

Research Article

Study of the Effects of 0.15 Terahertz Radiation on Genome Integrity of Adult Fibroblasts

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The applications of Terahertz (THz) technologies have significantly developed in recent years, and the complete understanding of the biological effects of exposure to THz radiation is becoming increasingly important. In a previous study, we found that THz radiation induced genomic damage in fetal fibroblasts. Although these cells demonstrated to be a useful model, exposure of human fetuses to THz radiation is highly improbable. Conversely, THz irradiation of adult dermal tissues is cause of possible concern for some professional and nonprofessional categories. Therefore, we extended our study to the investigation of the effects of THz radiation on adult fibroblasts (HDF). In this work, the effects of THz exposure on HDF cells genome integrity, cell cycle, cytological ultrastructure and proteins expression were

assessed. Results of centromere-negative micronuclei frequencies, phosphorylation of H2AX histone, and telomere length modulation indicated no induction of DNA damage. Concordantly, no changes in the expression of proteins associated with DNA damage sensing and repair were detected. Conversely, our results showed an increase of centromere-positive micronuclei frequencies and chromosomal nondisjunction events, indicating induction of aneuploidy. Therefore, our results indicate that THz radiation exposure may affect genome integrity through aneuploidic effects, and not by DNA breakage. Our findings are compared to published studies, and possible biophysical mechanisms are discussed. *Environ. Mol. Mutagen.* 59:476–487, 2018. © 2018 Wiley Periodicals, Inc.

Key words: THz radiation; human fibroblasts; spindle disturbance; DNA damage; chromosomal malsegregation

INTRODUCTION

Terahertz (THz) radiation is usually defined as electromagnetic waves oscillating at frequencies ranging from 10^{11} to 10^{13} Hz (sub-millimetre waves) and is located in the electromagnetic spectrum between the microwave and infrared regions. Until recent years, there has been little research in this frequency range, due to the limited availability of powerful sources and sensitive detectors. Nevertheless, many THz imaging and sensing technologies have been developed during the last decade with increasing applications such as military and civilian communication, security screening, quality control of food and pharmaceuticals, medical imaging, and diagnosis [Wilmsink and Grund, 2011].

The emergence of these new THz-based applications has resulted in an increased interest in the biological effects associated with this frequency range. Among other

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TABLE I. Irradiation Parameters

Frequency	Incident power density (IPD)	Micropulse duration	Micropulse interval	Pulse duration	Pulse rate	Irradiation Time
0.1–0.15 THz	0.4 mW/cm ²	50 ps	330 ps	4 μ s	2.5 Hz	20 min

studies, some investigations focused on the possible genotoxic effects of THz electromagnetic fields. So far, almost all researchers, evaluating chromosomal aberrations [Bogomazova et al., 2015], micronuclei frequencies [Zeni et al., 2007; Hintzsche et al., 2012; Hintzsche et al., 2013; De Amicis et al., 2015], phosphorylation of H2AX histone (γ -H2AX) alone and in association with p53-binding protein 1 (53BP1) [Bogomazova et al., 2015; De Amicis et al., 2015] or using the Comet assay [Hintzsche et al., 2012; Hintzsche et al., 2013; Angeluts et al., 2014; De Amicis et al., 2015], found no induction of DNA breaks. Nonetheless, some authors reported induction of aneuploidy [Korenstein-Ilan et al., 2008; Hintzsche et al., 2011; Angeluts et al., 2014; De Amicis et al., 2015], i.e., numerical abnormalities of chromosomes due to malsegregation or chromosome loss.

