Thermal Injury Causes DNA Damage and Lethality in Unheated Surrounding Cells: Active Thermal Bystander Effect

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Direct heat exposure to cells causes protein degradation and DNA damage, which can lead to genetic alteration and cell death, but little is known about heat-induced effects on the surrounding tissue. After burns or laser surgery, loss of viability in the surrounding tissue has been explained by a temperature gradient due to heat diffusion. This study shows that, in the absence of any direct heating, heat diffusion, or cell-to-cell contact, "bystander" cells that share the medium with heat-exposed cells exhibit DNA damage, apoptosis, and loss of viability. We coin this phenomenon "active thermal bystander effect" (ATBE). Significant ATBE was induced by fibroblasts exposed for 10 minutes to a temperature range of 44–50°C (all $P<0.011$). The ATBE was not induced by cells heated to lethality above 54°C and immediate medium exchange did not suppress the effect. Therefore, the thermal bystander effect appears to be an active process in which viable, heat-injured cells induce a signal cascade and/or mediator that damages or kills surrounding bystander cells. The ATBE may have clinical relevance for acute burn trauma, hyperthermic treatments, and distant tissue damage after localized heat stress.

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INTRODUCTION

The general definition of a cellular bystander effect is the induction of a biological stress response in cells that are not directly exposed to any kind of stress such as radiation, heat, chemical, or pH change (Dale, 1940; Nagasawa and Little, 1992; Dabrowska *et al.*, 2005; de Bruijn *et al.*, 2006). Various bystander effects have been observed for decades. Kettman and Skarvall (1974) reported immune responses in cells that were not directly stimulated. Radiation-induced bystander effects became of interest in the 1990s. Nagasawa and Little (1992) recognized genetic changes in cells not irradiated with α-particles, but sharing the medium with irradiated ones. These data contradicted an old dogma in radiation research, that heritable biological effects require direct damage to the DNA (Hall *et al.*, 1988). Therefore, DNA damage in bystander cells caused controversy. A series of *in vitro* studies confirmed the presence of radiation-induced bystander effects (Azzam *et al.*, 1998, 2003; Cheng *et al.*, 1999; Brenner *et al.*, 2001; Little *et al.*, 2002; Nagasawa and Little, 2002; Little, 2003; Schettino *et al.*, 2003; Sokolov *et al.*, 2005; Yang *et al.*, 2007). Despite many studies of the bystander effect in surrounding non-irradiated cells after X-ray or α-particle irradiation, the mechanism remains still unclear.

Various stresses such as α-particles, X-ray, chemical, and photodynamic therapy have been investigated to induce the bystander effect, but very little is known regarding possible bystander effects after heat exposure. A bystander effect induced by cell necrosis, including heat-killed cells, was shown by Dabrowska *et al.* (2005). However, heat-exposed cells can also suffer sub-lethal, repairable damage, which enables the exposed cells themselves to respond with a stress response (Miller and Ziskin, 1989). Ironically, heat shock was the first cellular stress response noted, but a thermal bystander effect generated by viable cells has not been described earlier.

We tested the possibility that sub-lethal heating of cutaneous fibroblasts might induce a bystander stress response in co-cultured but physically isolated, non-heated cells using a transwell culture system (Figure 1).

RESULTS

Representative temperature plot of the cell medium

The initial temperature of the medium, measured close to the cell layer in a ''mock'' insert/well, was always 37°C. Inserts containing the cells for heat exposure were heated for 10 minutes on a hot plate at various preselected temperatures from 37 to 70°C. For all subsequent data shown in this paper, the reported temperature is the maximum temperature achieved at the end of the heating period at the location of the directly heated cells (Figure 2).

