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Abstract. Induction of heat shock protein (Hsp) expression correlates with cytoprotection, reduced tissue damage, and accelerated healing in animal models. Since Hsps are transcriptionally activated in response to stress, they can act as stress indicators in burn injury or surgical procedures that produce heat and thermal change. A fast *in vivo* readout for induction of Hsp transcription in tissues would allow for the study of these proteins as therapeutic effect mediators and reporters of thermal stress/damage. We used a transgenic reporter mouse in which a luciferase expression is controlled by the regulatory region of the inducible 70 kilodalton (kDa) Hsp as a rapid readout of cellular responses to laser-mediated thermal stress/injury in mouse skin. We assessed the pulse duration dependence of the Hsp70 expression after irradiation with a CO_2 laser at 10.6 μm in wavelength over a range of 1000 to 1 ms. Hsp70 induction varied with changes in laser pulse durations and radiant exposures, which defined the ranges at which thermal activation of Hsp70 can be used to protect cells from subsequent stress, and reveals the window of thermal stress that tissues can endure. *© 2011 Society of Photo-Optical Instrumentation Engineers (SPIE).*
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1 Introduction

Induction of heat shock protein (Hsp) expression exerts a cytoprotective effect in cultured cells and appears to correlate with improved healing after tissue injury in animal models.[1,2] This family of proteins can also be used as an indicator of thermal stress for cases of burn injury or surgical procedures that produce heat and thermal stress. Therefore, genetic elements that regulate the transcription of Hsp's can be used to generate reporter genes that enable the study of the stress response by providing a visible readout for increased transcription. In addition, such a reporter allows for the study of these proteins as mediators of therapeutic effects and as a response to thermal stress. We previously described a transgenic reporter mouse in which luciferase expression is controlled by the regulatory region of the inducible 70 kilodalton (kDa) Hsp (Hsp70A1).[3] This mouse model allows for the measurement of the Hsp70-directed luciferase expression in live animals using *in vivo* bioluminescence imaging (BLI), and here, we tested responses to fast, high temperature stresses.

The protective effects of Hsp's are likely mediated by the ability of these proteins to function as molecular chaperones preventing inappropriate protein aggregation and facilitating the transport of immature proteins to target organelles for packing, degradation, or repair.[4] Expression of Hsp has been demonstrated after exposure to a variety of environmental and pathological stresses, and much of the focus has been on the 70 kilodalton protein, Hsp70.[4] Increased expression of Hsp70 in response to temperatures slightly above physiological temperatures (e.g., 42°C), for periods of several minutes has been studied as a potential treatment for indications such as ischemic heart disease, diabetes, neurodegeneration, cancer, and thermal damage.[4–7] High temperature, short duration thermal stresses have not been well-studied in mammalian tissues.

Ablation techniques that employ lasers for tissue removal or remodeling can result in very high temperatures for extremely short periods of time, and levels of Hsp expression in adjacent tissues may correlate with healing or may predict tissue damage. Understanding the patterns of Hsp expression in tissues surrounding ablation sites will reveal the nature of the accompanying stress and may aid in the development of more effective tissue ablation and regeneration strategies. To date, very little data involving the expression of Hsp70 in this rapid, high temperature regime has been obtained. Ferrando et al. first showed induction of Hsp70 in cells using a CO_2 laser with a 1 s pulse duration with similar parameters to those employed in this research.[8] Other authors have quantified the expression of Hsp70 using bioluminescence with a similar construct to the one used here in both cells and "raft cultures," a surrogate tis-

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sues model[3,9,10] after exposure to thermal stress applied by both water bath and laser.

In contrast to tissue stress responses at temperatures only slightly above physiologic levels, the effects of high temperature exposure for extremely short durations may lead to significantly different responses. This is because the stress response is determined by both the time of energy deposition (e.g., laser pulse duration), and the thermal diffusion time which can greatly exceed the energy deposition time at short pulse durations due to thermal confinement. Thus, the rate of heat transfer sets a limit to a biologically relevant duration of thermal exposure. Taking a typical value of heat conductivity for biological tissue of 0.6 $W\ m^{-1}$ K and cell size of 10 μm, the heat dissipation time from a single cell within living tissue is on the order of 100 μs.