In agreement with several of the abovementioned studies, we previously observed aneuploidy induction in fetal fibroblasts exposed to THz [De Amicis et al., 2015]. Fetal cells demonstrated to be a useful model, because these cells appear to be more sensitive to genotoxic insults than adult ones. In fact, several *in vivo* studies found higher levels of genotoxic damage in foetuses and newborn compared to their mothers, both in rodents and humans as reviewed in Udroui and Sgura [2016]. This greater sensitivity was also detected in studies in which lymphocytes sampled from human adults and newborns were treated *in vitro* with ethyl paraoxon [Islas-González et al., 2005] or X-ray [Floyd and Cassoni, 1994; Bakhmutsky et al., 2014]. Nonetheless, exposure of human foetuses to THz electromagnetic fields is extremely improbable, both because this radiation can penetrate no more than few millimetres of tissue [Alekseev et al., 2008] and because pregnant women are not expected to be exposed to them. However, THz irradiation of adult dermal tissue is cause of possible concern for some specific categories, like security and military personnel, material science and art conservation specialists, patients undergoing THz diagnostic imaging; more importantly, it is possible that in the near future a larger part of the population will be exposed to THz radiation, as this bandwidth is envisioned as the next frontier for vehicular communication networks [Mumtaz et al., 2017]. Therefore, we chose to investigate the consequences of 0.1 THz pulsed electromagnetic radiation (≈ 3 mm wavelength) on adult human fibroblasts, by evaluating its effects on cell cycle, genome integrity (DNA breakage and aneuploidy), cellular ultrastructural

morphology and expression of heat shock, cytoskeleton, and pro-survival signaling proteins. In addition, to better investigate the sensitivity of fetal and adult cells to this nonionizing radiation, we compared our recent findings with those reported in our previous paper [De Amicis et al., 2015].

MATERIALS AND METHODS

Cell Culture Technique

Human dermal fibroblasts, HDF (ECACC, passage 10–11) were cultured in DMEM medium (Euroclone) supplemented with 10% heat inactivated fetal bovine serum (Euroclone), 1% 2 mM L-glutamine, 1% penicillin/streptomycin (Gibco) and 1% nonessential amino acids (Euroclone). Cell cultures were grown incubated at 37°C, in humidified atmosphere, at 5% CO₂. Twentyfour hours before THz exposure, cells were seeded into 6 cm diameter polystyrene Petri dishes (Corning 3295), in 5 mL of medium at the density of 2.5×10^5 cells. The thickness of the culture medium results in 0.21 cm.

THz Exposure

Exposure was performed, as previously described [De Amicis et al., 2015], in the frequency band between 0.10 and 0.15 THz using the ENEA Compact Free Electron Laser (FEL) operating in the so-called “Coherent Spontaneous Emission” mode at a central frequency of 0.12 THz with a typical relative bandwidth of about 20%. For the experiments described in this paper, the Compact-FEL delivered 1 kW, 4 μ s duration, radiation pulses at a rate of 2.5 Hz. Each radiation pulse is composed of a “train” of short micropulses spaced 330 ps from each other (Table I). This peculiar temporal structure of the emitted radiation allows maintaining a low average power incident over the whole sample area, typically a few mW, thus avoiding heating effects [De Amicis et al., 2015].

The FEL radiation is transported to the exposure set-up through a special mm-wave transmission line composed of a copper light pipe with 25 mm clear aperture and appropriate delivery optics. A specific THz Delivery System (TDS) provides the necessary expansion of the THz beam needed to match the area of the Petri dishes used in the experiments and directly placed on the top of the TDS. The radiation reaches the sample from the bottom to avoid the shielding effects of the culture medium. Nonirradiated cells used as control samples were placed on the same working plate as the THz irradiated samples.

During the irradiation time (20 minutes), the temperature distribution over the sample area was monitored using an infrared camera FLIR A325 with 0.2°C resolution. The average incident power density of the radiation was 0.40 mW/cm² and the ambient temperature 20°C. Since the fibroblast cells formed a thin layer, of approximately 10 μ m thickness, adherent to the bottom of the Petri dish, a specific absorption ratio (SAR) in the range 15–20 mW/g was calculated according to the simple model described in previous work [De Amicis et al., 2015].

Cell cultures were subjected to at least three irradiation experiments for each assay (except for Western Blotting, two experiments in that case).

Unirradiated HDF cells, used as control, were analyzed using the same assays.