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Abbreviations: ATBE, active thermal bystander effect; DSB, double-strand break; MN, micronuclei; MTT, [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]

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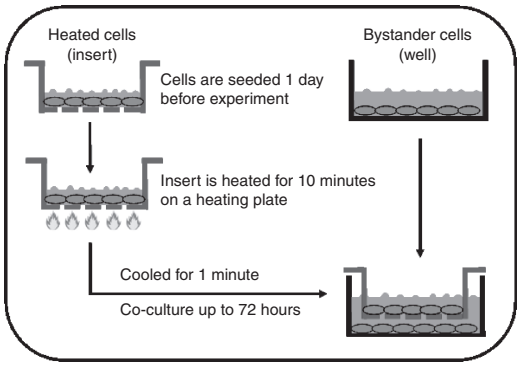

Figure 1. Experimental setting. Cells were seeded and cultured separately in inserts and wells. After heat exposure of the inserts, followed by a cooling off period, cells in inserts and wells were co-cultured for up to 72 hours.

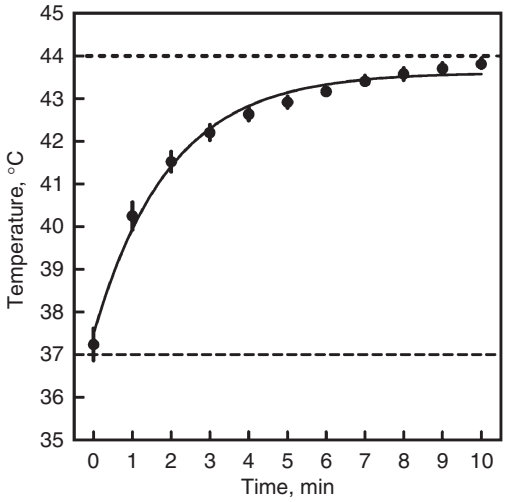

Figure 2. Representative temperature plot for the different heating settings. The selected plot resulted within the 10 minutes heating period in a maximum temperature of 44°C. Temperature of the heated cell medium was monitored every minute. The maximum temperature during the 10 minute heating period was used to characterize the heat exposure. Each data point represents up to 20 independent experiments.

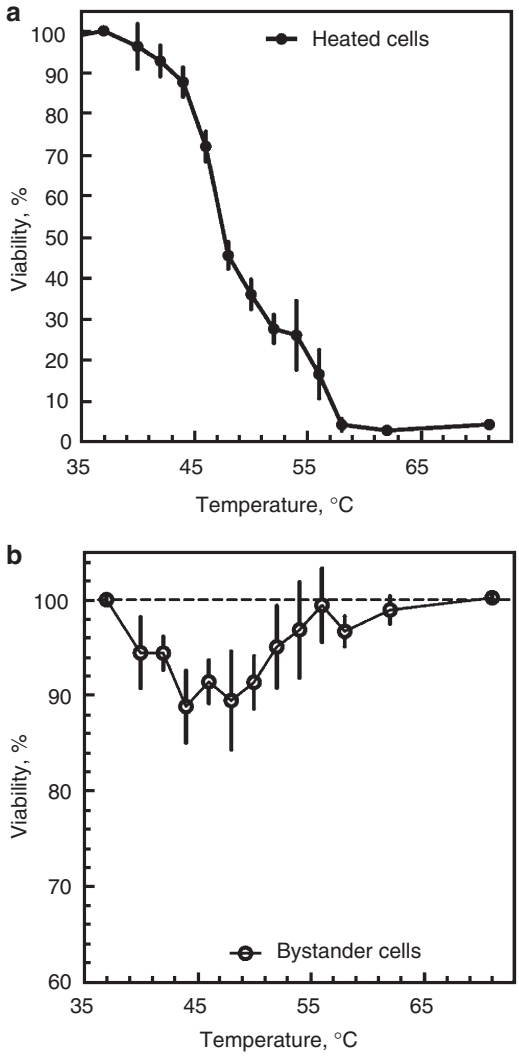

Figure 3. Cell viability. (**a**) Heated cells show a temperature depending decrease in viability with a shoulder at the beginning. (**b**) Bystander cells also show a loss in viability which is temperature depending up to 48°C. Above 48°C the viability of the bystander cells returns to control levels. All data represent 5–20 independent experiments for each temperature.