Cell layers are exposed to short pulses of heat during laser microsurgery procedures such as selective therapy of the retinal pigment epithelium,[11] or tissue surface treatments with a penetration depth of a few micrometers. Similar conditions can also occur in the vicinity of a plasma generated by femtosecond laser-induced dielectric breakdown.[12] To refine the laser parameters (wavelength, spotsize, pulse duration, radiant exposure) appropriate for laser surgery and tissue remodeling, as well as diagnostic laser applications, it is important to understand the spatial and temporal patterns of cellular responses to thermal insults up to, and including, the threshold for irreversible cellular damage or ablation in these short, high temperature pulses.

In a previous study,[3] we generated an Hsp70-luc reporter gene in NIH3T3 fibroblast cells and used these cells to determine the baseline and dynamic response of Hsp70 expression levels on a scale of minutes to seconds. Transfected cells were first treated at 42°C in a water bath for durations of 20 min, and the luciferase expression was monitored at 2 h intervals up to 10 h, and then at 24 h (Ref. 3). Shorter exposures to higher temperatures were studied using a temperature gradient from 50 to 64°C for exposure times from 5 to 35 s.[3] In this and other studies,[3,9] cells treated with shorter durations (on the order of seconds) were found to survive higher temperatures. In addition, this cell line has been used to determine if there is a cytoprotective effect in preshocking the cells to 43°C for 30 min prior to a 50 min heating of the cells at 45°C for comparison between Hsp70-deficient murine embryo fibroblast cells. It was found that the Hsp70 expression, as indicated by the reporter gene, contributes significantly to cellular thermotolerance.[13] However, extremely short durations, corresponding to thermal confinement times (on the order of microseconds), were not evaluated.

Other authors have developed transgenic (Tg) Hsp70 mice for thermal stress analysis.[14–17] We developed a transgenic model based on our previous Hsp70A1-luc construct, but with a dual reporter comprised of firefly luciferase and enhanced green fluorescent protein (eGFP) joined by the 2A "ribosome slippage site" from the foot and mouth disease virus (FMDV) (Hsp70A1-luc-2A-GFP).[3,9,18,19] This dual reporter construct was designed to enable macroscopic analysis of an Hsp70 expression *in vivo* via luciferase expression using BLI and microscopic assays using eGFP. The resulting Tg mouse line was previously used to assess the laser thermal stress and spatiotemporal patterns of the Hsp70 expression in the skin *in vivo* after 10.6 μm carbon dioxide (CO_2) laser irradiation at differing energy levels on a limited time scale of 1 s.[20] Microarray analysis was also performed using the same pulse duration and energy on this mouse to identify spatially distinct gene expression patterns responding to thermal injury.[21] This research compared the thermally damaged laser spot with the thermally stressed adjacent region of Hsp70-luc expression. It was found that 69 genes were up-regulated in the adjacent region and 145 genes were up-regulated in the laser irradiation region at the 7 h time point following laser irradiation.[21] It was found that the severe damage of the laser region did not compromise general transcriptional activity, since there were genes with increased expression. which supports the idea of a graded response corresponding to the extent of stress in this region. This mouse line has also been used to reveal Hsp70 expression patterns following the laser thermal stress/damage using a free electron laser at 2.94, 6.1, and 6.45 μm in wavelength[22] and a 1.85 μm pulsed diode laser.[23] In addition to laser related research, this mouse line has also been used to study the expression of Hsp70 following a high intensity focused ultrasound stimulation *in vivo*.[24]

The goal of this study was to determine the pulse duration dependence of Hsp70 expression by varying the pulse duration of a CO_2 laser at 10.6 μm in wavelength from 1000 to 1 ms in duration. We performed these studies in the skin of Tg mice at various energies. The differences in expression at the various energies were assessed using *in vivo* bioluminescence imaging, and were compared with thermal imaging and mathematical modeling to determine the energies that had the greatest effects on Hsp70 expression, and a complete analysis of the pulse duration dependence. By using bioluminescence imaging, we were able to analyze the Hsp70 response *in vivo* related to 5 different time points, 6 different pulse durations, and 15 different radiant exposures, which would not have been possible with traditional histology.

