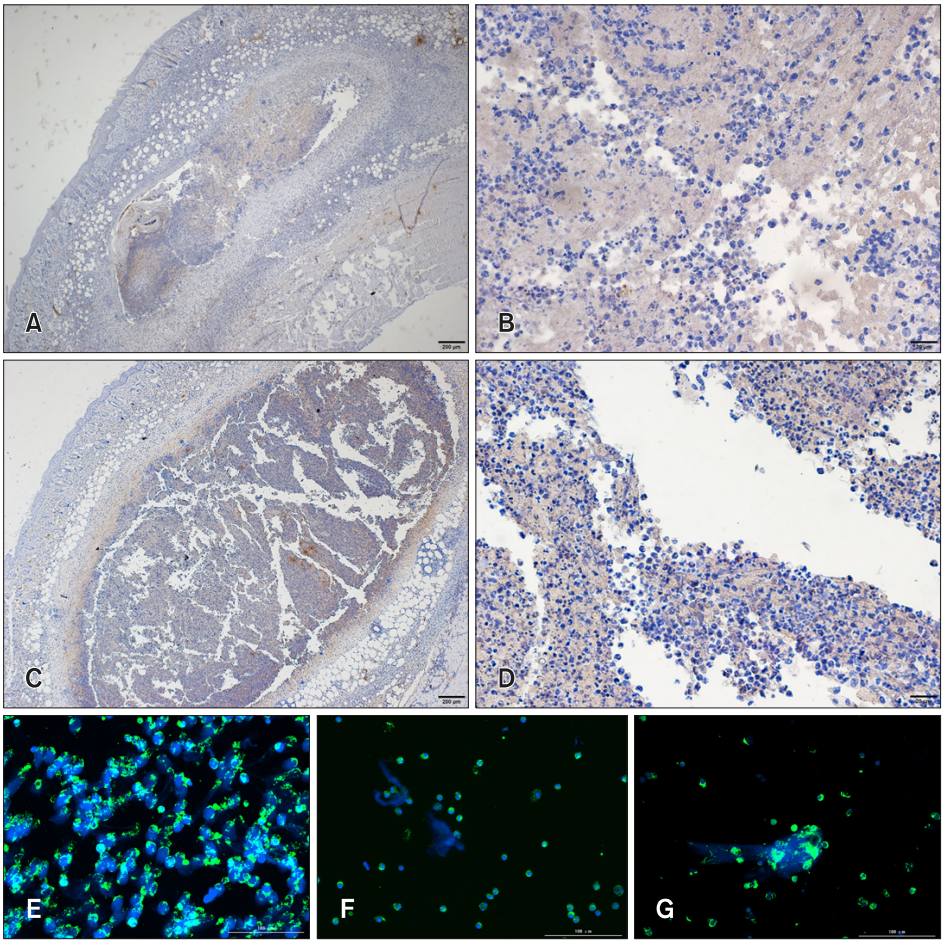

Fig. 4. Formation of neutrophil extracellular traps (NETs). (A~D) Demonstration of NETs by immunohistochemistry. (A, B) Hyperthermia group, after one week. (A) Original magnification: 40× (scale bar=200 μm). (B) Original magnification: 400× (scale bar=20 μm). (C, D) Control group, after one week. (C) Original magnification: 40× (scale bar=200 μm). (D) Original magnification: 400× (scale bar=20 μm). (E~G) Formation of NETs by immunofluorescence after stimulation with (E) phorbol myristate acetate, (F) yeast cells of *Sporothrix globosa* at 41°C or (G) yeast cells of *S. globosa* at 37°C.

obtained between two groups ($p>0.05$; Fig. 5E). Finally, we investigated the effects of PMA on yeast cell viability in the presence or absence of neutrophils and found no significant differences ($p>0.05$; Fig. 5F).

DISCUSSION

In general, hyperthermia has long been considered as an effective treatment for sporotrichosis[3]. However, this conclusion has been based on only a few case reports[10,11,13], and no randomized placebo-controlled trials or comparisons with other treatments have been conducted to assess the validity and reliability these local hyperthermia effects on sporotrichosis. Moreover, the findings that sporotrichosis can experience spontaneous cures[2,4], highlights the need for performing controlled experiments to evaluate these hyperthermia effects. Indeed, here we also found spontaneous cures of sporotrichosis in our *in vivo* model. However, in the controlled experiments performed within this report we do find that hyperthermia is clearly effective in enhancing the cure of this condition as indicated in a number of parameters related to the cure of sporotrichosis.

