Laser Induced Expression of Heat Shock Protein 47 (Hsp47)

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Background and Objectives: **To investigate the effect of contact cooling on heat shock protein 47 (Hsp47) expression in the dermis and associated structures of laser treated rat skin, and determine the significance of cooling temperature versus energy and pulse width.**
Study Design/Materials and Methods: **Twenty-three male Sprague Dawley rats were treated with a 1319nm Nd:YAG laser using a sapphire cooling plate attached to the handpiece (Sciton Inc, Palo Alto, CA). A 4 cm x 4 cm area on each side of the rat was treated with the same energy and pulse settings, but the contact cooling temperature varied for each treatment. Biopsies from the treated areas were harvested at 10 and 24 hours, 4, 14, and 28 days after laser treatment. Immunohistochemistry (IHC), Western blot and PCR were performed to evaluate the effects of superficial cooling on Hsp47 expression.**
Results: **The IHC revealed that changing the temperature of topical cooling had considerable effects on depth and quantity of Hsp47 expression within the dermis. These results were confirmed by Western blot and PCR.**
Conclusion: **Hsp47 is a collagen specific biomarker of heat stress, and its levels are proportional to collagen production. Varying the contact cooling temperature alters the expression of Hsp47 in the dermis and its associated structures. Hsp47 is a useful biomarker for determining areas of heat stress in skin, and is a significant tool in determining the effects of laser parameters on tissue heating.**

Key words: 1319 nm Nd:YAG; collagen; *Hsp47;* Immunohistochemistry; non-ablative; contact cooling; rat skin

INTRODUCTION

Nonablative lasers have been used over the past decade to stimulate collagen production to improve the appearance of scars, decrease wrinkles, remove fine lines, and tighten and rejuvenate photoaged skin. Fisher et al [1] reported that the alterations causing photoaged skin reside in the dermis, and the skin displays changes in the collagenous extracellular matrix. They also showed that collagen fibers are responsible for the strength and resilient properties of the skin. In 2002, a retrospective analysis of clinical studies that had used a variety of devices for nonablative photorejuvenation was done to evaluate the scientific evidence presented. The study concluded that there was inconsistent data on the efficacy of nonablative photorejuvenation [2]. This inconsistency is the cause of much of the debate about nonablative procedures and the different devices available.

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Several groups have evaluated a number of different laser wavelengths as well as other types of devices that have been used for nonablative treatments [3-8]. To date, there are at least 8 different wavelengths of lasers that have been used ranging from 532 nm to 1540 nm, and multiple intense pulsed light devices (IPL) that typically emit light in the range of 500 - 1200 nm. There has also been investigation of radiofrequency (RF) and plasmakinetic energy for nonablative procedures, and one of the newest technologies is based on an idea called fractional photothermolysis.

Using histopathologic and clinical observations, the effects of laser energy on skin and its associated structures has been evaluated. A complete profile of the mechanisms involved with these effects has not been determined though [5-7,9,10]. A full understanding of what is happening biologically in the dermis to cause these changes, and more importantly, the knowledge and ability to manipulate these effects still needs to be discovered. The significance of contact cooling temperatures, pulse duration and fluence used during laser treatments has not been completely investigated. Ross et al [10] used a 1540 nm Er:Glass laser to examine the effects of various pulse energies and pulse numbers on clinical outcome using a constant contact cooling temperature. But to date, no studies have looked at the use of heat sensitive biomarkers, i.e. heat shock proteins (Hsp) found in the tissues to determine the importance of these parameters. There has been investigation of Hsp in association with the effects of laser energy on wound healing [11,12], but none to determine what significance contact cooling versus pulse duration or fluence has on laser treated skin.

Heat shock protein 47 (Hsp47) is a collagen–specific, stress induced molecular chaperone residing in the endoplasmic reticulum of fibroblasts that produce type I collagen. It plays a vital role in collagen synthesis [13-16], and its levels are proportional to the rate of collagen synthesis [14-17]. This makes it a potentially valuable biomarker to assess the effects of laser energy on skin and collagen production. The theory behind laser – associated skin changes is that these effects occur due to heating of the tissue. One of the critical factors involved with assessing the effects of lasers on skin is the ability to monitor the areas of the skin that are being heated. Although not fully understood, the significant changes involve the dermis [3-5]. Denaturation of collagen in the dermis causes fibroblastic collagen synthesis [18], and damaged fibroblasts then multiply and after a few days migrate to the site of the thermal wound and produce type I procollagen [19]. By using heat induced biomarkers present in the skin, we can not only monitor the areas of the skin being heated. This will increase our understanding of the effects lasers have on tissue.

