

Original article

Temporal effect of local hyperthermia on murine contact hypersensitivity

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Keywords: fever; dermatitis, contact; Langerhans cells; immunization

Background The sensitization and elicitation phases are involved in the immunopathogenesis of contact hypersensitivity (CHS). Langerhans cells (LCs) are believed to play pivotal roles in the sensitization stage of CHS. Local hyperthermia on skin induces the migration as well as maturation of epidermal LCs. Although fever-range whole body hyperthermia and local hyperthermia at 43°C prior to sensitization were reported to suppress CHS, the effects of different temperatures and the timing sequence of local hyperthermia on CHS have not been tackled.

Methods Local hyperthermia was applied to murine dorsal skin 3 days prior to, concurrent with, or 2 days post sensitization with fluorescein isothiocyanate (FITC) in BALB/c mice. Local hyperthermia temperatures at 37°C, 39°C, 41°C and 43°C were applied to mouse dorsal skin and the severity of CHS was calculated by measuring the swelling response of the challenged ears.

Results Local hyperthermia at 39°C, 41°C and 43°C prior to sensitization reduced the severity of CHS, as compared with that at 37°C. The suppression of CHS was temperature dependant in that higher temperature had a stronger effect. On the contrary, the hyperthermia treatments, either concurrent with or post-sensitization, resulted in an enhanced temperature-dependant ear swelling response.

Conclusions The severity of murine CHS could be influenced by local hyperthermia at the sensitization stage in a temperature dependant manner. The temporal effect of local hyperthermia suggested a novel factor in interpreting the severity of allergic contact dermatitis.

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The classical paradigm of contact hypersensitivity (CHS) requires two spatially and temporally dissociated phases, e.g. the sensitization and elicitation. Langerhans cells (LCs) are believed to play pivotal roles in the sensitization phase by taking up, processing, and presenting the antigen to naive T cells in the regional lymph nodes. The process of antigen presentation by LCs is accompanied by its migration from epidermis to the regional lymph nodes via the afferent lymphatics. Epidermal LCs are liable to extrinsic stimuli. For example, the number of epidermal LCs was reduced by ultraviolet (UV)-irradiation, topical steroid, tape stripping and even stress.¹⁻⁴ When mice were sensitized under such preconditioned stimuli, the CHS responses were reduced.¹ Similarly, it has been observed that hyperthermia (local or whole body) induces migration of epidermal LCs to draining lymph nodes. Mild fever-range whole body hyperthermia and local hyperthermia at 43°C when applied before sensitization, were shown to suppress CHS. There was a positive correlation between the decreased epidermal LCs and degree of CHS when mice were treated with local hyperthermia before sensitization.^{5,6}

Exogenous hyperthermia to the mouse skin induces dynamic changes of epidermal LCs. We previously demonstrated that the number of LCs gradually decreased after temporary hyperthermia on the skin, and then the

number of LCs gradually returned to normal in 7–14 days.⁶ The decreased number of epidermal LCs was accompanied with an increase of LCs in draining lymph nodes. In addition, the percentage of LCs expressing maturation markers was higher in hyperthermia treated mouse than in the controls, suggesting that hyperthermia promoted the capacity of antigen presentation by LCs.⁶

Determinants that affect the severity of allergic contact reaction include the nature of haptens (ranging from strong to weak sensitizers), their concentrations, the conditions of exposure, and the irritating potentials of a hapten.⁷ In the present study, the mice were treated with local hyperthermia on fluorescein isothiocyanate (FITC)

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sensitized area using a protocol that hyperthermia was performed prior to, concurrent with and post the sensitization. The study was elaborated to determine if the local hyperthermia temporally influence the severity of CHS.

METHODS

Mice

Young adult female BALB/c mice ((20±2) g body weight, 8 weeks) were purchased from the animal department of China Medical University and housed at 25°C for 7 days to adapt to the conditions of the lab. Water and a standard diet were available ad libitum. All protocols involving these mice were approved by the Animal Study Committee of China Medical University.

Hyperthermia treatment

A patented local hyperthermia device with infrared emitting source (Patent number: zl200720185403.3; China Medical University) was used to deliver local hyperthermia at different temperatures. The heat generated by the device acted on the skin surface without direct contact. The surface temperature of skin could be controlled and stabilized at the desired degrees with an accuracy of ±0.1°C by a micro-circuit.^{8,9}

Counting epidermal LCs

Groups of 5 mice were subjected to local hyperthermia at 37°C, 39°C, 41°C and 43°C, respectively, for 20 minutes. Biopsies were taken at different time intervals after local hyperthermia.