The exact mechanisms involved with the beneficial effects of hyperthermia remain elusive. Notably, the number of yeast cells in abscess cavities were not significantly different between the hyperthermia and control group and our *in vitro* experiments also demonstrated that the number of CFU in the presence or absence of neutrophils at 41°C or 37°C showed no significant differences. Nonetheless, the number of yeast cells in these lesions did decrease over time in both groups. Based upon these findings, it can be concluded that hyperthermia does not contribute to fungi elimination via neutrophils. Besides, after three weeks of treatment, neutrophils, macrophages, lymphocytes were observed in the lesions of a third of the mice in both the hyperthermia and control group following PAS staining, but no fungus was found in the lesions. Li et al.[14] reported that the lesions and secretions from the itraconazole and Photodynamic therapy group were negative results for yeast cells, albeit a limited pus-like inflammatory focus could still be observed. It would appear that hyperthermia plays a role in reducing inflammation in the body to promote rash resolution. Li et al.[15] reported that the secretion of interleukin (IL)-8 in keratinocytes were up-regulated by yeast

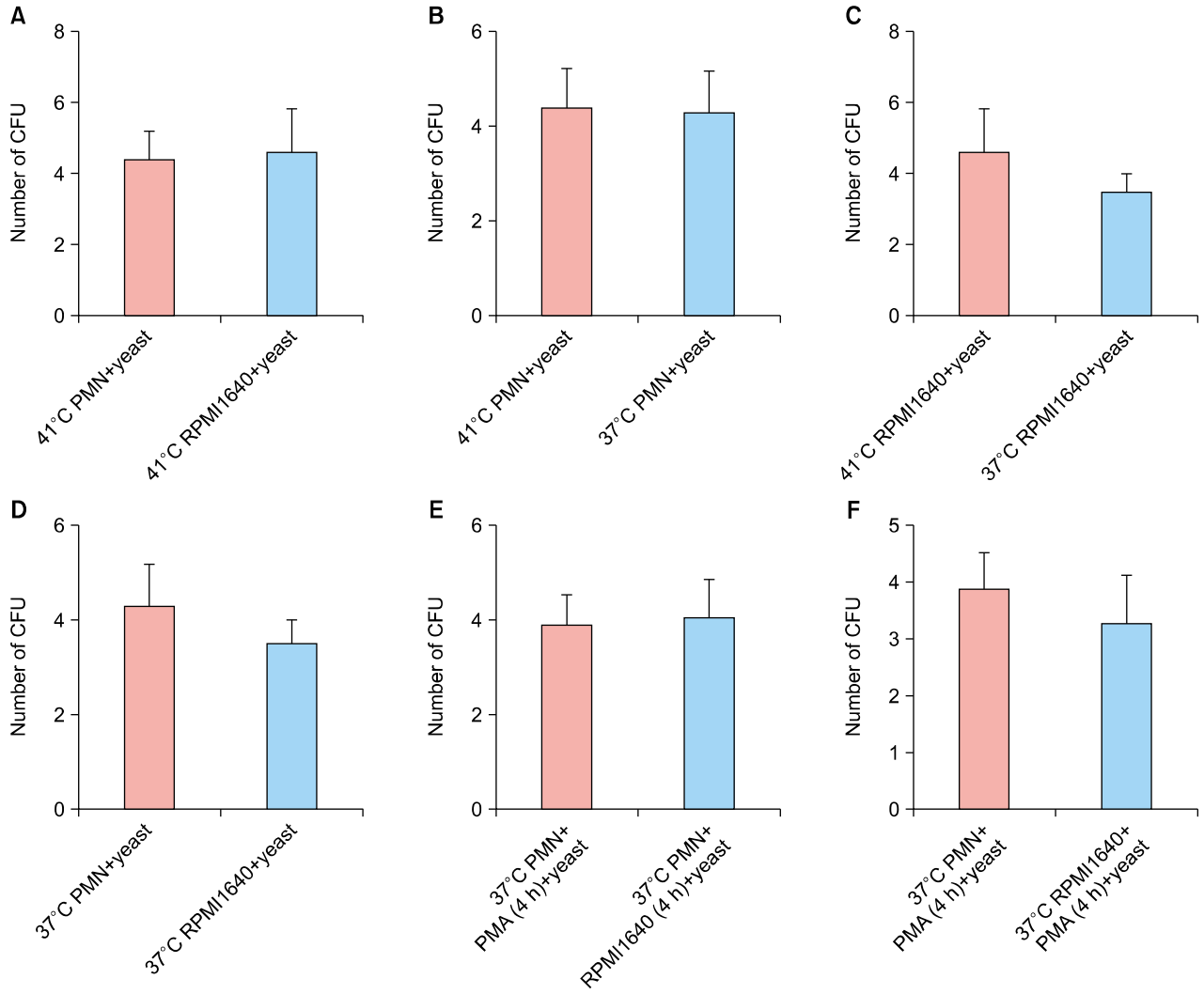

Fig. 5. Number of CFU in response to the different treatments. Values are presented as means±standard error of the mean. (A) Number of CFU in the presence or absence of neutrophils at 41°C ($t=-0.145$, $p=0.888$). (B) Number of CFU in the presence of neutrophils at 41°C or 37°C ($t=0.09$, $p=0.931$). (C) Number of CFU in the absence of neutrophils at 41°C or 37°C ($t=-0.819$, $p=0.436$). (D) Number of CFU in the presence of neutrophils or absence of neutrophils at 37°C ($t=-0.746$, $p=0.477$). (E) Number of CFU after yeast cells were co-cultured with neutrophils or neutrophil extracellular traps induced by PMA ($t=-0.144$, $p=0.889$). (F) Number of CFU after yeast cells were co-cultured with PMA in the presence or absence of neutrophils ($t=0.577$, $p=0.58$). CFU: colony forming units, PMA: phorbol myristate acetate.