2 Methods

2.1 *Transgenic Reporter Mouse*

The Tg mouse line (Hsp70A1-L2G) on the friend virus B (FVB) background has been described previously[20] and has been bred to homozygosity. Four to eight week old female mice were used for the *in vivo* experiments. The coding region for enhanced green fluorescent protein (eGFP, Clontech, Palo Alto, California) is included in this dual reporter gene L2G (Luc-2A-eGFP),[25] which consists of a modified firefly luciferase gene (pGL3, Promega Inc., Madison, Wisconsin) joined to 54 base pairs (bp) of the FMDV 2A sequence[19] and, via 24 bp of polylinker sequence, to the eGFP gene at the 3′ end. The GFP signals in these studies were weak and not reported in this study.

2.2 *Laser Thermal Stress Analysis*

The tissue response to thermal stress was generated with a 100 W CO_2 laser (PLX-100, Parallax Technology Inc., Waltham, Massachusetts) triggered in pulsed mode, and was evaluated in the Hsp70A1-L2G mouse. Using the CO_2 laser, the dorsal skin of the transgenic mice was irradiated at a wavelength of 10.6 μm. Mice were shaved and depilated 24 h — prior to irradiation with the laser; – 24 h was sufficient time to allow residual expression of Hsp70, caused by shaving, to return to the background.[20] Experiments were performed at 500, 100, 30, 10, and 1 ms pulse durations. Four irradiation spots were generated at separate locations on the back of each mouse in a grid pattern. An 6 mm diameter flat-top spot was used to generate an affected area large enough to provide adequate resolution

