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Millimeter-Wave Pulsed Heating in Vitro: Effect of Pulse Duration

Rosa Orlacchio , Yann Le Page, Yves Le Dréan , and Maxim Zhadobov , *Senior Member, IEEE*

Abstract—The aim of this work is to compare the response of A375 melanoma cells following 90 min of exposure to trains of 1.5 or 6 s millimeter-waves (MMW)-induced thermal pulses with the same temperature dynamics. Phosphorylation of heat shock protein 27 (HSP27) and activation of cleaved Caspase-3 were used as markers of cellular stress and apoptosis, respectively. Immunofluorescence was used to observe and precisely quantify the cellular response as a function of the spatial distribution within the exposed area. Results show that cellular response was stronger when cells were exposed to a train of 1.5 s compared to 6 s heat pulses despite the same average temperature dynamics. Cellular apoptosis induced by 1.5 s pulses was about 50% greater compared to the one induced by 6 s pulses in the area of maximal thermal stress. Similarly, HSP27 phosphorylation was approximately 20% stronger than the one induced by 6 s pulses, and mainly focused within a small area of a few mm². Cellular response to MMW induced by pulsed heating does not only depend on the peak, average, and minimum temperature. It is a function of combination of the pulse parameters, including duration, peak power, and period. MMW-induced heat pulses can be efficiently used to induce cellular stress and apoptosis in melanoma cells as a promising innovative tool for the treatment of superficial skin cancer. Adaptative therapies might be envisaged by tuning the pulse shape as a function of the desired effect.

Index Terms—Caspase 3, heat pulses, hyperthermia, HSP27, melanoma, millimeter waves (MMW), in vitro experiments.

I. INTRODUCTION

THERMAL treatment of cancer represents a promising alternative for damaging or killing tumoral cells with minimal injury to healthy tissues. In hyperthermia treatments, biological tissues are heated between 40 and 47 °C to sensitize cells to other treatments, such as radiotherapy and chemotherapy. During ablation, temperatures above 48 °C are applied for a short duration (between a few seconds and 4–6 minutes) to induce irreversible cellular injury. Three main effects can be induced at the cellular level depending on the thermal dose:

1) cytotoxicity [1] or

- 2) radiosensitization [2], both as responses to a heat shock typically > 43 °C, and
- 3) thermotolerance [3] appearing during continuous heating at temperatures < 43 °C or short exposure at temperatures > 43 °C followed by incubation at 37 °C [4], [5]. Relatively high activation energy behind these changes (within 120–146 kcal/mole) suggests that protein unfolding is the common transition triggering such effects [1].

Cellular thermotolerance is the ability of mammalian cells exposed to a non-lethal shock to acquire a transient resistance to subsequent exposures at elevated temperatures [3]. This phenomenon is mediated by overexpression of cellular chaperones and heat shock proteins (HSP) that assist heat-denatured proteins to refold into their native and functional conformations, thereby playing a protective role. Various forms of cellular stress, such as moderate heat, may dramatically increase the phosphorylation of small molecular weight chaperones, such as HSP27, to promote the recognition of client proteins in order to trap and protect the stress-induced misfolded proteins from aggregation [6]. On the contrary, when cells undergo a severe heat shock, the HSP response may be not able to cope with thermal stress, provoking critical denaturation of the proteins that leads to a direct cellular death [7], possibly mediated by activation of a group of cysteine proteases, called Caspases [8]. Heating of biological tissues may be achieved by means of different power sources such as ultrasound or electromagnetic waves, including radiofrequency (RF), microwave (MW), ultraviolet (photothermal therapy), and light (lasers). Our recent results suggest that the upper part of the millimeter-wave (MMW) band may be used for thermal treatment of superficial skin cancer, such as spreading melanoma, located within the MMW penetration depth (i.e., ~ 0.5 mm at 60 GHz) [9], [10], [11]. We showed that continuous wave (CW) MMW-induced heating at temperatures higher than 46 °C may minimize cellular thermotolerance while inducing apoptosis [10]. Moreover, MMW-induced heat pulses with duration of 1.5 s, period of 20 s, amplitude of 10 °C, and peak steady-state temperature > 48 °C were able to induce a significant cellular injury in melanoma cells compared to CW-induced heating with the same average temperature rise of about 42 °C [9], [11]. These results are in agreement with previous studies demonstrating that pulsed electromagnetically-induced heating leads to stronger cellular damage compared to continuous constant heating [12], [13]. During pulsed exposure, high peak temperature, necessary to kill cancer cells, can be rapidly achieved in the warm-up phase of the pulse, maintaining the average temperature under the lethal threshold. This results in avoiding a possible injury of

