

Heat Shock Proteins 47 and 70 Expression in Rodent Skin Model as a Function of Contact Cooling Temperature: Are We Overcooling Our Target?

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Background and Objectives: The degree of protective cooling required during laser therapy to achieve an optimal result is unknown. The expression of heat shock proteins, Hsp47 and Hsp70, were examined in the epidermis and dermis as biomarkers to quantify the degree and depth of tissue affected by non-ablative laser treatment using variable protective cooling parameters.

Study Design/Materials and Methods: Twenty-one male Sprague–Dawley rats were treated with a 1,319 nm Nd:YAG laser using a sapphire cooling plate attached to the hand piece. A 4 cm×4 cm area on each side of the rat was treated with the same energy and pulse settings, with variable contact cooling. Protective cooling parameters, for each degree increment, ranging from 0 to 25°C were studied. Immunohistochemistry (IHC), Western blot and PCR were performed to evaluate the effects of superficial cooling on Hsp47, and Hsp70 expressions.

Results: Our data showed the extent of topical cooling needed to produce a thermal effect at different depths in the dermis, quantified by the expression of Hsp47 and Hsp70. Significant Hsp expression was observed with cooling of 13°C and warmer; no identifiable cellular reaction was observed when cooling below 5°C. There was no evidence of epidermal injury when treating the skin with any protective cooling ranging from 0 to 25°C.

Conclusion: Our data would suggest contact cooling temperatures 5°C and below completely protects through the entire dermis. There was no evidence of epidermal injury with protective cooling at any temperature between 0 and 25°C. Warmer temperatures are safe and adequately protect the epidermis in this model. *Lasers Surg. Med.* 39:504–512, 2007. © 2007 Wiley-Liss, Inc.

Key words: 1,319 nm Nd:YAG; collagen; animal model; heat shock proteins; skin

INTRODUCTION

Thermally mediated therapeutic procedures have become increasingly popular for cutaneous resurfacing and remodeling [1–8]. Non-ablative photothermic laser therapy is based on directing and maximizing thermal

energy to a target chromophore (superficial vessels, hair shaft, or dermal water), while maintaining epidermal integrity. Conventional ablative therapy obliterates the superficial epidermis and papillary dermis, while attempting to spare the reticular dermis and deeper tissues. Epidermal disruption may result in morbidity; increasing the risk of infection, erythema, scarring, and hypopigmentation of the treated area [3,4,7–9].

The addition of contact cooling to non-ablative laser treatments allows for the protection and preservation of superficial skin structures [9]. Within the dermis the thermal energy dissipates throughout the tissue on account of energy absorption or scatter. Numerous investigators use a wide range of contact cooling protocols for epidermal protection [3,6,9–11]. Laser manufacturers recommend 0–5°C as the desired protective cooling temperature for non-ablative laser treatments [3,11].

Developed biological pathways within the skin and body attempt to prevent cells from becoming necrotic or apoptotic following a thermal challenge. A specialized family of proteins, the heat shock proteins (Hsp), are up-regulated when placed under thermal stresses above 4–6°C of normal skin temperatures [12–19]. Generally, Hsp members function by transferring damaged intra-cellular proteins (unfolding) to respective recycling or secretory sites. The inducible form of Hsp70 is expressed intracellularly in keratinocytes, fibroblasts and adipocytes [20,21], while Hsp47 expression is associated with fibroblasts, collagen and the extracellular matrix in the dermis [22,23]. Identifying these proteins enables us to examine tissue reaction and injury from a biological/cellular standpoint.

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Sapphire plate contact cooling and cryogen spray cooling are two of the better studied cooling modalities in practice [24]. We used the contact cooling system, which works by cooling a transparent medium (sapphire plate), that is placed in direct contact with the skin surface as the laser is delivered through it to reach its target. The cooling plate temperature is maintained by recirculating chilled water, which is set to a predetermined temperature, between two sapphire windows. The window is kept in direct contact with the skin for >2 seconds before the treatment begins and is manually removed at the completion of the therapy [3,10,11]. Contact cooling allows the physician to select the appropriate temperature for the desired protective “cooling” effect.

An animal-based experimental protocol was developed to examine the effect of variable contact cooling temperatures on Hsp47 and Hsp70, when undergoing a standard 1,319 nm Nd:YAG (Sciton, Inc., Palo Alto, CA) non-ablative laser procedure.

MATERIALS AND METHODS

Laser Treatment

Twenty-one commercially produced male Sprague–Dawley rats weighing 250–300 g were studied using a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Southwestern Medical Center at Dallas. Animals were housed in the vivarium (Animal Resources Center) and fed a commercial rat chow and water ad libitum. Pre-operatively, all animals were anesthetized using ketamine/xylazine intra-peritoneally and shaved over the dorsal-lateral aspects of the torso. All fine hair was removed using an epilating cream for 30 minutes.