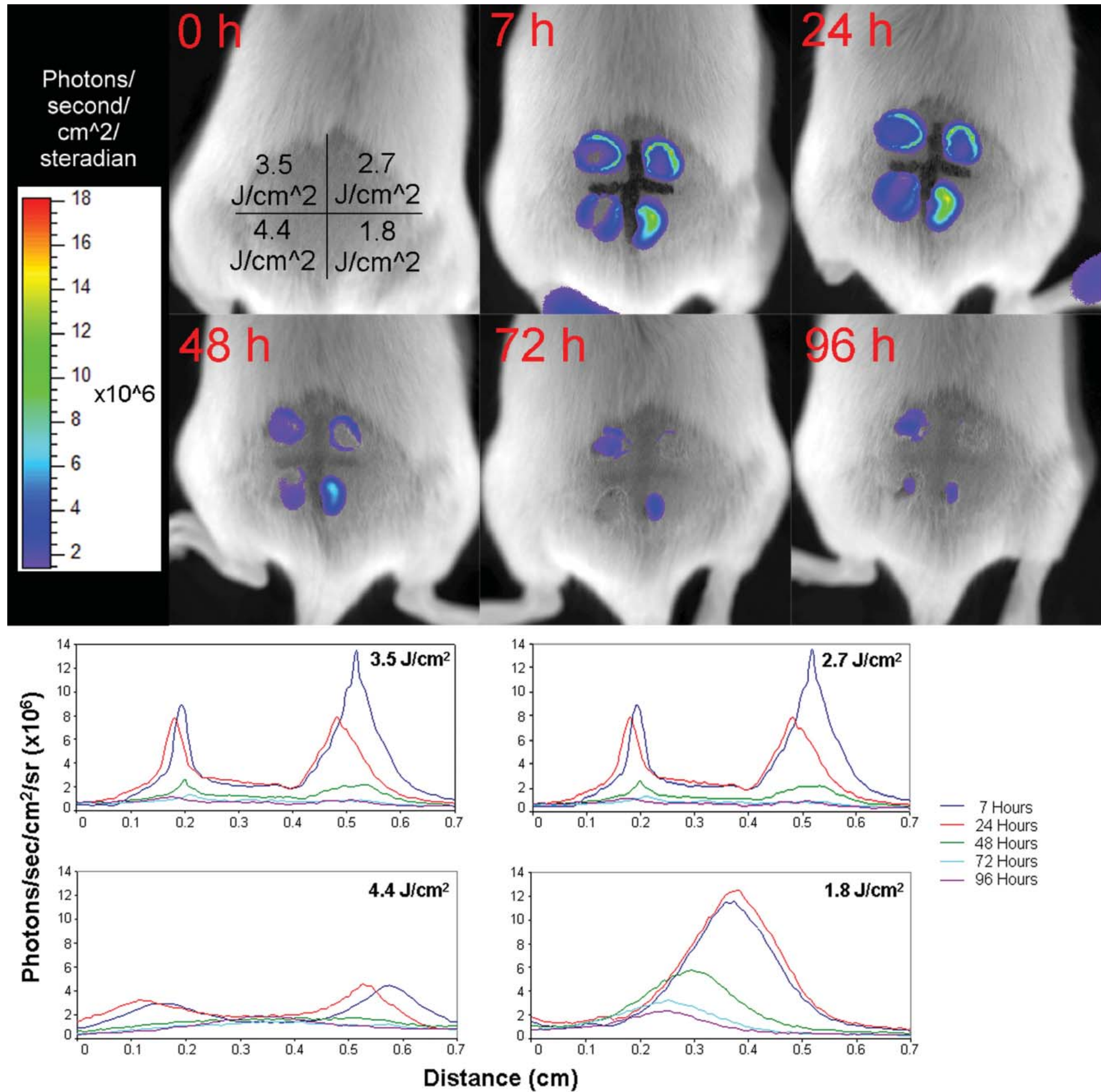

Fig. 1 Bioluminescent signals indicative of the *in vivo* thermal response of mouse skin following laser injury. (a) A 500 ms CO_2 laser pulse at $\lambda = 10.6\ \mu$m was used at four different radiant exposures (1.8, 2.7, 3.5, and 4.4 J/cm^2). One representative mouse is shown here with images taken at 7, 24, 48, 72, and 96 h post laser heating. The images are represented with a common false-color scale representing the emitted bioluminescence radiance from 2×10^6 to 1.8×10^7 photons/second/centimeter^2/steradian. All images were taken 10 min after IP injection of luciferin. Note the strong decrease of signal for the lowest radiant exposure after 48 h compared to the 7 h time point. (b) Each panel consists of a series of 7 mm long profile plots across the 6 mm laser lesion at each of the five time points and four radiant exposures for the 500 ms CO_2 laser pulse. A Gaussian pattern of expression is seen for the lowest radiant exposure, while a much different pattern of expression is seen for the highest radiant exposures. At the high radiant exposures, the center region of the pulse is unable to emit light at the early time points. The region immediately surrounding this center region emits light corresponding to high levels of Hsp70 expression at these time points. The surrounding region's expression declines to background levels by the 72 or 96 h time point, while the expression in the center region does not show any increase in signal during the entire time range.

to determine the expression profile using an IVIS 200 imaging system (Xenogen Product from Caliper Life Sciences, Alameda, California), with high resolution binning[4] and a 3.9 cm field of view to obtain a 60 μm resolution across each treated location. BLI was performed 10 min after intraperitoneal (IP) injection of 50 μl/10 g mouse of 30 mg/ml stock solution of D-luciferin (150 mg/kg body weight) (Biosynth L-8220; Staad, Switzerland) with a 1 min integration time. Imaging was performed at 7, 24, 48, 72, and 96 h post-irradiation with the laser on a minimum of three mice per experiment.