Cell viability of heated and non-heated (bystander) fibroblasts

Cells heated above (Figure 3) 40°C showed a steep temperature-dependent decrease in viability (Figure 3a). An apparent shoulder in the cell viability *versus* temperature curve was seen at about 48°C, and at temperatures above 56°C there was virtually complete loss of viability. Figure 3b shows viability of non-heated bystander fibroblasts after co-culture for 24 hours with the heated fibroblasts (see Figure 1). The non-heated bystander cells showed significant loss of viability ($P<0.011$), if the co-cultured cells were heated to a temperature between 44 and 50°C, with a maximum drop of about 10% viability at 44–48°C (Table 1). Above 50°C, no significant decrease in bystander cell viability ($P>0.18$) was detected. In addition, we compared the viability of 'washed' *versus* 'non-washed' bystander cells for preselected temperatures (37, 42, 46, 50, and 54°C; Figure 4). The 'washing' was performed approximately 5 minutes after heat exposure by replacing the medium of the heat-exposed cells with fresh DMEM medium. Both settings ('wash' and 'non-washed') showed a statistically significant bystander effect at 46°C (washed $P=0.009$, non-washed $P=0.001$) and at 50°C (washed $P=0.048$, non-washed $P=0.001$). There was no significant difference in the capability to induce a bystander effect between 'washed' *versus* 'non-washed' cells at 46°C ($P=0.168$) and 50°C ($P=0.158$). The two-way repeated-measures analysis of variance model (temperature and washed *versus* non-washed condition as factors) indicated a strong temperature effect on cell viability ($P<0.001$) for both washed and non-washed cells, and a nonsignificant effect of washing ($P=0.80$) on viability across the temperature range used.

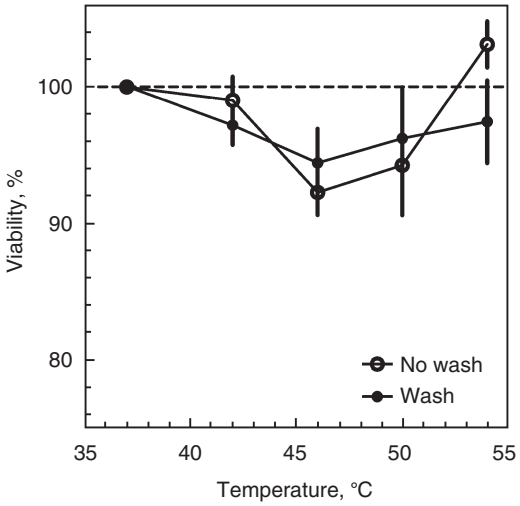

Figure 4. Cell viability in bystander cells with or without medium replacement ("cell washing"). Both bystander cells show a significant loss in viability at 46 and 50°C regardless of an additional washing step.

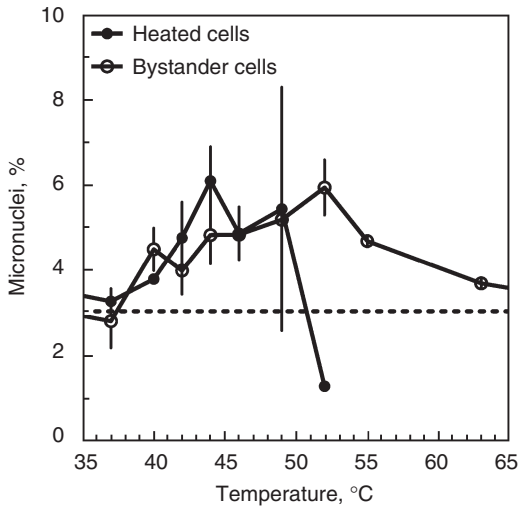

Figure 5. Micronuclei (MN). Heated and bystander cells show a temperature dependent increase of MN up to about 50°C, followed by decrease of MN. Both heated and bystander cells generate a twofold increase of MN compared with control. The dotted line is the normal background determined from the bystander and the heated cells data at 37°C (3.05 ± 0.34). No data could be acquired for heated cells at higher temperatures as all cells died and detached from the glass coverslip. Data represent at least three independent experiments for each temperature.