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healthy tissues surrounding the tumor, as opposite to continuous heating [14]. The biological rationale for the application of heat to treat melanoma is mediated by a high susceptibility of this cancer to elevated temperatures [15], [16]. In addition, activation of an apoptotic pathway mediated by activation of Casp-3,-6, and 7 [15] or by the endoplasmic reticulum [17] was observed following exposure to continuous heating within the 41–48 °C range or pulsed heating with a steady-state peak temperature of approximately 49 °C [9].

Conventional models of cellular response usually employ as a metric the steady-state temperature, overall exposure duration, and heat dose (in terms of degree \times minutes [18] or cumulative equivalent minutes [19]). However, during the pulsed heating the number of warm-up intervals may vary from one (for continuous constant heating) to tens or hundreds in case of pulsed periodic heating. The latter is characterized by cumulative metrics mentioned above as well as by intrinsic parameters of pulses, including pulse duration, duty cycle, peak temperature, average temperature, and temperature rise rate. While their role remains underexplored so far, these parameters of thermal pulses could specifically alter heat-sensitive cellular processes.

The main objective of this study is to extend the current knowledge about the heat-induced modifications in melanoma cells by MMW-pulsed heating as a function of the heat pulse duration for adaptative therapies. To this end, results previously obtained using short 1.5 s pulses [9], [10], were compared, for the first time, to longer pulses (6 s), with the same minimum, peak, and average temperature rise. Cellular response was quantified in terms of cellular injury after 90 min of exposure mediated by the activation of cleaved Casp-3 and compared to heat shock response mediated by phosphorylation of HSP27.

II. MATERIALS AND METHODS

A. Exposure Setup and Electromagnetic Dosimetry

The exposure system as well as the electromagnetic dosimetry were described in detail in [10], [20]. Briefly, a thin cell monolayer cultured in a well of a 12-well tissue culture plate was exposed from the bottom 5 mm away from an open-ended rectangular WR15 waveguide (WG) antenna (aperture size 3.81×1.905 mm²), used as a source of the MMW radiation. Experiments were performed inside an incubator (Memmert UNE400, Schwabach, Germany), which temperature was set to 32 °C to avoid cellular overheating due to the fast temperature elevation occurring in the beginning of exposure. This allowed to reach the desired steady-state temperature with a peak of about 49 °C and average around 42 °C in the center of the exposed area [20]. A high-power generator (QuinStar Technology, Torrance, CA) operating at 58.4 GHz with an output power up to 3.7 W was used as a narrowband source in CW or PW amplitude modulation regimes. Amplitude modulation of the MMW radiation was obtained through a programmable pulse generator (HMP 4040, Hameg Instruments, Hampshire, U.K.) allowing to adjust peak power and pulse duration.

Numerical simulations were performed using the finite integration technique (FIT) solver of CST Microwave Studio 2018 (Computer Simulation Technology [CST]; Dassault Systemes,

Darmstadt, Germany) as detailed in [10], [20]. Due to the shallow penetration of the MMW, the specific absorption rate (SAR) distribution in the exposed cell monolayer rapidly decreases along all the directions of the well [10], [20]. Computed local SAR in the cell monolayer, at the center of the exposure – aligned with the boresight axis of the open-ended WG – is equal to 19.8 W/kg per 1 W of incident power. Note that in a fraction of mm along the well height (~ 0.13 mm) SAR drops by about 50%.

B. Microscale Temperature Measurement

The temperature increase induced at the cellular level by the exposure to CW and PW MMW was measured using a K type thermocouple (TC) probe with the lead diameter of 75 μ m (RS Components, Corby, U.K.). Precise protocol and instrumentation employed for temperature acquisition and recording are detailed in [10]. Briefly, the sensor of the TC was located on the exposure beam axis with its leads lying on the bottom of the well perpendicular to the *E*-plane to avoid induction of currents in the TC and possible related artefacts [10], [21], [22], [23], [24]. Note that under the exposure conditions of this study, the presence of a small metallic TC (diameter < 1 mm) during the exposure introduces a local (within 0.1 mm around the TC) increase of SAR (along the *E*-plane), by about 5%, close to the TC tip that practically does not impact heating of the exposed sample (difference of heating dynamics with and without TC is less than 1%) [10]. Temperature measurements and cell exposures were performed in separate experiments. In this way, possible cellular damage, contamination, and local increase of SAR in the cell monolayer due to presence of the TC were avoided. Temperature measurements were performed at least in 3 independent experiments for each waveform.