Cell Cycle Analysis

Asynchronous, log-phase growing HDF cells (60% confluent, approximately 2.5×10^5 /well in 6-well plates) were exposed to THz radiation for 20 min. One and 24 hr after THz exposure, adherent as well as suspended cells were harvested, centrifuged at 1,500 rpm for 10 min and washed twice with cold phosphate-buffered saline (PBS). The cell pellets were resuspended in 70% ethanol and incubated for 1 hr at 20°C. The cells were then washed twice with cold PBS, centrifuged at 1,500 rpm for 10 min, incubated for 1 hr in the dark with propidium iodide (25 µg/mL final concentration in 0.1% citrate and 0.1% Triton X-100), and analyzed by flow cytometry using a FACSCalibur cytometer with CellQuest Pro 5.2 software (BD Biosciences, San Jose, CA) [Masuelli et al., 2013]. A minimum of 20,000 events were collected.

Micronucleus Assay with anti-Kinetochore Antibody (CREST)

After exposure, cytochalasin B (3 µg/mL final concentration, Sigma) was added to irradiated and control cultures to block cytokinesis. After 24 hr of incubation at 37°C, cells were harvested, treated with hypotonic solution (KCl 0.075 M), spotted on slides and fixed in absolute ice-cold methanol. The slides were processed for anti-kinetochore staining as previously described [De Amicis et al., 2015]. Briefly, CREST antikinetochore antibody (Antibody Inc., Davis CA) and FITC-conjugated secondary antibody (Sigma Immunochemicals, St. Louis) were used. Cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma Aldrich, St. Louis) in Vectashield (Vector Laboratories, Burlingame, CA). Micronuclei (MN) frequencies were assessed counting at least 1,000 binucleated (BN) cells for each experiment. Only MN that were not larger than one-third of the diameter of the main nucleus, did not overlap the main nucleus, and had distinct borders were included in the scoring, in agreement with the internationally agreed criteria [Fenech et al., 2003]. MN were classified for the presence (CREST-positive MN) or absence (CREST-negative MN) of kinetochore fluorescent signals under appropriate filters.

Fluorescence in Situ Hybridization (FISH) for Nondisjunction Analysis

BN cell preparations were obtained as described above. Fixed BN cells were dropped onto slides, air-dried and immediately utilized for FISH staining. Briefly, samples and probe were denatured simultaneously by heating on a hotplate at 75°C for 2 min. We used a probe containing a mix of repetitive sequences specific for the centromeric region of chromosomes 4 (Cy3, red), 10 (FITC, green) and 17 (Aqua, blue) (Metasystems, Germany) and hybridization was performed overnight at 37°C in a humidified chamber. Slides were washed in different buffer: (1) 2X SSC/0.05% Tween 20 (pH 7.0) for 30 min at room temperature, (2) 0.4X SSC for 2 min at 72°C, and then counterstained with DAPI (Sigma Aldrich, St. Louis) in Vectashield (Vector Laboratories, Burlingame, CA). A fluorescent microscope Axio Imager M1 (Carl Zeiss, Germany) equipped with a CCD camera was used. We considered normal BN, cells with 2 signals for each chromosome/fluorochrome in each nucleus of BN cell (2+2). BN cells with an unequal chromosome distribution of one of the fluorochromes, as 3+1 or 4+0, were considered aneuploid. Only BN cells with the correct number of hybridization signals were analyzed, technical artifacts were excluded [Kirsch-Volders et al., 1996]. About 1,000 BN cells were analyzed for each experiment.