Of the 21 animals, 18 animals were treated over a 4 cm × 4 cm area, with 1,319 Nd:YAG laser system (Sciton, Inc.) at 30 J/cm², using a 25 milliseconds pulse width at 2 Hz with a 6 mm spot size. The 18 animals were then divided into two groups. One group contained 12 animals, and the remaining 6 in the other. The group of 6 animals was treated using protective cooling of 5°C increments; 0, 5, 10, 15, 20, and 25°C, respectively. The second subset of 12 rats were treated at multiple sites using 1°C increments from 5 to 25°C contact cooling temperatures. Three animals served as controls as the cooling plate was applied at 5, 15, or 25°C in the absence of any applied laser energy. The skin was pretreated with the cooling plate for 2 seconds prior to each laser treatment and continued in contact with the skin throughout the duration of each treatment. Upon sacrifice, full thickness skin biopsies were harvested at 10 and 24 hours, and repeated at 4, 14 and 28 days post-treatment. Specimens were subsequently divided and transferred into storage vials with respective reagents for immunohistochemistry, Western blotting or ELISA determinations.

Immunohistochemistry

Biopsy sections were immediately fixed in 10% neutral buffered formalin, embedded in paraffin, cut 4–6 μm thick in serial horizontal sections and mounted on poly-L-lysine

slides. Slides were heated at 60°C for 1 hour, deparaffinized in xylene 3×5 minutes, rehydrated in serial ethanol dilutions and rinsed in double-distilled water (DDW) 2×5 minutes. Sections were treated with 3% hydrogen peroxide for 30 minutes and rinsed in DDW 2×5 minutes. Slides were then blocked with normal goat serum (Sigma, St. Louis, MO) diluted 1:10 in phosphate-buffered saline (PBS, pH 7.4) for 20 minutes. After rinsing in PBS 2×5 minutes, Hsp47 was labeled with a mouse anti-Hsp47 monoclonal antibody (#SPA-470, Stressgen Bioreagents, Victoria, BC, Canada) diluted 1:200 in PBS. The primary antibody was incubated at 4°C overnight. The slides were rinsed in PBS 2×5 minutes, and a horseradish peroxidase (HRP) conjugated goat anti-mouse IgG secondary antibody (#SAB-100, Stressgen Bioreagents) diluted 1:200 in 3% rat serum was incubated at room temperature (RT) for 1 hour. The slides were rinsed in PBS 2×5 minutes, and developed using a high contrast diaminobenzidine (DAB) kit (ScyTek, Logan, UT) according to the instructions. Slides were rinsed in PBS 2×5 minutes, counterstained with Gill #3 hematoxylin (Sigma), rinsed in DDW 3×2 minutes, dehydrated in ethanol and mounted using Cytoseal™ 60 (Richard–Allan Scientific, Kalamazoo, MI). The five different time samples were stained to evaluate the treated tissues for staining intensity.

Western Blotting

Biopsy sections were flash frozen in liquid nitrogen and stored at –80°C. Protein extraction was performed on biopsy sections collected at 14 days post-laser treatment. Sections were cut into weight equivalent pieces (approximately 100 mg) and homogenized in Hsp extraction reagent (Stressgen Bioreagents). Samples were electrophoresed by 10% SDS-PAGE, and transferred to a nitrocellulose membrane at 5°C. The membrane was blocked with 5% non-fat dry milk in PBS-Tween 20 (PBST) buffer for 1 hour at RT. A mouse anti-Hsp47 monoclonal antibody (#SPA-470, Stressgen Bioreagents) diluted 1:500 in 5% non-fat dry milk was added and incubated for 3 hour at RT. The blot was washed with PBST and incubated for 1 hour at RT with an HRP conjugated goat anti-mouse IgG secondary antibody diluted 1:10,000 (#SAB-100, Stressgen Bioreagents). Blots were washed, developed with ECL-plus, scanned, and image analyses were performed using ImageQuant v5.2 software.

ELISA

Enzyme linked immunosorbant assay (ELISA) is a biochemical technique used to detect the presence of protein through an antigen–antibody complex labeling pathway (Stressgen Bioreagents). We instituted this technique to quantify the amount of Hsp47 and Hsp70 in each specimen. The frozen sections were ground into a powder using a mortar and pestle, and the protein was extracted and processed for use in the assay according to the ELISA kit directions. Hsp47 and Hsp70 standards were made in serial dilutions from the recombinant standard provided with the kit. Samples, standards and blanks were

placed in the microtiter wells provided with the kit, and the assay was run according to the manufacturer's directions. The wells were placed in a Spectramax Plus microplate reader, and read at 450 nm using a correction wavelength of 570 nm. A standard curve was generated, and the concentration of the samples was then calculated.