Table 1. Fibroblast cell viability of non-heated cells based on temperature exposure of heated cells

Temperature, °C	Mean cell viability, %	95% CI	*P*-value
37	100.0	96.1–103.9	—
40	94.5	88.7–100.2	0.114
42	95.1	91.2–98.9	0.075
44	89.1	83.3–94.9	0.002[1]
46	91.7	87.2–96.2	0.006[1]
48	89.8	83.5–96.1	0.007[1]
50	91.8	86.8–96.8	0.011[1]
52	95.0	88.7–101.2	0.180
54	96.0	84.5–107.5	0.509
56	100.0	91.9–108.2	0.988
58	94.8	80.7–108.9	0.485
62	98.4	86.9–109.8	0.796
70	100.7	80.7–120.3	0.948

[1]Statistically significant compared with 37°C, repeated-measures analysis of variance to account for multiple wells per experiment. CI=confidence interval.

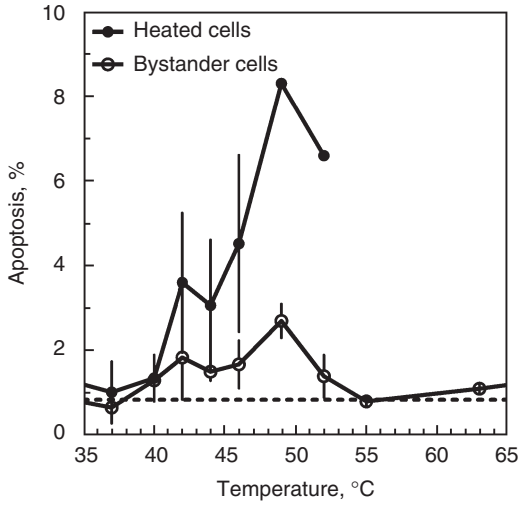

Figure 6. Apoptosis. Heated and bystander cells show a temperature depending increase of apoptosis up to 48°C, followed by a decrease in apoptosis. Heated cells generate an eightfold increase of apoptosis compared with a 2.5-fold increase for bystander cells. No data could be acquired for heated cells at higher temperatures as all cells died and detached from the glass coverslip. The dotted line is the normal background determined from the bystander and the heated cells data at 37°C (0.84 ± 0.39). The data represent at least three independent experiments for each temperature.

Micronuclei in heated and bystander fibroblasts

The background level of MN in non-treated fibroblasts was 3.05 ± 0.34% (Figure 5). The level of MN increased to a maximum of 6% at a temperature of 50°C in heated cells. Surprisingly, non-heated bystander cells showed a similar twofold increase of MN, induced by the co-cultured heated cells over the same temperature range. The dotted line represents the background level of MN at 37°C. Heat-killed cells *per se* did not induce MN in bystander cells; above 54°C, MN decreased to background level.

Apoptosis in heated and non-heated fibroblasts

Heat exposure caused an increase in apoptosis (Figure 6) (measured by apoptotic bodies and DNA condensation), in both the heated and the non-heated bystander cells. Heating at 40–50°C increased apoptosis in the heated cell population up to eightfold over the background level (at 37°C,

0.84 ± 0.389%, shown as dotted line in Figure 5). Apoptosis was similarly induced in non-heated bystander cells but to a lower extent (maximum threefold increase at 48°C). As previously shown, with lethality and MN, apoptosis in bystander cells was not significantly induced by cells heated to temperatures above 55°C.