The dissected skin was incubated with EDTA at 37°C for two hours. The epidermis, mechanically detached from the skin with fine forceps under a microscope, was fixed in acetone at room temperature for 10 minutes, and then washed using phosphate buffer saline (PBS). After that, epidermal sheets were incubated with biotin-conjugated mouse anti-mouse I-Ad monoclonal antibody (PharMingen San Diego, USA) overnight. Then the epidermal sheets were washed with PBS, mounted with Vectastain ABC reagent (SP-0024, China), and incubated in a dark humidified box at 37°C for 90 minutes. After incubation, epidermis sheets were washed and incubated with DAB. The LCs in an epidermal sheet were counted en face using a Olympus microscope with a total magnification of 400×. Those cells exhibiting microscopically a brightly stained cytoplasm were considered positive. Five fields were randomly chosen to count the number of LCs. The density of LCs was presented as the number of Langerhans (cells/mm²) ± standard deviation (SD).

Protocol of CHS induction

Briefly as previously described,¹⁰ mice were sensitized with 100 µl of 0.5% FITC (Fanbo Biochemicals, China) in a 1:1 solution of acetone: dibutyl phthalate on the shaved dorsal sides (about 1 cm² in size). Five days later, the mice were challenged by painting the dorsal surface of the right ear with the same FITC solution. Two days

later, ear thicknesses of both sides were measured with Quick Mini thickness-gauge calipers (Mitutoyo, Japan). The difference of thickness of painted and non-painted ears were calculated as the severity of ear swelling responses. Groups of 5 mice received the above treatments and served as sham control.

Hyperthermia treatment at sensitization stage

As shown in Figure 1, groups of mice were sensitized with FITC as described above, coupled with pre- (three days before FITC application), concurrent and post- (two days after FITC application) hyperthermia treatment at surface temperatures of 37°C, 39°C, 41°C and 43°C on FITC painted region, respectively. Each local hyperthermia treatment continued for 20 minutes. Then mice were challenged as described in 2.4.

Statistical analysis

All the data were analyzed using SPSS software (version V13.0, SPSS Inc., USA). Student's *t* test was performed to compare the number of epidermal LCs. One-way analysis of variance (ANOVA) was used to compare the difference between thickness of ear and local hyperthermia at different temperatures in each group. *P* values <0.05 were considered to represent statistical significant difference.

RESULTS

Effect of local hyperthermia on dynamic changes of epidermal LCs

As shown in Figures 2 and 3, the number of LCs (cells/mm²) in skin treated at 37°C was similar (838.8±26.0, 824.4±14.5, 828.4±15.9, 825.6±7.3) on each time point, and was not significantly different from that

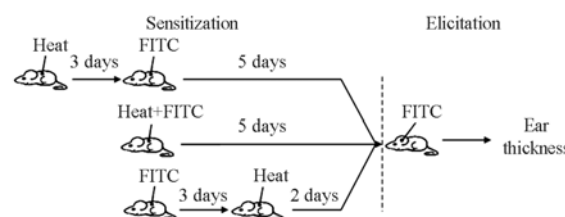


Figure 1. Schematic diagram of the experimental procedure.

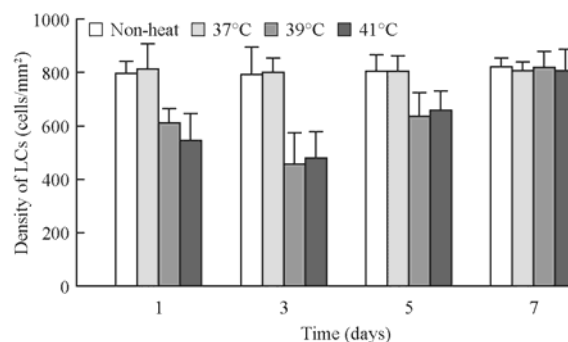


Figure 2. Dynamic changes of epidermal LCs after local hyperthermia. Number of LCs was expressed as mean value and error bars were shown, each group of data were drawn from five mice.