MATERIALS AND METHODS

Laser Treatment

Twenty – three commercially produced male Sprague – Dawley rats weighing 250 – 300g were used in this study. The rats were housed in appropriate facilities at The University of Texas Southwestern Medical Center Animal Resources Center. They were fed a commercial rat chow and water ad libitum. The rats were anesthetized using ketamine/xylazine intraperitoneally, and then shaved over the dorsal and lateral aspects of the torso. The remainder of the fine hair was removed using an epilating cream. Two rats were treated with a 1319 nm Nd:YAG laser with a sapphire cooling plate attached to the handpiece (Sciton Inc, Palo Alto, CA) using a fluence of 18J/cm^2, 50 ms pulse width at 2 Hz with a 6 mm spot size. One was treated with 5°C and the other with 25°C contact cooling. The cooling plate was applied to the skin for 3 seconds prior to firing the laser, and a 4 cm X 4 cm area was treated on each side of the rat. Biopsies were harvested 4 days after treatment from the center of each treated area. Multiple sections were taken from each rat and preserved for immunohistochemistry (IHC) and PCR.

The remaining 21 rats were treated with 30 J/cm^2, 25 ms pulse width at 2 Hz with a 6 mm spot size and a treatment area 4 cm x 4 cm. The first 6 rats were treated in 5°C increments from 0°C to 25°C contact cooling, and the other twelve rats were treated using 1°C increments from 5°C to 25°C contact cooling. Three of the 21 rats

were used as controls, and had the cooling plate applied at 5°C, 15°C or 25°C for the duration of the treatment without laser energy being applied. Full thickness skin biopsies were harvested at 10 and 24 hours, 4, 14 and 28 days post treatment from the center of the treatment area. Multiple sections were harvested from all rats for IHC and Western blot analysis.

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. Slides were heated at 60°C for 1 hour, deparaffinized in xylene 3 x 5 min, rehydrated in serial ethanol dilutions and rinsed in double–distilled water (DDW) 2 x 5 min. The sections were treated with 3% hydrogen peroxide for 30 min and rinsed in DDW 2 x 5 min. 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 min. After rinsing in PBS 2 x 5 min, 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 x 5 min, and a horseradish peroxidase (HRP) conjugated goat anti – mouse IgG secondary antibody (#SAB–100, Stressgen Bioreagents, Victoria, BC, Canada) diluted 1:200 in 3% rat serum was incubated at room temperature (RT) for 1 hour. The slides were rinsed in PBS 2 x 5 min, and developed using a high contrast diaminobenzidine (DAB) kit (ScyTek, Logan, Utah) according to the instructions. Slides were rinsed in PBS 2 x 5 min, counterstained with Gill #3 hematoxylin (Sigma, St Louis, MO), rinsed in DDW 3 x 2 min, dehydrated in ethanol and mounted using Cyotseal™ 60 (Richard–Allan Scientific, Kalamazoo, MI). The five different time samples were stained to evaluate the treated tissues for staining intensity.

Western Blot

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 100mg) and homogenized in Hsp extraction reagent (Stressgen Bioreagents, Victoria, BC, Canada). 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 hr at RT. A mouse anti-Hsp47 monoclonal antibody (#SPA – 470, Stressgen Bioreagents, Victoria, BC, Canada) diluted 1:500 in 5% non-fat dry milk was added and incubated for 3 hr at RT. The blot was washed with PBST and incubated for 1 hr at RT with an HRP conjugated goat anti-mouse IgG secondary antibody diluted 1:10,000 (#SAB–100, Stressgen Bioreagents, Victoria, BC, Canada). The blot was washed, developed with ECL-plus, scanned, and image analysis was done using ImageQuant v5.2 software.