γ-H2AX and 53BP1 Foci Analysis

Analysis of γ-H2AX/53BP1 foci was done by immunofluorescence staining to detect double strand breaks (DSBs). Since the induction of colocalized γ-H2AX and 53BP1 foci appears within minutes and reaches the maximum levels after 30 min, we chose three different time points: 30 min, 2 and 24 hr post exposure. After irradiation, HDF cells were spotted onto coverslips, fixed with 2% formaldehyde/PBS for 5 min, permeabilised with 0.5% Triton-X/PBS for 5 min and were blocked with 1% bovine serum albumin (BSA, Sigma) in PBS for 10 min. HDF cells were then incubated with a combination of 1:500 mouse monoclonal anti-γH2AX antibody (Merk Millipore) and 1:1,000 rabbit polyclonal anti-53BP1 antibody (Calbiochem) in 1% BSA/PBS for 45 min at room temperature in wet chamber. After three washing steps of 3 min in 1% BSA/PBS, cells were incubated in 1:500 anti-mouse Alexa Fluor 488 conjugated antibody (Molecular Probes, Life technologies), 1:500 anti-rabbit Alexa Fluor 555 Goat anti Rabbit IgG (Molecular Probes, Life technologies) for 30 min at room temperature in a wet chamber in the dark. Slides with DAPI counterstained cells were analyzed with an epifluorescence microscope (Imager Z1, Carl Zeiss) equipped with a CCD (charge-coupled device) camera. The image acquisition was performed with an automated approach using Metafer 4 software (version 3.6.9, MetaSystem). For each time point and condition 100–250 fields of each spot were selected and acquired by Metafer Autocapt module, using an immersion plan Apochromat oil 63× objective (Carl Zeiss). The foci scoring was performed using the free cell image analysis software Cell-Profiler (version 2.0, Broad Institute) as described by Carpenter et al. [2006].

Telomere Length Analysis

After THz exposure, cells in a complete medium were incubated at 37°C for 24 and 48 hr and then collected. Chromosome spreads were obtained as previously described [De Amicis et al., 2015]. After 6 hr of incubation in 5×10^{-6} M colchicine (Sigma Aldrich, St. Louis, USA), cell suspension was treated with a hypotonic solution (75 mM KCl) for 28 min at 37°C, followed by fixation in freshly prepared Carnoy solution (3:1 v/v methanol/acetic acid). Cells were then dropped onto slides, air dried, and utilized for cytogenetic analysis.

For Q-FISH staining, slides and probes (Cy3-linked telomeric, PANA-GENE, Korea and chromosome 2 centromeric Peptide Nucleic Acid probe generously provided by DAKO Cytomation, Denmark) were co-denatured at 80°C for 3 min and hybridized for 2 hr at room temperature in a humidified chamber. Slides were counterstained with DAPI (Sigma Aldrich, St. Louis) in Vectashield (Vector Laboratories, Burlingame, CA). Images were captured at a 63× magnification with an Axio Imager M1 (Carl Zeiss, Germany) equipped with a CCD camera. The telomere size was analyzed with ISIS software (MetaSystems, Germany). In particular, the software calculated telomere lengths as the ratio between the total telomeres fluorescence (T) and the fluorescence of the centromere of chromosome 2 (C). Centromere of chromosome 2 was used as the internal reference in each metaphase analyzed. Data were expressed as a percentage (T/C%) [Perner et al., 2003]. At least 10 metaphases were analyzed for each experiment.

Western Blotting

For immunoblotting analysis, twenty-four hours after THz exposure, cells were harvested, then washed twice with cold PBS and lysed in RIPA buffer as previously described [Masuelli et al., 2010]. Briefly, 50–80 µg of cell lysates were resolved in 10–15% SDS-PAGE and then transferred to nitrocellulose membranes. Equal loading of protein was verified by Ponceau staining of the membranes and by analyzing actin expression. The assay was then performed as previously described [Masuelli et al., 2014]. A densitometric analysis of autoradiographic bands was performed with ImageJ 1.42q software [Schneider et al., 2012] after blot scanning.

Transmission Electron Microscopy

Ultrastructural analysis was performed on HDF cells 1 and 48 hr after THz irradiation using transmission electron microscopy. Cells were fixed at 4°C in 2.5% glutaraldehyde in PBS pH 7.4, then were observed and photographed by inverted optical microscope. The samples were then

processed for transmission electron microscopy following routine procedure. Ultrathin sections were analyzed by a Philips Morgagni 268D [Masuelli et al., 2007].

Statistical Analysis

Statistical analyzes have been performed using different tests according to the assay. T test has been used for H2AX, FACS analyzes and differences in the intensity of immunoreactive bands and χ^2 for MN-CREST. The binomial probability test has been performed for nondisjunction analysis. Finally, the statistical analysis of the telomere length (Q-FISH) has been performed using the Mann-Whitney *U* test. Statistical significance was accepted for value $P < 0.05$.