Real-Time Polymerase Chain Reaction Assays

Biopsy samples for PCR were immediately put in RNAlater® (Qiagen Sciences, Valencia, CA) and stored at 4°C. Approximately 100 mg of tissue was homogenized in 1 ml of TRI reagent (MRC, Cincinnati, OH), and total RNA was extracted. The quality of each RNA preparation was checked by measuring optical density (OD) at 260/280 nm. First-strand cDNA was synthesized using a total of 2 µg RNA and an Omniscript Reverse Transcription kit (Qiagen Sciences). Primer sequences for PCR amplification were as follows: human Hsp47 [F] 5'-3' GCT GCT CGT CAA CGC CAT GT [R] 5'-3' CCA TCC AGG TCT TCA GCT GC.

Real-time PCR was performed in triplicate for each sample using an Mx3000P system and Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA). Reactions were run in a 96-well plate using volumes of 50 µl/well. Amplification was performed at 95°C for 10 minutes for the initial activation, and then 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minutes, and elongation at 72°C for 30 seconds. Message levels for Hsp were quantified by relative quantification. The expression levels of treated samples were compared to control samples after gene normalization, and values are reported as mean and SD of results in a single experiment. To confirm the specificity of the PCR reaction, products were separated by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining.

RESULTS

Hsp47

Minimal Hsp47 staining was observed at 10 and 24 hours time points in the dermis after treatment with 30 J/cm² at 25 milliseconds pulse width at 2 Hz. (data not shown). At 4 days post-treatment without the application of protective cooling, Hsp47 staining was observed throughout the dermis. At 14 days post-treatment, Hsp47 staining intensity increased with staining of hair follicles and fibroblasts throughout the dermis (Fig. 1). No obvious differences in staining intensities between 14 and 28 days post-treatment were noted.

The effects of contact cooling temperatures were examined on dermal Hsp47 levels. Based on the previous time course results, the 14 days post-laser time period was chosen for examination. Using 1°C increments of increased cooling temperature from 5 to 25°C, very low Hsp47 protein staining was associated when cooling to 0–5°C (Fig. 2).

Hsp47 Western blot analysis confirmed the immunohistochemical data at day 14 post-treatment (Fig. 3a,b) Hsp47 mRNA expression was evaluated using real-time PCR in skin specimens at post-treatment day 14, with contact cooling temperature of 5 and 25°C at 18 J and 50 milliseconds pulse width with the 1,319 Nd:YAG laser. These alternate laser settings were used to help quantify the Hsp47 mRNA expression, which was present at both contact cooling temperatures, but demonstrated a 10-fold increase in expression at 25°C cooling when compared to 5°C (Fig. 4a).

Hsp70

As with Hsp47, Hsp70 protein expression following laser treatment was initially determined using immunohistochemistry without any protective cooling applied. In

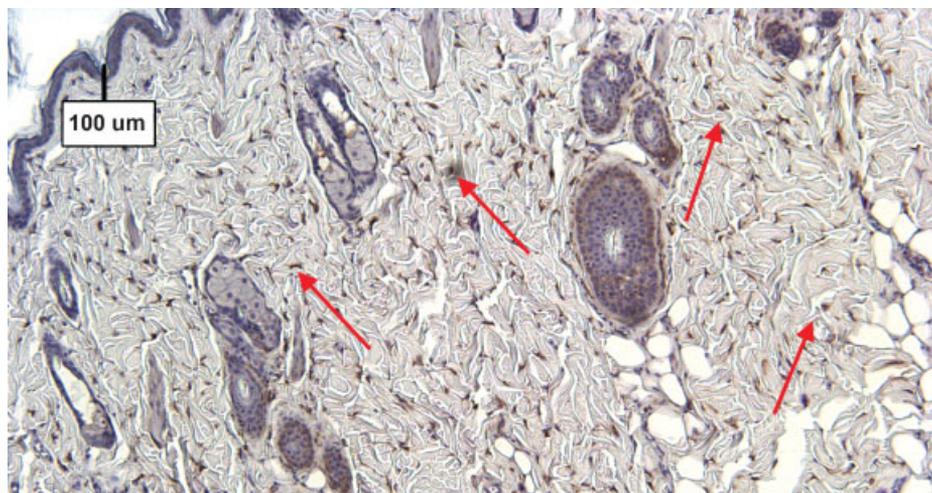


Fig. 1. Histological section 14 days post-Nd:YAG 1,319 non-ablative laser treatment. No protective cooling was applied to this tissue prior to treatment. Protein was identified using monoclonal mouse anti-47 (Stressgen Bioreagents) and stained using DAB chromogen (brown pigment). The specimen was then counterstained with hematoxylin (blue pigment) to

enhance visualization of the protein in the tissue section. At 30 J/cm² significant Hsp47 staining (arrows) was observed throughout the dermis (brown pigment). Immunohistochemistry provides actual visualization of the protein after the laser induced thermal stress.