PCR

Biopsy samples for PCR were immediately put in RNAlater® (Qiagen Sciences, Valencia, CA) and stored at 4°C. Approximately 100mg of tissue was homogenized in 1ml 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 2µg RNA and an Omniscript Reverse Transcription kit (Qiagen Sciences, Valencia, CA). 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 50µl/well. Amplification was performed at 95°C for 10 min for the initial activation, and then 40 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 1 min, and elongation at 72°C for 30 sec. 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

Immunohistochemistry

Multiple sections from each biopsy sample were stained to determine the Hsp47 staining intensity at each time point for the same temperature setting (15°C). The sections collected at 10 and 24 hr showed little staining in all areas of the dermis. At 4 days post laser, an increase in staining was seen throughout the dermis. At 14 days post laser, the staining intensity increased more showing heavier staining around the hair follicles and other structures within the dermis. There was also an increase in the number of individual fibroblasts in the dermis that were staining positive for Hsp47. There was little visual difference in staining intensity between the slides from 14 days post laser and the slides from 28 days post laser.

The 14 day sections were used for IHC to evaluate the effect of temperature on Hsp47 levels. The initial group of six slides in 5° increments from 0°C to 25°C showed almost a complete lack of staining at 0°C, with the heaviest staining at 25°C. The 5°C slide had little increase in staining over the slide at 0°C. There were differences seen in the quantity and location of staining in the dermis of the slides between 10°C and 20°C, with 10°C having the majority of staining in the deep layers of the dermis. The 15°C slide showed an increase in staining throughout the dermis, but the staining superficially was scattered. At 20°C, there was a higher concentration of staining in all levels of the dermis.

The group of 12 slides in 1° increments showed subtle changes even between slides that were treated with 1° difference in cooling temperature. The sections that were treated at 21°C and above showed marked Hsp47 staining in the dermis and hair follicles at all levels of the dermis. Around 16°C, there was a decrease in staining intensity in the dermis with the superficial dermis still showing moderate staining, but only the superficial hair follicles were staining. There were subtle differences in the staining patterns at temperatures between 11°C and 15°C, and staining intensity began to increase in the deeper levels of the dermis. At 10°C, there was staining primarily in the deeper dermis, and only hair follicles in the deep dermis were staining. At 5°C, the staining began to decrease to the level of the 0°C slides. This would indicate that the tissue was being cooled to a point that the heat increase within the tissue was not enough to stress the cells, and therefore collagen was not being denatured.

Western Blot

Western blot analysis was performed on the 6 sections taken from the rats treated in 5° increments. The protein concentrations at day 14 post laser confirmed the impressions made from examination of the IHC slides. There was a significant increase in Hsp47 levels at 25°C and a significant decrease at 0°C as was seen on the IHC slides. There was also a linear increase in Hsp47 levels between 5° and 20°C which would appear to correlate with the Hsp47 staining at those same temperatures.

PCR

PCR was performed on biopsy samples from the two rats treated at 25°C and 5°C using 18J/cm^2 and a 50 ms pulse width. RT-PCR was performed to examine mRNA expression, and real time PCR was then performed to quantitate mRNA levels. Both RT and real time PCR showed a significant difference in Hsp47 expression between 25° and 5°C indicating the lack of sufficient heating of the tissue using 5°C contact cooling. These results mirrored those seen using a fluence of 30J/cm^2 and a 25 ms PW at the same cooling temperatures.

DISCUSSION

Nonablative laser procedures have the advantage of being less invasive and having less down time with fewer potential

complications compared to ablative procedures. The disadvantage of the nonablative techniques at this time is that they are not as effective as the ablative procedures in achieving consistent and significant improvement in skin texture, tone and wrinkle reduction. Numerous laser wavelengths and other types of devices have been used to stimulate collagen production and rejuvenate photoaged skin.

Multiple studies have evaluated this technique using lasers, IPL and RF devices, and all have shown some degree of improvement. The problem associated with these studies is the inconsistent way in which they are carried out as well as the inconsistent results obtained. Leffell [2] came to the conclusion that the 11 studies he reviewed had failed to present consistent data on the efficacy of nonablative procedures. This is just one of the problems associated with nonablative techniques and the studies that have examined them. The lack of a thorough understanding of what is causing the changes that are seen with nonablative procedures and how to manipulate these effects are also problems that have yet to be solved.