RESULTS

Thermal Conditions

Infrared images of the Petri dish taken during the THz exposure showed a uniform temperature distribution over the exposed area (Fig. 1).

During the 20 minute irradiation time, no measurable temperature increase of the samples was observed by means of an infrared camera with a temperature resolution of 0.2°C. This result is in agreement with a maximum temperature increase $T_{MAX} = 0.3^\circ\text{C}$ calculated in the adiabatic approximation (radiation power entirely dissipated in a thermally isolated sample) as described in the Appendix of our previous work [De Amicis et al., 2015].

Cell Cycle Analysis

To evaluate the effect of THz exposure on the cell cycle distribution, FACS analysis of DNA content was performed on HDF cells exposed to THz radiation for 20 min (Table II). No significant changes in the different phases of the cell cycle were observed comparing the values of irradiated cells to those obtained with control cells, neither 1 hr nor 24 hr after THz exposure.

Micronucleus Assay with anti-Kinetochore Antibody (CREST)

To evaluate if THz exposure was able to induce chromosome instability, and to distinguish chromosome loss (aneugenic effect) from chromosome break (clastogenic

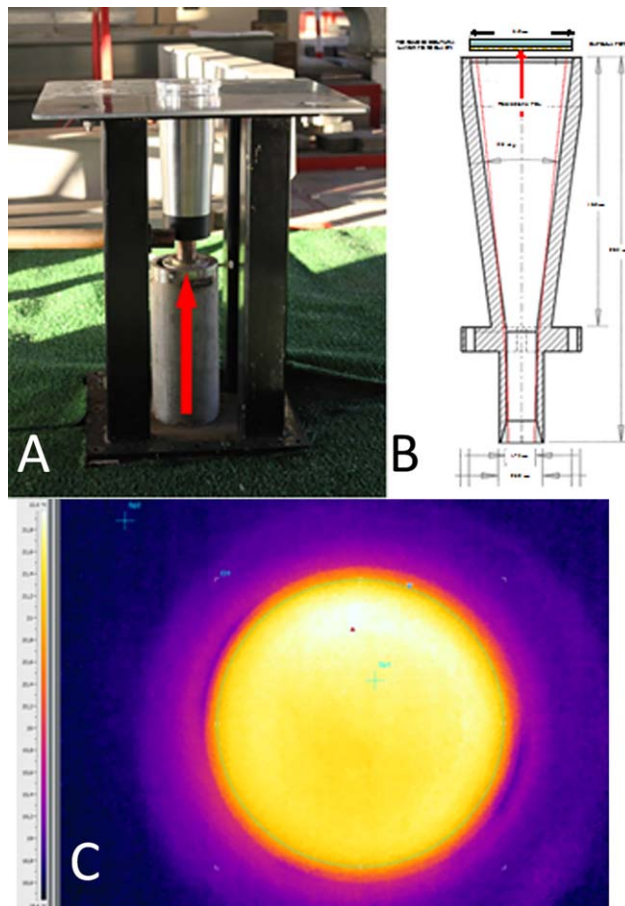


Fig. 1. Experimental set-up. (a) Photo of the exposure set-up, showing the THz Delivery System (TDS) with the Petri dish containing the cells on top of it. The red arrow, shows the propagation direction of the FEL beam (from [De Amicis et al., 2015]). (b) Mechanical drawing of the TDS. The radiation in the TDS is first focused to a 1.75 cm aperture and then let expand by diffraction to match the irradiation area of the Petri dish (5.5 cm internal diameter). (c) Infrared image of the Petri dish at the start of the THz exposure. It shows a uniform temperature distribution within 0.4°C around 21°C over the exposed area.