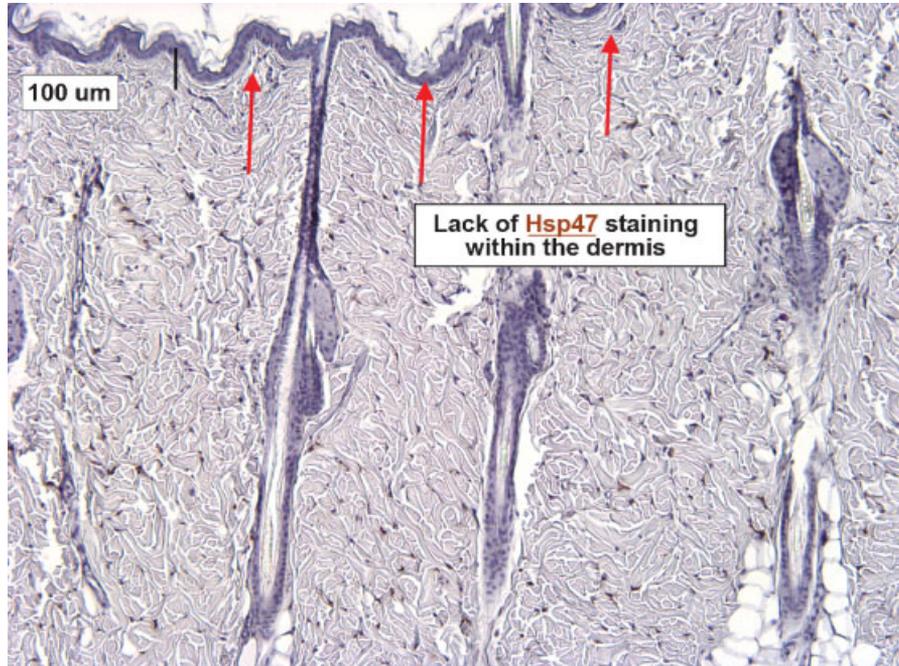
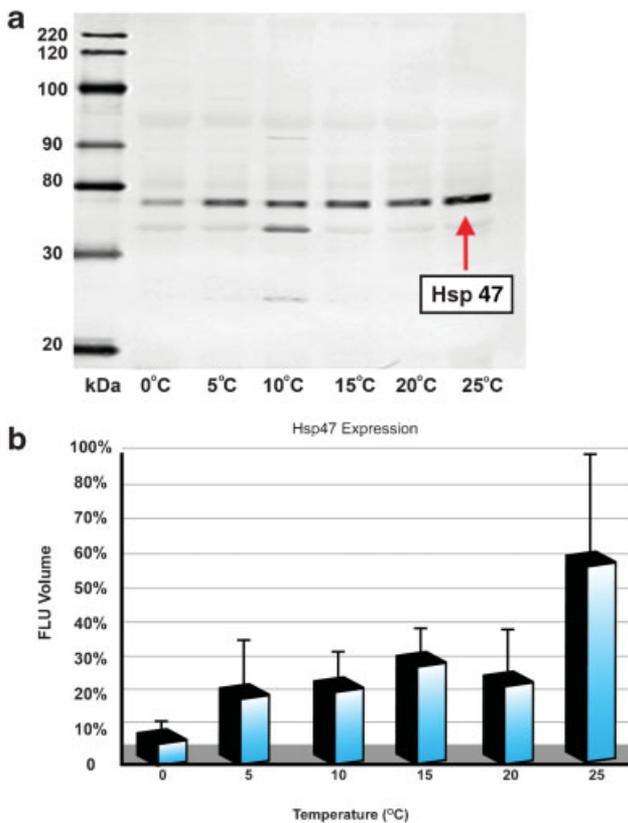


Fig. 2. Histological specimen with Hsp47 staining using protective cooling of 5°C with the 1,319 Nd:YAG laser. Staining was performed as described in Figure 1. Minimal Hsp47 staining (arrows) was observed throughout the tissue when cooling with 5°C. Minimal thermal tissue stress was observed under these protective cooling parameters.



contrast to the long time interval required for maximum Hsp47 expression, increased Hsp70 protein staining was present within 2 hours of the laser treatment. To confirm these results, Hsp70 protein levels were determined by ELISA at 2 hours intervals from 0 to 24 hours after a single laser treatment (data not shown). It should be noted that basal Hsp70 levels were detectable in normal untreated rat skin. However at time 0, low Hsp70 levels were detected with 20°C contact cooling, and were found to pick up significantly by 8 hours (Fig. 5). Increased Hsp70 levels were observed from 4 to 12 hours post-treatment corresponding with what is reported throughout the literature [25–28]. Hsp70 levels peaked approximately 12 hours after laser treatment (Fig. 6). Mean Hsp70 protein levels decreased from 12 to 24 hours, while no changes in Hsp70 levels were observed in non-treated control specimens. Hsp70 protein levels remained at low levels after 24 hours and returned to baseline at 14 days.