It is well understood that the effects of nonablative procedures are due to heat deposition within the tissue, specifically the dermis, but which factors are most significant. Pulse duration, fluence and contact cooling all play a vital role in the treatment outcome, but what is the significance of each. The results seen with the 2 slides treated with 18J/cm^2 and a 50ms PW were almost identical to the results of the slides treated with 30J/cm^2 and a 25ms PW at the same temperatures. The cooling temperature had the most significant effect on heat deposition in the tissue.

Other factors such as treatment time play a role in heating of the tissue, and help demonstrate the importance of cooling temperature. The longer the amount of time that laser energy is delivered to the tissue, the lower the overall temperature has to be for collagen to be denatured. For millisecond deliveries, such as those used in nonablative systems, the temperature threshold is likely in a range from 90°-120°C for collagen to be denatured [20,21]. What effect does topical cooling have on this relationship? Ross et al [10] found that the area of the dermis affected in their study was between 400 -1300 μm deep in areas without epidermal necrosis, and for optimal results the affected area of the dermis may need to be more superficial.

The importance of cooling temperature was not determined by these findings, but the need for a better method to evaluate the area of tissue that is being heated was shown. To date, most studies have used gross histologic evaluation to determine the area affected. What is not well understood is what is happening to those areas that do not show gross clumping of collagen or fibroplasia. This study demonstrates the ability to examine heat deposition in the tissue with the use of Hsp47 as a biomarker of heat stress and collagen production.

Hsp47 is significant because we can examine the role cooling plays versus energy or pulse width. Not only were we able to determine the significance of cooling, but we also discovered there is a marked difference between the amount and location of heat deposited in the tissue among individual cooling temperatures used. The area of the dermis that absorbs heat and demonstrates heat stress can be altered by changing the cooling temperature by as little as 2-3°C. We demonstrated that when using very low cooling temperatures, (<5°C) the tissue may not be heated sufficiently to cause denaturation of collagen or stimulate the production of collagen by fibroblasts. Even with cooling temperatures at 10°C, the heat deposited in the tissue was only in the deeper layers of the dermis.

We can monitor the amount of collagen being made using Hsp47 levels and its relationship to collagen production [14-17]. We also know when Hsp47 levels are at their peak. Keagle et al [17] showed that Hsp47 levels peaked around 14 days after creating a wound in normal rat skin, and these levels were still high at 28 days. This was demonstrated in our study by the highest staining intensity of Hsp47 at 14 days, and the persistence of increased staining at 28 days. These results create a time frame for monitoring Hsp47 levels, and allows for the evaluation of treatment parameters in a short time frame following laser treatment.

The goal of nonablative therapy is to induce the stimulation of fibroblasts and

cause contracture of collagen without damaging the epidermis. We have shown that this can be done effectively using a 1319 nm Nd:YAG laser and contact cooling in a precise temperature range. The primary target for the laser energy in treating photoaged skin is the superficial dermis. The collagen in the upper 400 μm of the dermis needs to be affected for significant improvements in facial rhytids and fine lines to be made. We have shown the ability to affect not only the superficial layers of the dermis, but the deep layers of the dermis as well. These results demonstrate the ability of the 1319 nm Nd:YAG to deposit heat in different layers of the dermis by using specific cooling temperatures.

Finally, protection of the epidermis is one of the key advantages to nonablative procedures. The results obtained in this study were achieved with no epidermal disruption or damage. Mild erythema was seen in the rats treated at 25°C for a few days after treatment, but the epidermis was intact at all biopsy times from 10 hr to 28 days. This was evident by evaluation of H&E stained histology slides as well as IHC slides. We were able to safely deliver laser energy sufficient to increase Hsp47 expression in all levels of the dermis, and this increase in Hsp47 also correlates to an increase in collagen production. Heat could be deposited in the dermis in independent layers or throughout the entire dermis by varying the cooling temperature using multiple energy and pulse settings.