DISCUSSION

This study shows that sub-lethal heat injury causes 'bystander' cells (nearby, but not heated cells) to show evidence of DNA damage and suffer significant lethality. To our knowledge this is previously unreported. The so called 'bystander effect' occurred when the inducing cells were moderately heat stressed, but not at higher temperatures causing complete thermal necrosis of such cells. This suggests that an 'active' cellular process is involved in the heated, inducing cells, in contrast to a 'passive' mechanism such as leakage of cellular content because of thermally induced membrane damage and cellular disintegration. To further investigate this, we performed a 'washing' experiment where we exchanged the DMEM medium 5 minutes after heat exposure and compared with 'non-washing' (Figure 4). We confirmed for both protocols (wash and no washing) a statistically significant bystander effect at 46°C (washed $P = 0.009$, non-washed $P = 0.001$) and at 50°C (washed $P = 0.048$, non-washed $P = 0.001$). The media exchange did not significantly affect the bystander effect when 'washed' and 'non-washed' bystander cells were compared at 46°C ($P = 0.168$) and at 50°C ($P = 0.158$). This experimental data can be seen as a strong support that the bystander effect is not induced by rapid release of cellular content or debris as expected after acute membrane damage. These findings rather suggest that the thermal bystander effect is an active process in which viable, heat-injured cells induce a delayed signal cascade and/or mediators that damage or kill surrounding bystander cells. Therefore, we like to call this process active thermal bystander effect (ATBE). The putative mediator(s) of the ATBE remain unknown. Although all data strongly support the notion that the thermal bystander is mainly caused by an active cellular process, at this point it cannot exclude with certainty that at least partially some passive process (for example, nonspecific release of catabolic enzymes from dead cells) might be also involved in the bystander effect for the investigated temperature range.

The mechanisms for DNA damage induced by heating are not fully understood. Micronuclei (MN) are widely considered to be a marker for unrepaired DNA double-strand breaks (DSBs) (Fimognari *et al.*, 1997). Takahashi and Ohnishi (2005) described DSB production by comet assay and yH2AX after heat exposure, but these results are somewhat controversial as moderate heat was thought not to produce DSBs. Our results (Figure 5) show that heat generates DSB in both heated and bystander cells, which correlates with cell death (Figure 3) but may not be causally related. As nearly the beginning of medical hyperthermia treatments, it was assumed that heat can cause DNA breaks (Dikomey, 1982; Dikomey and Franzke, 1992) due to DNA damage increase measured with the alkaline unwinding assay. Later, Dahm-Daphi *et al.* (1997), Kampinga *et al.* (1985), and Kampinga and Laszlo (2005) stated that DNA damage is rather induced indirectly because of alteration of the activity of DNA repair enzymes. Hyperthermia is sometimes used in cancer radiotherapy to sensitize the tumor. Before irradiation, tumor tissue is heated to 40–43°C to inhibit DNA repair, causing more damage to the cancer cells upon radiation exposure. This inhibition also results in an accumulation of endogenous DNA damage which shows as an increase in damage after heating alone, as in Figure 5. At temperatures above 46°C, mammalian cells generally die by necrosis instead of generating DSBs and apoptosis induction (Harmon *et al.*, 1990). In our study, the decrease in DSBs and apoptosis at higher temperatures could also be partially explained by experimental limits, as at higher temperature the heated cells lose their attachment to the glass slide.

We found that the heated and the non-heated bystander cells show a similar amount of DSB for the same temperatures (Figure 5). Intriguing is the idea that DNA damage even in the directly heated cell population may be a bystander effect, where a sub-population of heated cells could be responsible for inducing DNA damage in adjacent heated cells. It might be possible that the DNA damage in heated and bystander cells is because of the same mechanism. DSBs in the bystander cells could be in theory caused by the induction of apoptosis, as active DNA lysis occurs during apoptosis. However, our study shows that the extent of apoptosis is different in heated and bystander cells (Figure 6), while the extent of MN (Figure 5) is similar, suggesting that there might be another mechanism explaining the similar amount on DSB found for heated and bystander cells. The absence of the ATBE at higher temperatures suggests the involvement of an active response pathway in the bystander cells, which induces DNA damage and apoptosis.

Higher temperatures that produce cell necrosis and/or lysis can cause a passive bystander effect. Dabrowska *et al.* (2005) described a bystander effect in human cancer cells, after extremely high heat exposure of 75°C for 10 minutes. Direct thermal cell necrosis because of high temperatures might result in the release of cellular debris, including lysosomes into the extracellular matrix, possibly damaging the surrounding cells, comparable with an inflammatory process. This is distinct from the ATBE of our study, which is mediated by thermally damaged, but viable cells. The mediators created with either ABTE or with high, necrotic temperatures, such as those used by Dabrowska *et al.* remain to be determined.