Fig. 3. **a,b:** Western blotting of Hsp47. **Panel A** depicts individual Hsp47 staining bands at various cooling contact temperatures, and the increase in signal at warmer protective cooling parameters (arrow). **Panel B** shows a quantitative analysis of the Western blot at the various cooling temperatures. The Flu volume is a quantitative average of the protein that is present as compared to the control. Increasing protein levels were observed in accordance with warmer protective cooling parameters from 5 to 25°C.

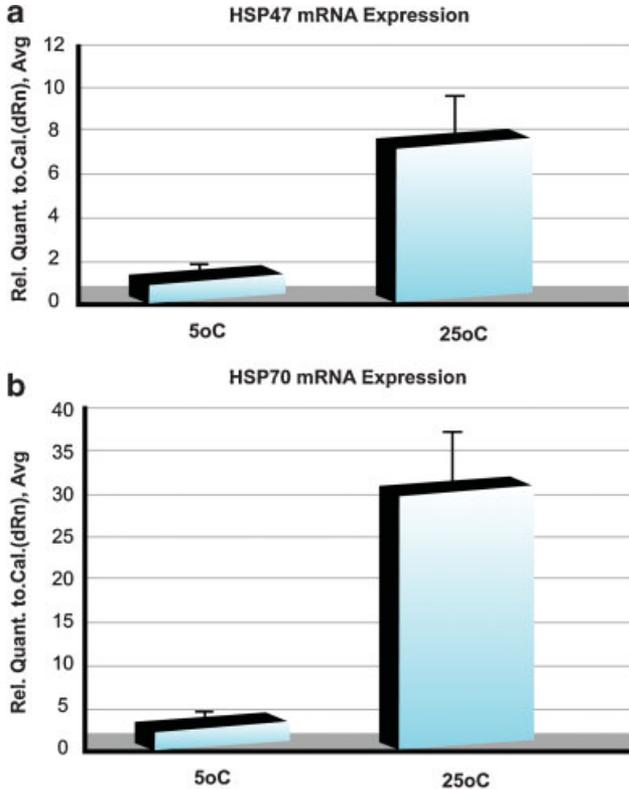


Fig. 4. **a,b:** RT polymerase chain reaction with 5 and 25°C. Both RT-PCR and real-time PCR were performed to quantify Hsp47 (a) and Hsp70 (b) mRNA expression. mRNA expression was present at both contact cooling temperatures for each of the respected proteins and exhibited a 10× fold significant increase in expression at 25°C compared to 5°C.

The effect of variable contact cooling temperatures and Hsp70 expression was then examined. At contact cooling temperatures below 10°C, minimal staining was observed at any level of the dermis or epidermis. However at less protective cooling temperatures, Hsp70 staining was observed in specific dermal levels. For example, individual hair follicles in the dermal level were not significantly stained when cooling at 10°C, but partially stained at 14°C and further intensified at 25°C (Fig. 7).

To support our immunohistochemical findings an mRNA expression profile was also performed for Hsp70. Similar to Hsp47, with real-time PCR Hsp70 mRNA levels were very low and almost undetectable at 5°C, but exhibiting a 10–12 fold increase with contact cooling of 25°C (Fig. 4b).

DISCUSSION

Our results demonstrate increased levels of Hsp47 and Hsp70 within the dermis after non-ablative laser treatments with and without protective cooling. [Their expression was found to be time dependent with Hsp70 demonstrating a more rapid increase early on, and Hsp47 increasing more progressively over time.]

Members of the Hsp family play a role in a multitude of different diseases and cellular processes. Located throughout the cytosol, mitochondria, endoplasmic reticulum, and nucleus, heat shock proteins as a group are thought to provide a unified cellular response mechanism to direct and indirect challenges [13–16,29,30]. Hsp have been reported to be up-regulated during ischemic/hypoxic changes, cardiac hypertrophy, fever, inflammation, metabolic poisons, tissue trauma, and even cancer [17,18,30,31]. Extensive research into cellular response to injury has resulted in an expanding identification and knowledge of more members of the Hsp family (e.g. Hsp60, Hsp32, Hsp47, Hsp90, Hsp70, etc.) and their respective participation in a number of essential inter- and intra-cellular processes.