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REFERENCES

1. Fisher GJ, Wang ZQ, Datta SC, Varani J, Kang S, Voorhees JJ. Pathophysiology of premature skin aging induced by ultraviolet light. N Engl J Med 1997; 337:1419-1428.
2. Leffell DJ. Clinical efficacy of devices for nonablative photorejuvenation. Arch Dermatol 2002; 138:1503-1508.
3. Kelly KM, Majaron B, Nelson S. Nonablative laser and light rejuvenation. Arch Facial Plast Surg 2001; 3:230-235.
4. Kim KH, Geronemus RG. Non-ablative laser and light therapies for skin rejuvenation. Arch facial Plast Surg 2004; 6:398-409.
5. Goldberg DJ. Non–ablative subsurface remodeling: Clinical and histologic evaluation of a 1320-nm Nd: YAG laser. J Cutan Laser Ther 1999; 1:153-157.
6. Schmults CD, Phelps R, Goldberg DJ. Nonablative facial remodeling: Erythema reduction and histologic evidence of new collagen formation using a 300-microsecond 1064-nm Nd:YAG laser. Arch Dermatol 2004; 140:1373-1376.
7. Goldberg DJ, Sarradet D, Hussain M, Krishtul A, Phelps R. Clinical, histologic, and ultrastructural changes after nonablative treatment with a 595-nm flashlamp pumped dye laser: Comparison of varying settings. Dermatol Surg 2004; 30:979-982.
8. Goldberg DJ, Cutler KB. Nonablative treatment of rhytids with intense pulsed light. Lasers Surg Med 2000; 26:196-200.
9. Levy JL, Trelles M, Lagarde JM, Borrel MT, Mordon S. Treatment of wrinkles with the nonablative 1,320-nm Nd:YAG laser. Ann Plast Surg 2001; 47:482-488.
10. Ross EV, Sajben FP, Hsia J, Barnette D, Miller CH, McKinlay JR. Nonablative skin remodeling: Selective dermal heating with a mid-infrared laser and contact cooling combination. Lasers Surg Med 2000; 26:186-195.
11. Souil E, Capon A, Mordon S, Dinh-Xuan AT, Polla BS, Bachelet M. Treatment with 815-nm diode laser induces long-lasting expression of 72-kDa heat shock protein in normal rat skin. Br J Dermat 2001; 144:260-266.
12. Capon A, Souil E, Gauthier B, Sumian C, Bachelet M, Buys B, Polla BS, Mordon S. Laser assisted skin closure (LASC) by using a 815-nm diode-laser system accelerates and improves wound healing. Lasers Surg Med 2001; 28:168-175.

13. Yasuda K, Hirayoshi K, Hirata H, Kubota H, Hosokawa N, Nagata K. The kruppel – like factor Zf9 and proteins in the Sp1 family regulate the expression of HSP47, a collagen–specific molecular chaperone. J Biol Chem 2002; 277:44613-44622.
14. Nagata K. HSP47 as a collagen-specific molecular chaperone: function and expression in normal mouse development. Semin Cell Dev Biol 2003; 14:275-282.
15. Nagata K, Hirayoshi K, Obara M, Saga S, Yamada KM. Biosynthesis of a novel transformation-sensitive heat-shock protein that binds to collagen. J Biol Chem 1988; 263:8344-8349.
16. Wang JF, Olson ME, Winkfein RJ, Kulyk WM, Wright JB, Hart DA. Molecular and cell biology of porcine HSP47 during wound healing: complete cDNA sequence and regulation of gene expression. Wound Rep Reg 2002; 10:230-240.
17. Keagle JN, Welch WJ, Young DM. Expression of heat shock proteins in a linear rodent wound. Wound Rep Reg 2001; 9:378-385.
18. Kuo T, Speyer MT, Ries WR, Reinisch L. Collagen thermal damage and collagen synthesis after cutaneous laser resurfacing. Lasers Surg Med 1998; 23:66-71.
19. Mordon S, Capon A, Creusy C, Fleurisse L, Buys B, Faucheux M, Servell P. In vivo experimental evaluation of skin remodeling by using an Er:Glass laser with contact cooling. Lasers Surg Med 2000; 27:1-9.
20. Maitland DJ, Walsh JT Jr. Quantitative measurements of linear birefringence during heating of native collagen. Lasers Surg Med 1997; 20:310-318.
21. Sankaran V, Walsh JT Jr. Birefringence measurement of rapid structural changes during collagen denaturation. Photochem Photobiol 1998; 68:846-851.