Mild and moderate heat exposure is well known to cause stress response called heat shock in mammalian cells (Page and Shear, 1988; Miller and Ziskin, 1989). Depending on temperature, heating time, cell type and culture conditions, heated cells can generally follow three different pathways. At a survivable combination of temperature and heating time, heat-shock proteins are activated to protect the cells. Heat-shock protein protection follows complex pathways including stabilization of denaturated cytoplasmic and membrane proteins, nuclear structures, and inhibition of apoptosis. Above a certain combination of temperature and heating time, apoptosis is induced despite the heat-shock

response. At still higher temperatures, cells die acutely by thermal necrosis, which releases cell debris. Harmon *et al.* (1990) detected in murine mastocytoma cell cultures an increase of apoptosis after heating the cells for 30 minutes up to 45°C, whereas higher temperature of 46 and 47°C showed only necrotic cell death. Membrane changes in heat-exposed cells seem to be an important alteration, highly correlated with cell lethality (Calderwood and Hahn, 1983; Konings and Ruifrok, 1985; Majda *et al.*, 1994; Coss and Linnemans, 1996).

Although radiation-induced bystander effects have been extensively described in the literature (Mothersill and Seymour, 1997; Azzam *et al.*, 1998; Nagasawa and Little, 1992, 1999; Zhou *et al.*, 2000; Little *et al.*, 2002; Schettino *et al.*, 2003; Smilenov *et al.*, 2006; Yang *et al.*, 2007), an active thermally induced bystander effect has not been previously described to our knowledge. In our study, we used temperatures up to 70°C. The detected ATBE was significant between 44 and 50°C, within a range where cells remain viable and therefore able to participate in cell metabolism. There are many speculations about the mechanisms involved in the radiation bystander effects. Blocking of gap junction communications between adjacent cells results in a decrease of the radiation bystander effect (Azzam *et al.*, 1998). On the other hand, adding the radical scavengers catalase or SOD to the medium also reduces the bystander effect (Yang *et al.*, 2007). In our study, communication by gap junctions was excluded because of the physical separation of the heated and the bystander cells. Therefore, the ATBE is most likely induced by molecular mediator(s), which may include specific proteins, heat-shock proteins, enzymes, lipid products, other macromolecules, or small signaling radicals such as nitric oxide (Hei *et al.*, 2008). The pore size of the membrane used in our experiment to separate the heated and bystander cells was 1 μm, which does not exclude molecules based on their molecular weight. In further studies we will identify the molecules and response pathways involved.

It is stunning that a single, relatively minor application of heat can cause DNA damage and cell death not only in directly heated cells but also in distant bystander cells. The clinical impact of this finding from our cell culture study is unclear, and deserves further evaluation. A skin condition called erythema ab igne because of chronic, repeated heat exposures is an example of atrophy and enhanced carcinogenesis because of thermal injury (Page and Shear, 1988). Thermal injury is also known to potentiate tumor formation in animal models of radiation, ultraviolet and chemical carcinogenesis (Rylander *et al.*, 2006). The ATBE might also be involved in the delayed progressive damage that occurs in the zone of stasis 24 hours after burn injury (Jackson, 1953). This hypothesis is also consistent with the surgical observation that the removal of burned tissue within the first 48 hours post-burn lessens the final extent of burn injury (Zimmerman and Krizek, 1984), but it should be mentioned that the extent of the zone of cells contributing to the ATBE maybe different from the zone of stasis. The ATBE may play an important role in certain laser applications. In particular, treatments that cause thousands of microscopic thermal injuries in tissue (Anderson and Parrish, 1983; Manstein *et al.*, 2004) produce a complicated interface between the directly heat-damaged and the surrounding ''bystander'' tissue. Further studies related to such clinical implications are warranted.

MATERIALS AND METHODS

Cell culture and transwell system

The HFF1 is a human foreskin fibroblast cell line that was purchased from ATCC (Manassas, VA). Cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in DMEM (Invitrogen, St Louis, MO) supplemented with 10% fetal calf serum (Invitrogen), 100 μg ml^{-1} streptomycin and 100 U ml^{-1} penicillin. Cells were used at passage 3–8 and plated separately in six-well plates and inserts (Becton Dickinson, Franklin Lakes, NJ) with or without glass coverslips depending on the assay. The six-well plates and inserts are made of plastic and the bottom of the inserts is a membrane with a 1 μm pore size allowing molecules below this threshold size to diffuse freely within the culture medium.