C. Cell Culture, Exposure, and Immunofluorescence

The human malignant A375 melanoma cell line was purchased from American Type Culture Collection (ATCC, Molsheim, France). Cells were cultured as previously described [9] in a standard humidified incubator at 37 °C and 5% CO₂. Experiments were performed at earlier passages between 4 and 10, to avoid a drift of the cellular population. For exposure, cells were seeded in 3 wells of a 12-well tissue culture plate at a density $> 3 \cdot 10^4$ cells per well. One well was exposed to MMW and the other two served as a control (negative control and positive control with chemical treatment to induce heat shock response or apoptosis [data not shown]) [9]. Cells were exposed to MMW for 90 min as previously described [9]. Sham exposures were performed under identical experimental conditions, but with the generator switched off. Since heat shock response may appear after certain delay after heat [25], cells were incubated at 37 °C for 6 hours after exposure. Finally, cells were fixed before proceeding to immunofluorescence (IF) analysis. The IF protocols were described in detail in [9], [26]. In brief, phosphorylated HSP27 and cleaved Casp-3 were detected using specific primary antibodies diluted at 1:200 (Phospho-HSP27 (Ser82), ref 2406, Cell Signaling Technology, Danvers, MA) and 1:500 (Cleaved Caspase-3 (Asp175), ref 96645, Cell Signaling), respectively. Revelation was carried out using fluorescent

secondary antibodies at 1:1000 dilution. Nuclei counterstaining was performed (Hoechst 33342, 10 $\mu\text{g}/\text{mL}$, Sigma-Aldrich) to identify individual cells. 121 square pictures (each of 0.25 mm^2) of cells were automatically taken, and fluorescence of each cell was quantified using a Cellomics ArrayScan VTI HCS Reader (Terro Fisher Scientific) at ImPACcell technological platform (Biosit, University of Rennes 1, Rennes, France). A total area of 30.25 mm^2 was covered within each well. This corresponds to the central area of the exposure, i.e., the zone of the maximal thermal stress. Each picture contained in average about 100 cells. Data of the cellular fluorescence within the nucleus and cytoplasm were analyzed cell-by-cell as a function of the distance from the center of the well, where distance equal to 0 mm was assigned to all cells belonging to the picture taken in the center of the well aligned to the open-ended WG axis as detailed in [10].

D. Thermal Dose

The thermal dose during CW and PW MMW-induced heating was calculated with the cumulative equivalent minute at 43 $^{\circ}\text{C}$ ($\text{CEM}_{43}^{\circ}\text{C}$) [18]:

$$\text{CEM}_{43}^{\circ}\text{C} = \sum_i^n t_i R^{(43-\bar{T})} \quad (1)$$

where n represents the number of intervals in which the duration of the exposure has been divided, t_i is the i -th interval over which temperature is averaged (in minutes), \bar{T} is the average temperature during the time interval t_i , 43 $^{\circ}\text{C}$ is the reference temperature, and R is related to the temperature dependence of the rate of cellular death above and below the reference temperature. Here, $R(T < 43^{\circ}\text{C}) = 0.233$ and $R(T > 43^{\circ}\text{C}) = 0.428$, as experimentally derived in [27] for human skin cells *in vitro*. The averaging interval was equal to 0.3 s to consider the fast temperature variations during the pulse exposure.

E. Statistical Analysis

All experiments were performed in three independent experimental series. Data are presented as mean values and standard error of the mean (SEM). Significance of measured differences were tested with the non-parametric Mann-Whitney Rank Sum test (SigmaPlot Statistics) for significance levels of $p < 0.05$.

III. RESULTS

A. Electromagnetic and Thermal Pulses

Square-wave amplitude modulation was used to create ON/OFF electromagnetic pulses with the duration of 1.5 s and 6 s. This choice is motivated by the fact that using this combination allowed us to obtain the same temperature dynamics, both in terms of peak amplitude of the heat pulse and minimum and maximum temperature rise at steady state, by varying only period and power. This facilitates the comparison and interpretation of results by reducing the number of variables. The pulse parameters are summarized in Table I. The peak power at the open-ended WG input was adjusted for each pulse duration to obtain a peak temperature in a pulse of 10 $^{\circ}\text{C}$ (Fig. 1(a)).