For the 500 ms pulse duration, radiant exposures of 1.8, 2.7, 3.5, and 4.4 J/cm^2 were used [Figure 1(a)]. The radiant exposures used for 500 ms were the same as those previously published using a 1 s pulse duration.[20] Radiant exposures of 0.4, 0.9, 1.8,

and 2.7 J/cm^2 were used for the bioluminescent analysis of 100 and 30 ms pulse durations. Radiant exposures of 2.24, 2.98, 3.72, 4.48, 5.22, 6.22, and 7.46 J/cm^2 were used for the 10 ms pulse duration analysis. Radiant exposures of 0.75, 1.49, 2.24, and 2.98 were used for the 1 ms pulse duration.

2.3 *Laser Temperature Change Determination*

Analysis for the laser temperature change was performed in two ways. The first method performed was by using a thermal camera (A20M Researcher, FLIR Systems, Boston, Massachusetts) to analyze the skin temperature following the laser pulse. Since the thermal camera was sensitive to the 10.6 μm wavelength of the CO_2 laser, it was necessary to delay the camera detection by 6 ms with a shutter, following the end of the laser pulse to both prevent damage to the camera chip and to obtain useful results. This thermal camera was used to analyze the four radiant exposures at 1 s, 500 ms, and 100 ms. In addition, the temperatures were also measured at 0.4, 0.9, and 1.8 J/cm^2 at 30 ms. It was not possible to detect an accurate temperature change with the thermal camera for any pulse durations shorter than 30 ms due to the limitations of the 30 Hz repetition rate of the thermal camera, and the required 6 ms delay in detection following the end of the laser pulse.

To obtain accurate numbers for comparison, mathematical analysis of the temperature was performed using FEMLAB (Comsol Inc., Burlington, Massachusetts). The specific radiant exposures and pulse durations as described before were used for the mathematical analysis of skin tissue for comparison to the thermal camera results. The epidermis was assumed to be 40 μm given that the average epidermal thickness usually ranges from 20 to 60 μm in thickness depending upon the age of the mouse, the older the mouse, the thinner the epidermis. Dermal thickness ranges from 150 to 170 μm, so 160 μm was used, which gave an average skin thickness of the epidermis and dermis at 200 μm. The light absorption of the epidermis was taken as the water absorption reduced proportionally to the water concentration in the epidermis (60%). While the absorption of the dermis is stronger, there is not much light that can make it to that depth. A 5 mm Gaussian beam was used for the FEMLAB modeling.

3 Results

BLI was used as an indirect measure to determine the relative levels of the Hsp70 expression at various times following laser injury to the skin. This imaging approach enabled macroscopic analysis of individual lesions in live mice with a temporal resolution that is relevant to Hsp70 induction [Fig. 1(a)]. The relatively short half-life of luciferase (~2 h, Refs. 26 and 27) allows for the study of both the activation and cessation of expression, and its dependence on adenosine triphosphate (ATP) for activity, which can be used as an indicator of cell viability. The representative image in Fig. 1 reveals the temporal responses of the heat shock reporter to the irradiation of skin with a CO_2 laser over a range of energies. Profile plots of each lesion generated by the laser were obtained at each of the five imaging times for comparison [Fig. 1(b)] by placing a 9 mm line across the center of each lesion using Living Image Software (Xenogen Product from Caliper Life Sciences) and examining the profile of expression across this line. The profile plots are from the same images used in Fig. 1(a).