Experimental setup

Cells were seeded either in six wells (7.5×10^4) or in inserts (5×10^4) 1 day before the experiment (Figure 1). For the experiment, inserts with seeded fibroblasts were put into one six-well plate with fresh medium and heated for 10 minutes on a temperature controlled heating plate. For precise temperature monitoring (Figure 2), one additional insert was outfitted with a thermocouple (Type T, Omega, Stamford, CT) and connected a digital thermometer (HH23 Thermocouple Microprocessor Thermometer, Omega) recording the temperature of the culture medium immediately at the level of the insert. After 10 minutes of heating, the insert was removed, and cooled down for 1 minute to 37°C. The insert was then placed in one-well of a six-well plate seeded with bystander cells. Heated cells and bystander cells did not have any direct cell contact (2 mm apart from each other), but shared the same medium where molecules could diffuse freely through the porous membrane of the insert. Subsequently, the six-well plates with the inserts were incubated for 24 or 72 hours depending on the evaluation method planned. A separate set of experiments was performed to compare the effects of medium replacement approximately 5 minutes after heat exposure ('washed' *versus* 'non-washed') at preselected temperatures (37, 42, 46, 50, 54°C).

MTT assay

One day before the experiment, cells were seeded in a six-well plate and in the corresponding insert. Inserts were heated, added to the well after a 1 minute down cooling period without or with medium replacement (cell washing) and were incubated for 24 hours at 37°C. Inserts and wells were separated after a 24 hours co-incubation, medium was discarded and cells were washed with phosphate-buffered saline. MTT (Sigma, St Louis, MO) was added to reach a final concentration of 500 μg ml^{-1} and incubated for 2 hours. MTT/phosphate-buffered saline solution was aspirated, DMSO (Sigma) added, and cells were mixed for 30 minutes to dissolve the converted dye. A volume of 200 μl of the solution was pipetted out of each well into a 96-well plate and absorbance was measured at 570 nm using a multiwell scanning spectrophotometer (ELISA reader).

Micronuclei and apoptosis assay

Micronuclei and apoptosis formation was measured using the cytokinesis block technique (Fenech and Morley, 1986). Within 5 minutes after heat exposure, the inserts were put into the wells with non-heated cells, and cytochalasin B (Sigma) was added to a final concentration of 1.5 $\mu g\,ml^{-1}$. After 72 hours of treatment, the cells were washed with phosphate-buffered saline and fixed with methanol:acetic acid (3:1, v/v). After drying, the cells were rehydrated with phosphate-buffered saline, stained with 4′, 6-diamidimo-2-phenylindole (Sigma) solution (10 $\mu g\,ml^{-1}$) and evaluated under a fluorescence microscope. At least 500 binucleated cells were analyzed for each sample. Apoptosis and MN were determined within the same cells. Both, apoptosis and MN were analyzed as the percentage of cells that contained typical morphological appearance of apoptotic bodies or binucleated cells with a micronucleus, respectively.

Statistical analysis

Cell viability was evaluated as a function of direct temperature exposure of heated fibroblast cell cultures as well as co-cultured non-heated cells using repeated-measures analysis of variance, which accounted for multiple wells per experiment. Two-degree temperature intervals were chosen to assess percent cell viability compared with the control at 37°C. A compound symmetry covariance structure was used to handle the repeated measurements and fit the data well (Vittinghoff *et al*. 2005). A two-tailed $P<0.05$ value was considered statistically significant, with the Fisher least significant difference method being used for multiple comparisons relative to control. To estimate precision of cell viability, 95% confidence intervals were calculated for the temperature intervals for both heated and non-heated cells. The temperature range in which non-heated cells show significantly reduced cell viability compared with control will be defined as the temperature predictive of achieving an ATBE. Statistical analysis was performed using the SPSS software package (version 16.0, SPSS Inc., Chicago, IL).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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