TABLE I
PARAMETERS OF THE ELECTROMAGNETIC PULSES FOR THE TOTAL EXPOSURE DURATION OF 90 MINUTES

| | Pulse duration (s) | |
|---|---------------------|---------------------|
| | 1.5 | 6 |
| Temperature rise rate ($^{\circ}\text{C}/\text{s}$) | 6.7 | 1.7 |
| Period (s) | 20 | 27.9 |
| Duty cycle (%) | 7.5 | 21.5 |
| Number of pulses | 270 | 194 |
| Peak power (W) | 3.7 | 1.6 |
| Average power (W) | 0.275 | 0.334 |
| Pulse energy (J) | 5.55 | 9.6 |
| $\text{CEM}_{43}^{\circ}\text{C}$ (min) | 502.67 ± 128.42 | 1021.67 ± 401.5 |

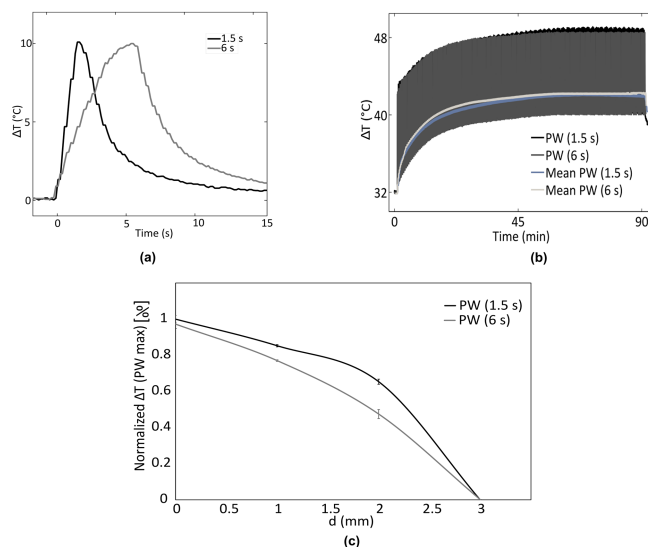


Fig. 1. Temperature dynamics for 1.5 and 6 s MMW heat pulses measured at the cell level on the exposure beam axis. (a) Measured single heat pulses with duration of 1.5 and 6 s. (b) Train of 194 and 270 heat pulses of 1.5 and 6 s, respectively, measured during 90 min of exposure. (c) Spatial distribution of the PW peak temperature rise at steady state (normalized to the temperature at the center of the well, i.e., 0 mm) along the well radius.

Peak power was set to 3.7 W and 1.6 W, corresponding to peak SAR levels of 73.6 kW/kg and 31.8 kW/kg in the cell monolayer, for the 1.5 s and 6 s pulses, respectively. The corresponding temperature rise rate was 6.7 $^{\circ}\text{C}/\text{s}$ and 1.7 $^{\circ}\text{C}/\text{s}$, respectively. Note that for longer durations of the thermal pulse, convection arising in the culture medium strongly impacts the temporal pulse profile decreasing the peak amplitude and increasing the cooling rate resulting in a 6 s heat pulse less sharp than the 1.5 s [20]. Indeed, high SAR gradients caused by local power absorption at MMW in the exposed liquid are responsible for the occurrence of convective currents that drive the heated liquid at the bottom to the top of the well [20]. The period of the pulses was adjusted to 20 s for 1.5 s pulses and 27.9 s for 6 s pulses to maintain the same average temperature dynamics (mean PW in Fig. 1(b)) around 42 $^{\circ}\text{C}$. Average temperature was calculated using a moving average filter with 75 s span through the smooth function of MATLAB.

The average temperature around 42 °C was chosen as it is within the typical temperature range used in the clinical practice of thermotherapy [28]. Note that in order to obtain the same temperature dynamics for both pulse durations, the average power for 6 s pulses (0.334 W) was higher by about 20% compared to the 1.5 s pulses (0.275 W). This allows to compensate for the effect of thermal convection which tends to decrease the peak amplitude and increase cooling of longer thermal pulses [20]. For 90 min of exposure, the pulse train was composed by 270 and 194 pulses, for the 1.5 s and 6 s pulses, respectively. The steady state was reached after about 40–50 minutes from the beginning of exposure.