of *Sporothrix schenckii*. IL-8 is an important chemokine that recruit and prime neutrophils during inflammation. Mice that were exposed to 40°C for 1 hour showed strongly reduced IL-8-induced neutrophilic skin inflammation[16]. Moreover, heat treatment can induce 3D-like cell proliferation of normal human fibroblast[17], and improve wound healing[18]. In summary, these results indicate that hyperthermia functions by ameliorating the inflammation and promoting tissue repair.

Curtiellas-Piñol et al.[19] reported that *S. schenckii* viability did not decrease appreciably after 2 hours of interaction with neutrophils, which is consistent with our *in vitro* results. In contrast, a significant increase in the killing rate of neutrophils at 40°C, but no effect of hyperthermia on the phagocytosis rates, have also been reported[20]. This inconsistency may be related to the duration of the treatments. For example, in the studies described above, the killing rate potential of neutrophils was tested at 40°C for 2 or 4 hours, whereas in our study, PMNs interacted with yeast cells at 41°C for only 30 minutes followed by a return to culture at 37°C for 6 hours. In this way, the actual duration of hyperthermia exposure was quite limited in our study. However, we also observed no differences in spore numbers between the hyperthermia and control groups as

tested *in vivo* and when we examined the viability of yeast cells cultured at 41°C or 37°C in the absence of neutrophils, we also found no differences. There are reports indicating that growth of all isolates was inhibited at 40°C[21] and some species even failed to grow at 37°C[1,22]. Such findings are similar to our current results and suggest that hyperthermia can inhibit the growth of yeast cells, but not eliminate fungi.

It had been reported that NET formations were observed in some lesions of fixed cutaneous sporotrichosis[7]. Formation of NETs has been observed under conditions where neutrophils were co-cultured *in vitro* with yeast cells of *S. schenckii*, but such morphological deformations occurred at very low frequencies of $<2\%$[19]. Similarly, we found that NETs were formed in both the hyperthermia and control groups as shown *in vivo*, and confirmed *in vitro*. *Aspergillus fumigatus*[23,24], *A. nidulans*[25], and *C. albicans*[26] have all been reported to induce the formation NETs, but only *A. nidulans* and *C. albicans* were reported to be susceptible to NETs, which suggests that not all fungi are responsive to NETs. When examining the effects of NETs on *S. globosa* we found that there were no differences in yeast cell viability when these cells were co-cultured with neutrophils, PMA or NETs induced by PMA. Taken together, it seems that NETs lack the ability to kill the yeast cells. In contrast to our results, Kajiwara et al.[27] reported that systemic infections and a more rapid onset of death were observed in mice with chronic granulomatous disease as compared with wild-type mice after subcutaneous injection of *S. schenckii*. Such results indicate that neutrophils play an important role in the evolution of sporotrichosis[27]. Moreover, calprotectin, a component of neutrophils, also plays an important role against *Candida* spp, *A. nidulans* and *C. neoformans*[25,28,29]. However, this effectiveness of calprotectin *in vivo*, involves concentrations of 1～20 mg/ml in abscess fluid from human abdominal abscess, which represents a very high concentration[30]; and, *A. nidulans* hyphae were not killed by NETs using low concentrations of calprotectin *in vitro*[25]. Taken together, these results indicate that neutrophils can clear fungus in a concentration-dependent manner, but this capacity requires relatively high levels to be effective. In our experiment, the concentrations of neutrophil or NETs as tested *in vitro* were apparently too low to exert any effects upon the viability of yeast cells. A large number of NETs were observed within abscess cavities of mice in both the hyperthermia and control groups, and it was confirmed that hyperthermia had no effect on the formation of NETs *in vitro*. Taken together, it can be concluded that hyperthermia does not contribute to fungi elimination via NETs formation.

In conclusion, we demonstrate that fixed cutaneous sporotrichosis can be cured spontaneously, but hyperthermia promotes an earlier onset and more complete recovery of this condition. Although hyperthermia serves as an effective therapy for fixed cutaneous sporotrichosis, this ability does not involve any fungicidal effects of NETs.

SUPPLEMENTARY MATERIALS

Supplementary data can be found via http://anndermatol.org/src/sm/ad-33-037-s001.pdf.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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DATA SHARING STATEMENT

Research data are not shared.

ORCID

Ya-Lan Wang, https://orcid.org/0000-0001-9534-2078
Rui-Qun Qi, https://orcid.org/0000-0002-5856-4584
Jing Lan, https://orcid.org/0000-0001-5002-4762
Zheng-Xiu Li, https://orcid.org/0000-0001-8898-1293
Xing-Hua Gao, https://orcid.org/0000-0001-8809-8564

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