Hsp's protect the cell by preventing inappropriate protein aggregation and mediating their intra-cellular transport. They enhance the cell's ability to withstand accumulation of abnormally folded proteins, either by facilitating the refolding or assisting in synthesizing new proteins to replace those damaged by the inciting event [13,16,18] (Fig. 8a,b).

The Hsp70 family (Hsp72, Hsp73) has shown to be expressed in all cells and represents the most highly induced member of the stress protein family [20,28]. It has been assumed that Hsp70 is involved in essential roles in the protection and adaptation to a broad variety of environmental stressors [32]. Hsp70 has been hypothesized to be a direct barometer to the amount of protein denaturation in a cell which increases at the time of stress or "heat shock" [19,20,31]. Current investigators are aggressively pursuing the anti-proliferative and anti-inflammatory effect of Hsp70 and its potential influence on wound healing [33–35].

Nagata [23] reported that a second protein, Hsp47, is the only heat shock protein with the ability to bind extra cellular matrix proteins. It has been shown that Hsp47 is found predominantly in fibrocytes, chondrocytes, smooth muscle cells of the gastro-intestinal tract, endothelial cells of blood vessels, and the basal layer of the epidermis [22,23,36–39]. Consistent with our immunohistochemical findings, these proteins are found to reside in the endoplasmic reticulum of fibroblasts and cells specifically expressing type I collagen. Hsp47 is intricately involved in the biosynthesis of collagen and its level of expression is directly proportional to the rate of collagen formation [23,37].

Keagle et al. [40] demonstrated the levels and timeframe of Hsp47 expression in the rat model which can be very useful for following skin tissue stress. We tracked the expression of HSP47, 70 with immunohistochemistry, PCR, and Western Blotting at individually set temperatures allowing us to measure tissue reaction at variable protective cooling parameters.

We recognize that wavelength, pulse width, energy, and spot size are all intricately involved in determining the appropriate parameters to adequately treat the desired target. The last variable which should not be ignored is the amount and duration of protective cooling. Ideal cooling temperatures for specific targeted tissues is unknown. Are

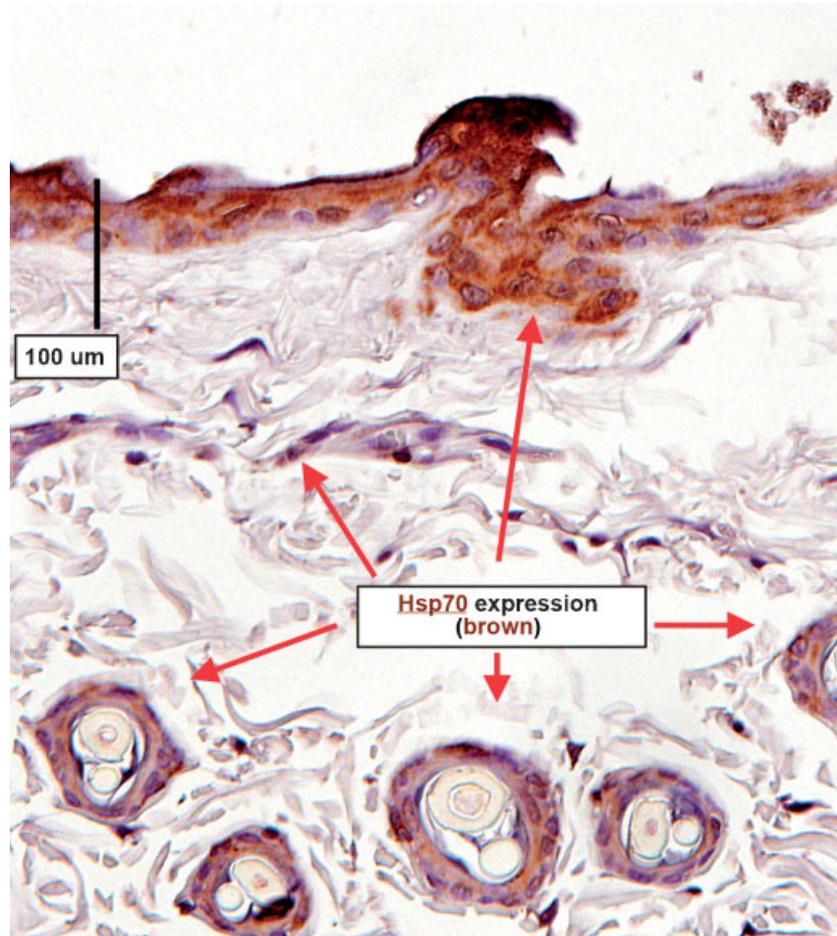


Fig. 5. Immunohistochemical staining for Hsp70 at 8 hours post-1,319 Nd:YAG laser treatment (no protective cooling) (arrows). Similar staining technique of Hsp47 was used for Hsp70 by substituting monoclonal mouse anti-Hsp70 (Stressgen Bioreagents). Hsp70 staining was observed more acutely and was evident in untreated tissue. A significant increase in Hsp70 staining (brown pigment) was observed within 4 hours post-laser treatment when compared to untreated skin.