Maximum thermal dose in the center of the well bottom, corresponding to the highest temperature induced in the cell monolayer, was 502.67 ± 128.42 min and 1021.67 ± 401.5 min for 1.5 s and 6 s PW exposures, respectively (mean \pm standard deviation). CEM43 °C for CW at the center of the exposure was 23.9 ± 9.7 min. Temperature decreases as moving away from the WG axis. Temperature peak, reached at the steady state, during exposure to 1.5 s pulses was equal to 49.2 ± 0.1 °C, 49 ± 0.1 °C, 48.3 ± 0.3 °C, and 46 ± 0.23 °C, respectively on the well axis and at 1, 2, and 3 mm from it. For 6 s pulses convection triggered in the culture medium slightly decreases the peak temperature (differences less than 10%) compared to 1.5 s pulses (Fig. 1(c)). Variation along the radius of the cell monolayer with respect to maximum value at $d = 0$ mm of the PW peak temperature is shown in Fig. 1(c). The maximum relative deviation between 1.5 s and 6 s pulses is of 27% at $d = 2$ mm. Average temperature for both exposure conditions was of 41.7 ± 0.3 °C in the center and it decreased by about 1 °C at 3 mm from the central point towards the lateral sides of the well. Measurements performed along the H -field direction, i.e., perpendicular to the E -field direction, and electromagnetic numerical simulations suggest a similar temperature distribution as the one recorded along the E -field direction in the area of interest.

B. Induction of Apoptosis

Apoptosis is a highly regulated process primarily orchestrated by the activation of a family of proteases named Caspase. The latter, pre-exist in cells as catalytically inactive zymogens (pro-caspase) precursors, activated by cleavage in response to a variety of cell death stimuli. Initiation of apoptosis occurs through either intrinsic or extrinsic pathways both leading to the activation of the major effector caspases, such as Casp-3, which coordinates the cleavage of key proteins, leading to demolition of cellular structures and organelles [8]. In order to quantify the cellular injury, we analyzed the activation of cleaved Casp-3 as a marker of cellular death following the exposure to 1.5 and 6 s PW MMW-induced pulses in the A375 melanoma cell line. As in [9], we calculated the percentage of dead cells as the percentage of cells above the level of background noise of activated Casp-3, labelled through high-content fluorescence microscopy analysis. Results were compared with CW heating at about 42 °C, i.e., the average heating induced during PW exposure.

The spatial distribution of apoptotic cells, in the area of interest, 6 hours after exposure is illustrated in Fig. 2(a). It

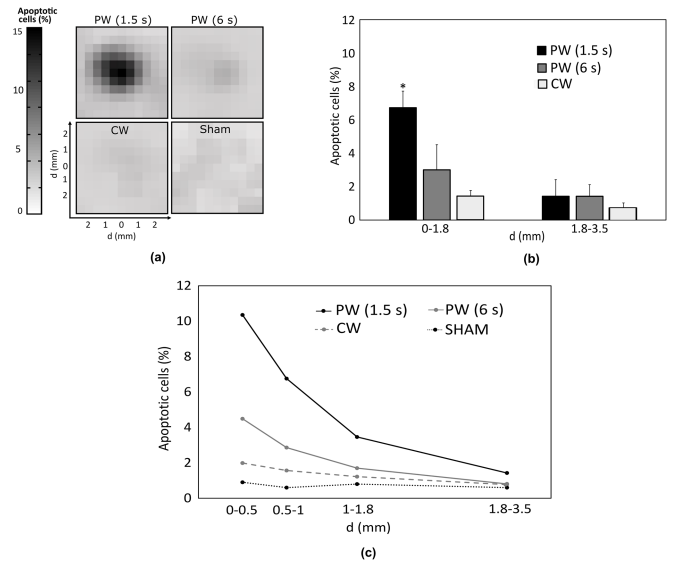


Fig. 2. Percentage of apoptotic cells after 1.5 and 6 s PW exposure compared to CW heating with the same average temperature. (a) Spatial distribution of apoptotic cells for PW (1.5 s), PW (6 s), CW, and sham. (b) Apoptotic response analyzed cell-by-cell 6 h after exposure shown as mean values ($n = 3$) \pm SEM (standard error of the mean) normalized to sham. The data are averaged over two areas around the center of the well. (*) indicates statistical significance compared to CW at $p < 0.05$. (c) The same data shown for the averaging with a higher spatial resolution.