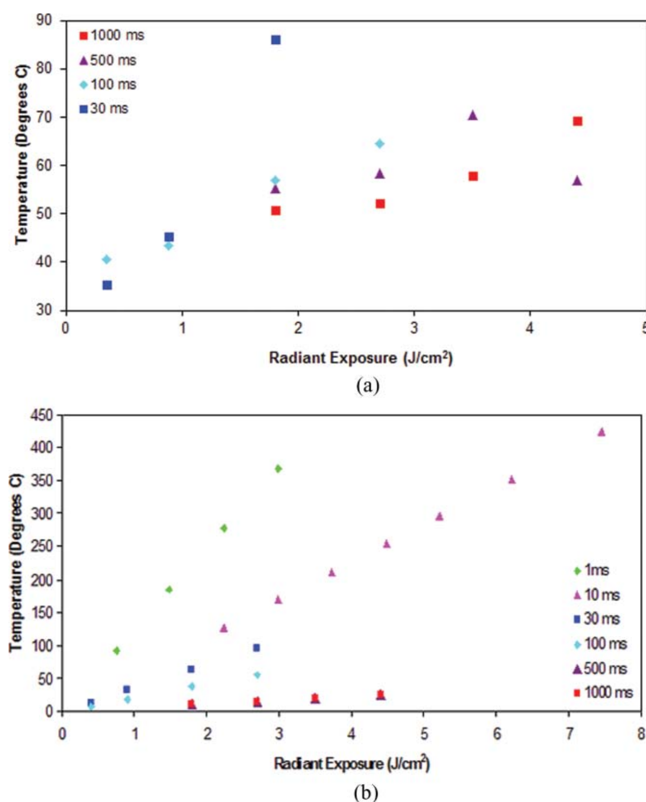

Fig. 2 (a) The measured temperatures from a thermal camera taken 6 ms after the end of the laser pulse on the mouse skin for 1000, 500, 100, and 30 ms pulse durations. (b) The FEMLAB calculated temperatures for 1000, 500, 100, 30, 10, and 1 ms pulse durations.

The image analysis shows a qualitative peak of expression at 7 h post-irradiation, which is apparent up to 96 h. Intermediate energy levels caused reduced expression. The results are more clearly demonstrated by the linear profile plots across the center of each lesion. By contrast, the highest radiant exposure images show a ring-like expression contour surrounding an inner region of reduced expression which is most likely correlating with decreased cell survival. The luciferase activity is dependent on cell viability due to its energy requirement (ATP). This expression contour is seen as two distinct peaks at either edge of the lesion in the profile plots. Intermediate energy levels produced a Gaussian profile similar to those obtained for the lowest radiant exposures.

To allow for an accurate comparison of the bioluminescence data, it was essential to compare the temperature changes, due to the varying laser pulse durations and radiant exposures. Figure 2(a) shows the results obtained from the thermal camera from 1000 to 30 ms in pulse duration. Consistent rises in temperature between 1000 and 100 ms in pulse duration were seen with increasing temperatures from 35°C to 70°C. Given the starting temperature of 23°C of the mouse's skin, this correlates to a 12 to 47°C temperature change. Differences were seen using the shortest pulse durations with the radiant exposures that we used. The first change was seen using 1.8 J/cm^2 at 30 ms in pulse duration. This showed an 86°C temperature peak (a 63°C increase in skin temperature).

The FEMLAB analysis is shown for comparison in Fig. 2(b). Similar trends are seen for the comparison with the thermal camera results; however, the temperatures for the laser pulses used from the mathematical modeling were consistently lower than those measured with the thermal camera. This difference was due to the limited assumptions in the FEMLAB analysis. However, given the large increases in temperature change that are seen with the 30, 10, and 1 ms pulse durations, the correlation of bioluminescence data with the thermal analysis methods can be observed (Fig. 2).

In addition to the thermal analysis, a measure of peak radiance was obtained in calibrated units of photons/second/centimeter^2/steradian from each image, as shown in Fig. 3, with a minimum of $N = 3$ mice for each data point. The peak radiance was obtained by placing a region of interest (ROI) with a 7-mm diameter around each irradiated spot of each mouse image, and measuring the peak radiance using LivingImage Software (Caliper Life Sciences). The data obtained at 7 h following the laser treatment is shown in Fig. 3(a). Figure 3(b) shows the results at 24 h, and Fig. 3(c) shows the results obtained at 48 h following the laser treatment. The bioluminescent signals showed an average 1.2 fold decrease at 72 h and an average 1.9-fold decrease at 96 h, relative to the 48 h time point for all pulse durations (data not shown).