our inconsistencies seen with non-ablative laser technologies the fault of the technology itself or our lack of understanding of the significance of cooling and how it directly effects not only the superficial structures but also the deeper ones we are trying to target?

As the therapy is delivered to the target tissue certain bimolecular and cellular alterations will occur. We know that beyond a certain threshold, epidermal temperature will continue to rise causing nonspecific thermal injury to the surrounding tissue causing collateral or bulk damage. To the best of our knowledge, the appropriate protective cooling temperatures needed for a given wavelength or energy has not been established. With the use of heat shock protein markers, we are able to observe, from a cellular basis, the depth of tissue invasion and degree of tissue reaction to the laser therapy [12,31].

We have assumed the degree and depth of cooling in accordance with the sapphire contact cooling apparatus

provided by Sciton, Inc. Through a series of software based and mathematical analyses, the temperature differential throughout the tissue was calculated as a function of depth and time with a cooling plate of either 0 or 5°C.

When the contact cooling plate was set to less than 13°C, we found minimal tissue effect, or heat shock response, within the dermis or epidermis. As previously stated, current protective cooling modalities are set to more extreme cooling levels (0–5°C). Our findings suggest this degree of protective cooling may in fact overprotect the targeted tissue or structure, by overcooling through the entire skin thickness.

This animal study would suggest that more consistent improvement with photothermic laser therapy may be achieved using “warmer” protective cooling temperatures. If these findings are able to be translated to the clinical forum physicians may be able to achieve better results with fewer treatments, at lower energies, and decrease

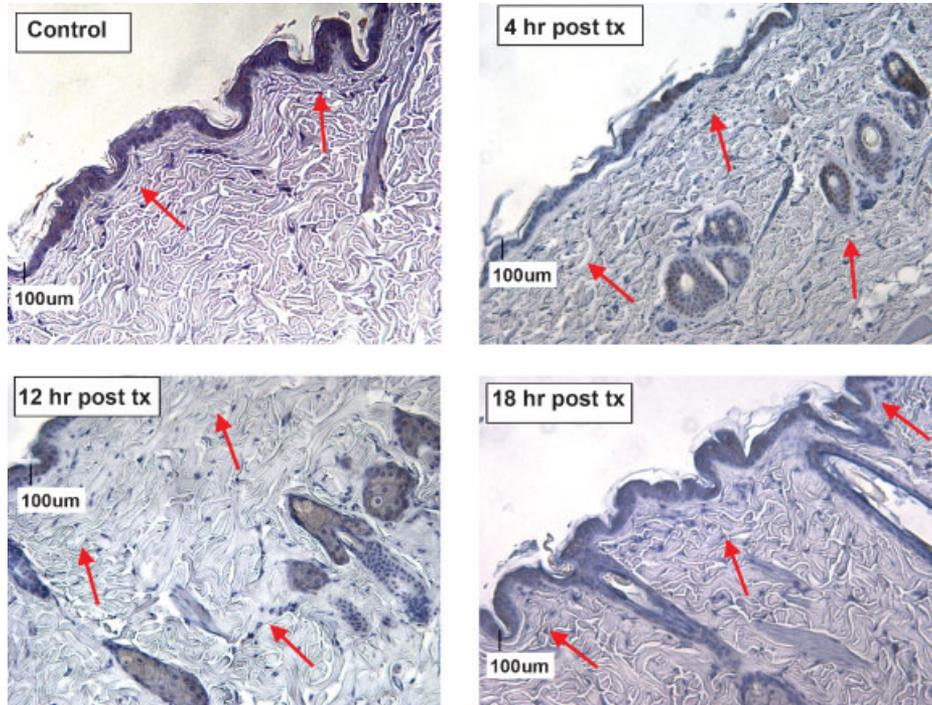


Fig. 6. Immunohistochemical sections showing increased staining post-treatment over time (control, 4, 12, 18 hours) with 20°C of protective cooling. Maximal staining was observed between 8 and 16 hours post-laser treatment, and began to taper off after 20 hours (depicted by arrows).

associated morbidities (hyper/hypopigmentation, scarring). Understanding that these results need to be further investigated and confirmed in an appropriately controlled clinical setting.