qualitatively shows that 1.5 s pulses induce stronger localized apoptosis compared to 6 s pulses and CW heating. Cellular death is mainly observed in the central area of the well within 1.8 mm-radius region (Fig. 2(b)) where cellular response following the application of 1.5 s pulses is higher compared to the other exposure conditions. Specifically, percentage of apoptotic cells was 7%, 3%, and 1.4% for 1.5 s, 6 s, and CW exposures, respectively. However, for $d > 1.8$ mm, where SAR and peak pulse temperature, are below 77% and 86%, in respect to the peak on the axis, respectively, the number of apoptotic cells rapidly decreases to $< 1.5\%$, i.e., comparable to sham values. Note that from 0 to 3.5 mm (SAR and thermal pulse amplitude drop of about 50%), the percentage of apoptotic cells was reduced by almost 7 times for 1.5 s pulses and 5.5 times for 6 s pulses (Fig. 2(c)).

Our data suggest that when cells are exposed to heat pulses reaching a maximum peak temperature between about 48 °C and 49 °C ($0 \text{ mm} < d < 1.8 \text{ mm}$), for certain parameters of pulses (i.e., pulse duration, temperature rise rate, peak power, etc.), they are not able to cope with the external stress fully activating repair processes, resulting in initiation of an apoptotic pathway as visualized by the cleavage of Casp-3. The purely thermal mechanism (based just on the consideration of minimal, mean, and peak temperatures) would imply similar results induced by 1.5 s and 6 s pulse trains with the same minimal, mean, and peak temperatures. However, here we observe that in the center of the exposed area, 1.5 s heat pulses, with the smaller thermal dose (about 50% less than 6 s) induce 55% stronger response compared to 6 s pulses. These results suggest that the sensitivity of the melanoma cells also depends on other properties of the

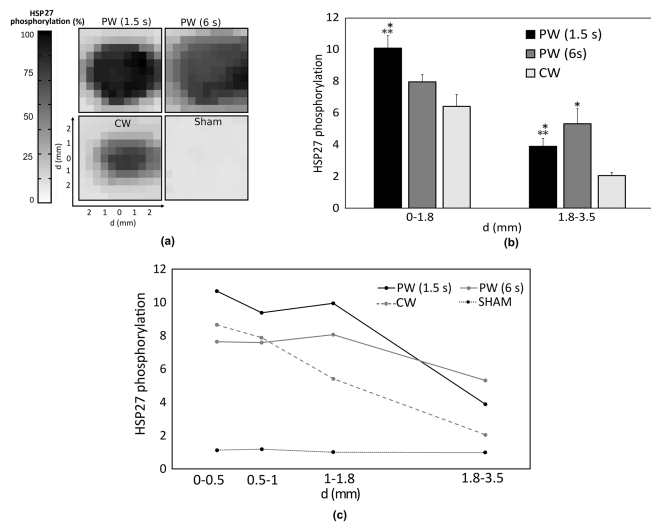


Fig. 3. Phosphorylation of HSP27 after PW and CW exposure. (a) Spatial distribution of the normalized intensity of the HSP27 phosphorylation for PW (1.5 s), PW (6 s), CW, and sham. (b) Phosphorylation of HSP27 analyzed cell-by-cell 6 h after exposure shown as mean values ($n = 3$) \pm SEM (standard error of the mean) normalized to sham. The data are averaged over two areas around the center of the well. (*) indicates statistical significance at $p < 0.05$ compared to CW, (**) indicates statistical significance at $p < 0.05$ compared to 6 s pulses. (c) The same data shown for the averaging with a higher spatial resolution.

thermal pulses, such as duration, period, or temperature rise rate (see Discussion).

C. Phosphorylation of HSP27

HSP27 phosphorylation was quantified to correlate the heat stress induced by different types of heat pulses with the level of effective cellular damage. Phosphorylation is the most widespread post-translational modification in eukaryotic cells, involved in all principal cellular processes, including signal transduction, cell cycle, growth, apoptosis, membrane protein-protein interactions, etc. [29], [30]. HSP27 phosphorylation has been related to several physiological and pathological processes [31] as well as to chaperone activity [32]. Following phosphorylation, HSP27 reorganizes itself into smaller oligomers able to trap and restore the heat-stressed misfolded polypeptides through cooperation with the well characterized ATP-dependent “foldase” chaperone machinery refolding activity [6]. In addition, phosphorylation of HSP27 may inhibit the Caspase-dependent apoptosis by repressing the Casp-3 activation [25]. Herein, the phosphorylation of HSP27 was used as a marker of cellular stress and a reliable tool to spatially map the response induced by the exposure to 1.5 and 6 s PW MMW-induced pulses in the A375 melanoma cell line. Results were compared to CW heating at 42 °C, similarly to the analysis of the MMW-heating induced apoptosis.