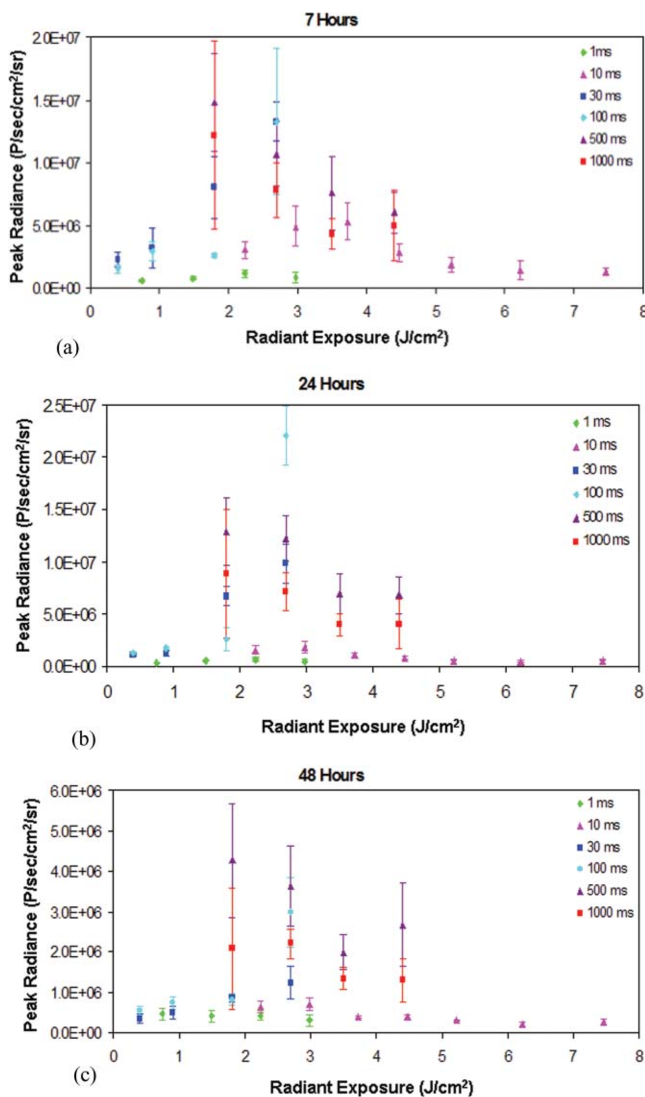

Fig. 3 The average radiance in photons/second/cm^2/steradian of a 7-mm diameter ROI around each laser heated spot is shown for each of the radiant exposures used in this experiment at the 7 h time point (a), the 24 h time point (b), and the 48 h time point (c). Each data point is for a minimum of N = 3 mice and the error bars represent the standard error of the mean. A general decrease in the bioluminescence and thus the Hsp70 expression is seen over time, while a late recovery in the center region of the highest radiant exposure region is seen at later time points.

4 Discussion

The specific comparison between radiant exposures and pulse durations can be primarily explained by thermal stress and thermal damage. Thermal stress relates to the Gaussian profile as seen in Fig. 1(b) at 1.8 J/cm^2 in radiant exposure. We observed concomitant decreases in the thermal profile and Hsp70 expression. By the 96 h time point, the response to thermal stress has almost completely diminished—as assessed by the *in vivo* luciferase activity measures. Thermal damage, however, is more obvious for the 3.5 and 4.4 J/cm^2 radiant exposures.