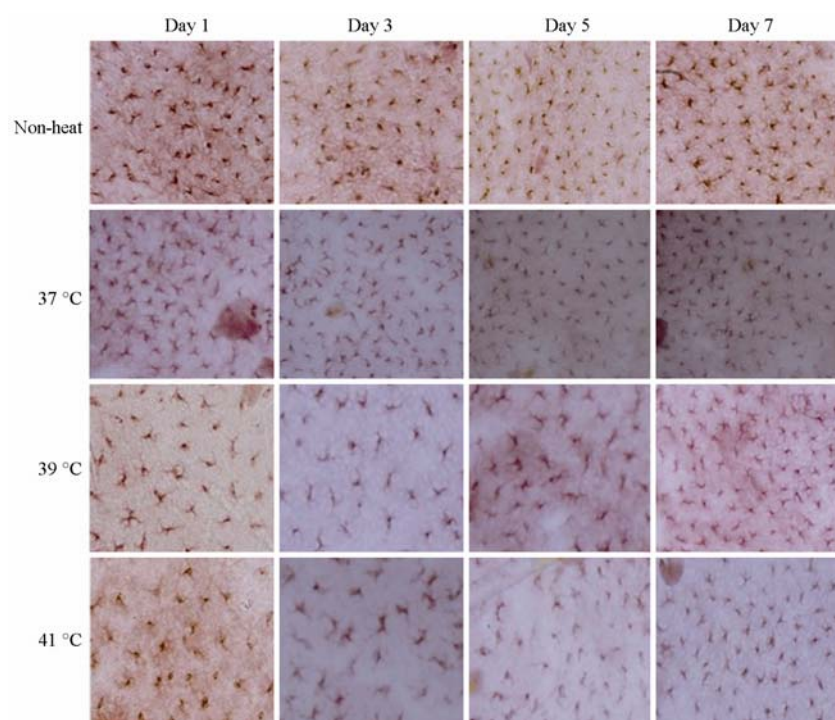


Figure 3. The SP staining of LCs. Non-heat treated skin or skin treated at 37°C, 39°C or 41°C after days 1, 3, 5 and 7 (Original magnification $\times 400$).

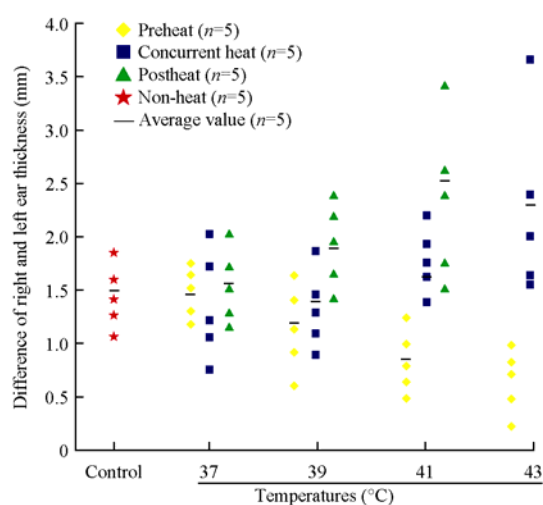


Figure 4. By One-way ANOVA analysis, the ear thickness decreases with elevation of the hyperthermia temperatures in the preheated group, $P=0.003$. On the contrary, the ear thickness showed a decrease with an increase in hyperthermia temperatures in concurrently heated group, $P=0.038$; similar findings were shown in two-day-post sensitization heated group, $P=0.030$. Non-heat control group showed no statistical difference between 37°C heated groups, $P<0.05$.

of sham control (826.2 ± 12.7 , 819.8 ± 28.5 , 828.2 ± 16.4 , 834.6 ± 8.3). The numbers of LCs in skin treated at 39°C were 631.6 ± 15.7 , 470.4 ± 32.7 , 656.0 ± 25.5 , 834.8 ± 16.8 , respectively, after days 1, 3, 5 and 7. The numbers of LCs in skin treated at 41°C were 566.4 ± 28.7 , 495.2 ± 26.6 , 678.8 ± 20.8 , 832.0 ± 21.2 , respectively, after days 1, 3, 5 and 7. Local hyperthermia treatment decreased the number of epidermal LCs in a temperature dependant manner. The number of epidermal LCs reached the lowest

three days post hyperthermia, then returned to the normal levels in a week.

Sensitization with pre-hyperthermia suppressed the severity of CHS

It was assumed that low number of epidermal LCs during the sensitization stage render fewer numbers of LCs to present antigen to T cells in the draining LNs, subsequently ameliorating the severity of CHS at the elicitation phase. As described above, three days post hyperthermia substantially reduced the number of epidermal LCs to the lowest level. Therefore, this time point was selected to pre-heat the mice before sensitization. The severities of CHS (mm) were 1.20 ± 0.32 , 0.84 ± 0.26 , 0.68 ± 0.28 , in mice pre-heated at 39°C, 41°C and 43°C, respectively. The severity of CHS in mice preheated at 37°C was 1.46 ± 0.21 , similar to sham controls (1.44 ± 0.27), $P>0.05$. There was a suppression of CHS by pre-hyperthermia at sensitization site, and the effect was more prominent in mice treated with higher local hyperthermia

temperature ($P<0.01$) (Figure 4).