Fig. 3(a) represents the spatial distribution of the fluorescence intensity of cells following PW, CW, and sham exposures in the central area of the cell monolayer. It qualitatively shows that both PW exposure conditions and CW exposure induce phosphorylation of HSP27. Inside the 1.8-mm radius area, 1.5 s pulses result in levels of phosphorylation of HSP27 significantly

higher compared to 6 s pulses and CW heating (Fig. 3(b)). Within this area, the induction of HSP27 phosphorylation was 10.1, 7.9, and 6.4-fold in respect to the sham for 1.5 s, 6 s pulses, and CW, respectively. It is interesting to note that heat induced by 1.5 s pulses is mostly concentrated around the center of the exposure while 6 s pulses-induced response spreads almost over the whole region of interest. We assume that different HSP27 spatial distribution is possibly due to the different thermal diffusion time of thermal pulses (see Discussion).

The drop of phosphorylation of HSP27 from 0 to 3.5 mm is much lower compared to the decay of cellular death. Heat shock response decays by a factor of 4.2, 2.7, and 1.4, following CW, 1.5 s, and 6 s PW heating, respectively (Fig. 3(c)) showing a plateau between 0 and 1.8 mm in the case of heat pulses (Fig. 3(c)). This may be related to the saturation of the refolding system not able to cope anymore with the intense thermal stress in the 1.8 mm-radius area (peak PW temperature rise is between 47.7–49.2 °C [9]) as suggested by the substantial increase of apoptosis observed in this region (Fig. 2(c)). We observed that the strongest cellular death occurred for 1.5 s heat pulses (Fig. 2) is correlated to the highest level of phosphorylation of HSP27 (Fig. 3). This is not surprising considering that the protein redistribution in small oligomers due to excessive phosphorylation negatively regulates the chaperone-like activity of HSP27 leading to a significant decrease in their protective function [33].

IV. DISCUSSION

In this study, two aspects of the complex response activated by MMW-induced heat pulses in the A375 melanoma cells have been analyzed, namely the cellular damage, quantified as the number of cells undergoing to apoptosis, and the heat shock response mediated by the phosphorylation of HSP27. Responses induced by 90 min of exposure to a train of 1.5 s and 6 s pulses, with the same thermal profile, have been compared. Results demonstrated that peak, minimum, and average temperature elevation, are not the only parameters determining the cellular response.

Pulse duration may play a fundamental role in determining the spatial extension of the cellular response in terms of thermal stress. Thermal diffusion τ represents an important parameter to consider during photothermal therapies:

$$\tau = \delta^2/4\alpha \quad (2)$$

where δ (mm) is the penetration depth of the incident radiation and α (mm^2/s) is the thermal diffusivity of the exposed tissue [34]. If the duration of the pulse is shorter than the heat diffusion time, the distribution of the thermal energy is limited within the target zone. Pulses longer than the thermal relaxation time lead to diffusion of heat outside the target zone causing in practice the damage of the neighboring healthy tissue. Under exposure conditions of this study, $\tau = 0.12$ s with $\delta = 0.26$ mm and $\alpha = 0.143$ mm^2/s . Even though τ is lower than the pulse duration, the ratio between 1.5 and 6 s heat pulses and τ is different (12.5- and 50-fold lower than the duration of the 1.5 s and 6 s heat pulses, respectively). This could explain the fact that heat pulses with shorter duration deliver high peak power to the target area

restricting the heat affected zone to a localized region as opposite to the long pulse duration where heat is more widespread. This suggests that short thermal pulses are more suitable for localized ablative therapies compared to longer pulses despite the similar thermal profile.