It is important to note this study is not without criticism. While we did thoroughly explore the role of cooling temperatures and thermal tissue effect with non-ablative therapy, it is in an animal model. Further evaluations of cooling parameters in the human model are currently underway.

Appropriate staining for Hsp47 and 70 can have variability and is dependent on the appropriate antibody used to recognize the specified protein of interest. We are confident that our technique, after numerous modifications in antibody and staining, gives an adequate representation of heat shock protein expression in the treated tissue, and is further strengthened with the use of DNA and protein analysis (e.g. Western Blot, ELISA). Further investigation into the precise temperatures in the deeper tissue (thermocouplers, heat probes, etc.) using the variable protective

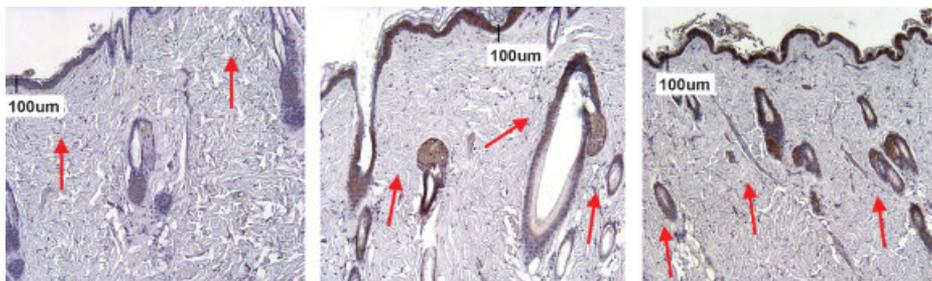


Fig. 7. Immunohistochemical staining of Hsp70 (arrows) using 10C, 14C, and 25°C of protective contact cooling with the 1,319 Nd:YAG laser, respectively. At protective cooling temperatures below 10°C, minimal Hsp70 staining was observed at any level of the dermis or epidermis and progressively increased with the application of warmer protective cooling temperatures (14, 25°C).

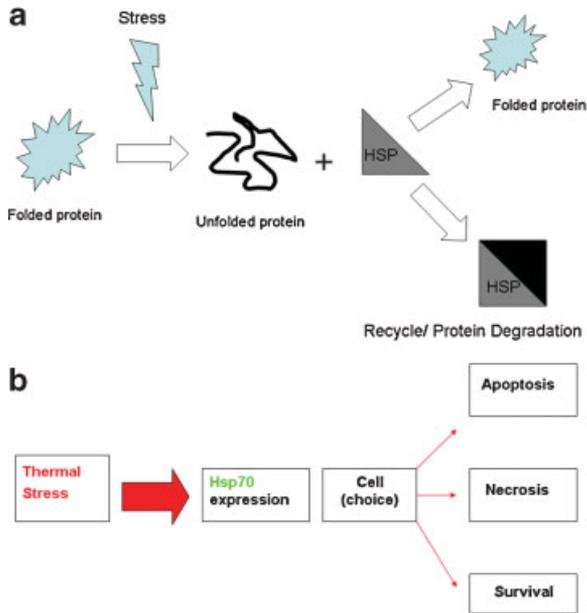


Fig. 8. **a,b**: Heat shock protein cartoon (**A**) and schematic (**B**). Heat shock proteins are intricately involved in the biomechanics of protein assembly and cell survival when placed in a stressful environment. They enhance the cell's ability to withstand accumulation of abnormally folded proteins, either by facilitating the refolding or assisting in synthesizing new proteins to replace those damaged by the inciting event [32]. B: Heat shock proteins are felt to play a major role in determining the future state of that cell.

cooling parameters is needed in order to make accurate conclusions regarding the degree of tissue protection to the skin layers.

However, our findings suggest the possibility of achieving better results with fewer treatments, lower energies, and a decreased morbidity to patients by using less protective cooling.

CONCLUSION

The contact cooling had a profound effect on the level of expression of the Hsp's and their localization throughout the skin in the rat model. Colder temperatures had significantly decreased expression of Hsp47 and 70 in protein, immunohistochemistry, and RNA expression. To reemphasize, 5°C, the cooling standard by most current manufacturers, showed almost no protein expression in the treated tissue. Clear planes of cellular staining and expression were observed in the immunohistochemistry sections at specific temperature increments. We hope that this technique of analysis will allow us to better understand the photothermal effects of both ablative and non-ablative lasers. It may serve as a model to determine the ideal parameters of laser devices in a more predictable and precise manner. These findings may help to further define and predict the depth of treatment with use of the heat shock proteins as markers of cellular injury, and look

further into the extent of protecting cooling with the variable laser technologies available today.

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