Sensitization with concurrent and post-hyperthermia enhanced the severity of CHS

Our previous study showed that local hyperthermia promoted the migration as well as maturation of LCs.¹¹ It is assumed that sensitization with concurrent local hyperthermia renders more efficient antigen presentation by LCs to T cells in the regional LNs. The severity of CHS (mm) was 1.24 ± 0.11 , 1.32 ± 0.30 , 1.70 ± 0.27 , 2.32 ± 0.78 in mice heated at 37°C, 39°C, 41°C and 43°C during sensitization, respectively. Again, there was no difference of between 37°C and sham control mice (data not shown). Sensitization with concurrent local hyperthermia enhanced CHS, and that the severity was significantly stronger in mice exposed to higher hyperthermia temperatures ($P<0.05$). When local hyperthermia was applied two days after sensitization, the severity of CHS (mm) was 1.54 ± 0.32 , 1.84 ± 0.34 , 2.28 ± 0.70 , 2.54 ± 0.67 in mice heated at 37°C, 39°C, 41°C and 43°C, respectively. There was a temperature dependant enhancement of CHS. The extent of CHS was even stronger than those treated with concurrent local hyperthermia (Figure 4).

DISCUSSION

Contact dermatitis is a frequent inflammatory skin disease induced by skin exposure to low molecular weight chemicals, endowed with both proinflammatory and antigenic properties.^{12,13} The occurrence of allergic contact hypersensitivity is mostly dependant on the nature of the exposed allergens.⁷ However, whether

environmental factors play a role in CHS has not been sufficiently documented. In real life, there are situations when contact sensitization is coupled with the temperature changes in environment, either before, during or after contact sensitization. Exogenous hyperthermia, either within fever range or above (39°C–43°C), is temporarily tolerated by human tissue without irreversible damage. One common definition of the thermal dose (D) is calculated in the formula of $D = tRT - 43$, where t = exposure time, T = given temperature, with $R = 2$ for temperatures <43°C, and $R = 4$ for temperatures $\geq 43^\circ\text{C}$.¹⁴ In our previous study on whole organ culture of human skin, we noticed that hyperthermia at temperatures ranging from 39°C–43°C for 30 minutes was effective in promoting the migration of LCs,¹¹ and these thermal doses were subjectively well tolerated in patients with HPV infected skin.⁸ As the mouse skin is much thinner than that of human subjects, which make the transmission of heat to deep layer more quickly, we reduced the duration of hyperthermia to 20 minutes. Under such a condition, we observed apparent effects as displayed in the present paper. Moreover, we noticed that hyperthermia ranging between 39°C–43°C for 20 minutes did not cause apparent clinical and histologic damage to the targeted sites. The evaluation of effects of different thermal doses on CHS remains an interesting topic for further investigation.

In a time-course study, Yoshioka et al⁶ observed that local hyperthermia before the contact sensitization suppressed the severity of CHS, and the suppressive effect was statistically significant in 1–4 days before sensitization. Our study demonstrated that local hyperthermia decreased the number of epidermal LCs in a temperature-dependant manner with the strongest reduction occurring at day 3 after the hyperthermia. We postulated that when the number of epidermal LCs was low, fewer LCs were able to prime the T cells and thus reduced the severity of immune responses. Indeed, local hyperthermia three days before sensitization suppressed the severity of CHS, and the suppression of CHS was inversely correlated with the degree of temperatures applied.

Contrary to the above findings, when local hyperthermia was applied during and two days after sensitization, the severity of CHS was enhanced in a temperature-dependant manner. In a whole skin culture system, hyperthermia was shown to promote the migration of LCs away from the epidermis, and temperature dependantly increased number of LCs expressing CD83 in the emigrations.¹⁵ Kimber et al¹⁶ have demonstrated that, following topical application of a chemical sensitizer to mouse skin, LC began to migrate from epidermis within 30 minutes and arrived in the draining lymph nodes about 4 hours post-sensitization. The maximum influx of LC into the lymph nodes occurred at 18–24 hours. We propose that sensitization with concurrent or post- hyperthermia renders more

mature antigen – loaded LCs to prime the T cells in the draining LN.

Although epidermal LCs are believed to play a pivotal role in induction of CHS, dermal dendritic cells were also shown to take a role in antigen presentation.¹⁷ Dermal dendritic cells may directly contact the keratinocytes or, more likely, may acquire Ag from the migrating LCs as they traverse the dermis on their way to the draining lymph nodes.¹⁸ Recently, the role of LCs in CHS has been challenged in several transgenic mouse models, and it was shown that LCs probably take regulatory, rather than stimulatory functions (or both) in CHS.¹⁹ *In vivo* and *in vitro* studies have documented that haptens can activate skin cells and induce the production of several cytokines, by stimulating the migration of skin LCs to lymph nodes, play a key role in sensitization.^{20,21} The role of dermal dendritic cells was unable to be determined in the present study. However, our study suggested that the antigen presentation by LCs be influenced by local hyperthermia applied at different times, and that the temporal application of hyperthermia regulate the severity of CHS. In addition, there is a possibility that T cells and inflammatory components are also involved in the hyperthermia-regulated CHS. It would have been interesting to analyze T cell responses with regard to the impact of local hyperthermia on effector T cell or regulatory T cell generation, and measure pro- and anti-inflammatory cytokines in the skin treated with hyperthermia in future.

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