Moreover, the higher heat shock response observed after 90 min of exposure to long pulses, in cells located at points beyond 1.8 mm, may be associated to thermodynamic phenomena occurring in the exposed liquid. Indeed, thermal convection, facilitated by the long thermal pulses [20], may be also responsible of a stronger movement of the liquid bulk, that may affect cellular response through indirect mechanisms, such as a local modification of the concentration of oxygen or nutrients transported by the culture medium [35], and possibly determine different cellular response compared to the cells exposed to shorter pulses, with the same temperature dynamics.

Peak power of pulses can also influence cellular response. For example, Zorec et al., [36] investigated transdermal drug delivery of compounds possessing different molecular weights by means of YAG laser, analyzing the effect of duration, power, and energy of laser pulses. They found that the energy of the pulse mostly determined the size of the laser ablation, while the duration of the pulse the extent of thermally stressed tissue. In particular, the study showed that shorter pulses with higher power allowed the achievement of higher ablative zone reducing undesired thermal effects on tissue. This is in agreement with results of our experiments where 1.5 s pulses with higher peak power (57% higher than 6 s pulses) provided higher damage of cells focused within a smaller region compared to 6 s pulses. Moreover, we showed that the extent of the heat stressed area is higher in 6 s pulses having 46% stronger energy than 1.5 s pulses.

Number of pulses may also influence cellular response. For example, in electroporation stronger cellular injury can be achieved by increasing the number of pulses for the same exposure conditions [37]. The higher number of short pulses (28% > compared to 6 s pulse train), associated to higher peak power, might also be responsible for the stronger response observed in this study both in terms of thermal stress and induced apoptosis.

Temperature rise rate represents another important parameter that could lead to different changes at the cellular level as suggested by several studies [14], [38], [39].

Despite the direct action of temperature rise rate on the enzymatic reaction has not been studied so far, we hypothesize that the observed cellular responses could depend on it. Previous studies showed that different spatio-temporal activation profiles of the same signaling proteins may result in different physiological responses, including cellular apoptosis [40]. Thermal stimulation of a cell induces activation of several downstream kinases, leading to the phosphorylation of key proteins. Faster temperature rise of the thermal train of 1.5 s pulses (6.7 °C/s) might abruptly denature proteins that do not have the time to associate with protective chaperone proteins. On the contrary, a train of 6 s thermal pulses (1.7 °C/s), might allow cells to cope with thermal stress by properly exercise their function of molecular chaperones when the heat stress is stronger.

The role of **pulse period** or **pulse repetition rate** is unclear. Some studies show that it might play a role in defining the cellular response [41]. For example, the study of the swelling response of cells as a function of pulse number and repetition rate, demonstrates that cell swelling increases when the pulses repetition rate is higher [42]. However, in another study, a decreased effect, in terms of cell viability, was observed at a higher pulse repetition rate [43].

Overall, we can hypothesize that MMW induced heat pulses can modulate cellular response and signaling depending on the pulse parameters. This is supported by a recent study [44] showing that calcium oscillation can be induced or inhibited through high voltage μ s pulsed electric fields as a function of the pulse parameters.

Note that the discrimination of the effect due to each parameter of the pulse is not straightforward. Indeed, the observed cellular response appears as a complex combination of the pulse characteristics rather than a function of a single pulse parameter. Therefore, further research is needed to isolate the effects of each parameter on cellular response, by for example using pulses of different shapes or varying other pulse parameters while maintaining the same pulse duration. The deeper insight into the impact of each pulse parameter on cell death and stress may be of importance to adaptively optimize treatment conditions by tuning the pulse parameter depending on the specific requirement.

V. CONCLUSION

MMW-induced heat pulses allow reaching high-peak temperature while keeping the average temperature rise at a relatively low level therefore avoiding, in real-case scenarios, overheating of healthy tissues surrounding the tumor. In this study, the cellular response of a malignant melanoma cell line is investigated following the exposure to 1.5 s and 6 s PW MMW-induced heating with the same temperature dynamics through high-content fluorescence microscopy image analysis. Our results demonstrated that 1.5 s heat pulses led to a stronger cellular response, both in terms of cellular apoptosis and stress, suggesting that cellular behavior is determined by a combination of pulse parameters, including peak temperature, number of pulses, temperature rise rate, pulse energy, and period. In particular, we have shown that the use of 1.5 s pulses is advantageous as it may provide a high selectivity within the irradiated zone accompanied by a dose level which is below the typical thermal threshold reported for human skin (i.e., around 600 CEM43 °C [45]). Further investigation aiming to discriminate the role of each parameter of pulses on the cellular response represents the main perspective of the study.

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