The difference seen between these two energies can be explained by thermal damage. At the 3.5 J/cm^2 radiant exposure, the cells at the center of the laser spot have been damaged or killed, and the luciferase activity is only apparent in the surrounding cells, as seen by the two peaks. Levels of expression return to near normal levels by 48 to 72 h. At the highest energy used for this pulse duration, at 4.4 J/cm^2, there is a less observable signal from the surrounding cells, likely due to cell death or damage from thermal diffusion from the site of the laser spot. As the injury heals and new cells are recruited, these cells would not likely express Hsp70, since they have remained at body temperature.

The FEMLAB results showed thermal diffusion over time. The differences that were seen could be accounted for by evaporation, which plays an important role at the higher temperatures seen with the shorter pulse durations. In addition, thermal diffusion also becomes an issue for the longer pulse durations on the order of 500 and 1000 ms. Since the temperature was not constant during the laser pulse, it was not possible to compare this data with a direct theory. To fit the data with the Arrhenius law, the effective pulse length was introduced as the length of an imaginary constant temperature heat pulse with a temperature equal to the maximum temperature of the real pulse, which produces the same amount of damage as a real pulse. The peak temperature at the beginning of thermal diffusion was used for comparison with the thermal camera results. Given the 1 s delay of the thermal camera detection and the limiting assumptions that were necessary for operating FEMLAB, there were consistent differences seen between the data obtained with the thermal camera and that from FEMLAB; however, the overall thermal analysis of the laser parameters used were fairly consistent.

There is a clear correlation between Hsp70-luc expression and the laser pulse durations. The highest bioluminescent signal was observed for the longest (1000 to 500 ms) pulses. This is most likely due to the radiant exposures used, as well as the thermal diffusion. The overall temperatures achieved at these pulse durations show the thermal limit of cell damage/death with regard to time and temperature. The bioluminescent

signal is less for each pulse duration/radiant exposure until the lowest is seen for the shortest (1 ms) pulse. For the 100 and 30 ms pulse duration, the rise in signal jumps drastically for the 30 ms pulse duration as the radiant exposure increases. This shows that we had not reached the complete death threshold for the cells at these energies. Even though the temperatures were much higher, due to the short pulse duration, the cells survived high temperatures for short durations. The 100 ms pulse duration showed a greater bioluminescent signal at the 2.7 J/cm^2 regime at the 24 h time point [Fig. 3(b)], compared to the 7 h time point [Fig. 3(a)]. This may be explained by a delay in Hsp70 expression due to significant thermal damage to the cells. This was also seen for the 1000 ms pulse as described previously.[20] By the 48 h time point, the overall signal at each pulse duration had decreased from that of the earlier time points. At this time, the signals from the shorter pulses had returned to near baseline levels, while the signals from the longer pulses show a continual decline up to the 96 h time point (data not shown).

By performing *in vivo* bioluminescence imaging, we were able to analyze the long term response to thermal stress and damage following a large number of pulse durations and radiant exposures that would not have been possible using traditional histology. The highest pulse duration analyzed showed the greatest increase in Hsp70-mediated luciferase expression at the 7 h time point. While shorter pulse durations achieved a much higher temperature, both quick thermal diffusion at lower energies, and cell death or cellular damage at higher energies may have prevented strong increases in Hsp70 expression. By the 72 to 96 h time point, Hsp70 levels, as indicated by luciferase expression, had returned to near baseline levels for all of the pulse energies used. The cytoprotective effects of increased Hsp70 are likely greater for the longer pulse durations, and the lower radiant exposure at shorter pulse durations may prevent irreparable cellular damage and cell death.

5 Conclusions

In this study, we have used a transgenic mouse line that, in conjunction with BLI, enables the rapid analysis of cellular responses to laser-mediated thermal injury. The extent and duration of Hsp70 induction varied with changes in laser pulse durations and radiant exposures of the skin and these data serve to define the ranges at which thermal activation of Hsp70 can be used to protect cells from subsequent stress, and reveals the window of thermal stress that tissues can endure.

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