TABLE II. Effects of THz Radiation on the Cell Cycle of HDF Cells 1 and 24 hr After Exposure

	Sub-G1 ^a	<i>P</i>	G0/G1	<i>P</i>	S	<i>P</i>	G2/M	<i>P</i>
1 hr after exposure								
CTRL	0.66 ± 0.30	NS	85.32 ± 0.16	NS	6.52 ± 2.56	NS	7.95 ± 2.33	NS
THZ	0.65 ± 0.08	NS	88.50 ± 2.20	NS	5.38 ± 0.47	NS	5.71 ± 2.72	NS
24 hr after exposure								
CTRL	1.98 ± 0.03	NS	81.89 ± 0.58	NS	9.34 ± 0.11	NS	7.23 ± 0.53	NS
THZ	1.28 ± 0.44	NS	80.92 ± 3.40	NS	8.71 ± 1.61	NS	9.47 ± 2.46	NS

^aPercentage of cells in the sub-G1, G0/G1, S, and G2/M phases were calculated using Cell Quest software. The results are expressed as the means ± SD of three independent experiments performed in duplicate. NS: not significant ($P > 0.05$).

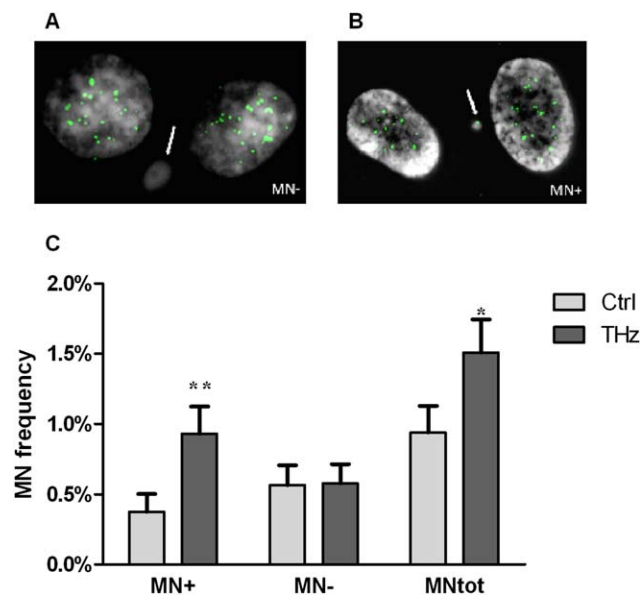


Fig. 2. Analysis of MN with CREST staining: (a) Example of BN cell staining by CREST. White arrow indicate the MN-. (b) Example of BN cell staining by crest with a MN+ indicated by the white arrow. (c) Bar-plots of data obtained by CREST staining. Micronuclei frequencies represent the percentages of binucleated cells with micronuclei per binucleated cells. Results showed a significant increase of the frequency of MN+ ($P < 0.01$) and MNtot (MN+ plus MN-) ($P < 0.05$) in exposed samples respect to the control ones. Bars indicate the standard error. ** $P < 0.01$, * $P < 0.05$ by χ^2 test.

effect), we performed CREST analysis (Fig. 2). Results showed in Figure 2c indicated that there were both a significant increase ($P < 0.05$) of total MN (i.e., the sum of CREST-positive and CREST-negative MN) and a significant increase ($P < 0.01$) of MN+ in irradiated cells compared to control. In addition, we observed no differences in CREST-negative MN frequency in exposed and control samples. These results indicated that the increase of total MN is due to the increase of CREST-positive MN.

Nondisjunction Analysis

To further assess whether THz exposure induces aneuploidy in HDF cells, we analyzed chromosomes nondisjunction for three different homologous pairs (chromosomes 4, 10, and 17). Figure 3 shows the percentage of nondisjunction (ND) for each homologous examined between the two daughter nuclei. Our results showed a significant increase ($P < 0.001$) of total nondisjunction (ND 4 + 10 + 17) in irradiated samples respect to the control ones. We also observed a significant increase ($P < 0.01$) of chromosome 10 malsegregation in irradiated samples respect to the control ones, indicating that this chromosome is the most involved of the three investigated in chromosome nondisjunction. The differences observed in events of ND for the other two homologous analyzed (chromosome 4 and 17) were not statistically significant. Nullisomy and tetrasomy (4 + 0) was not

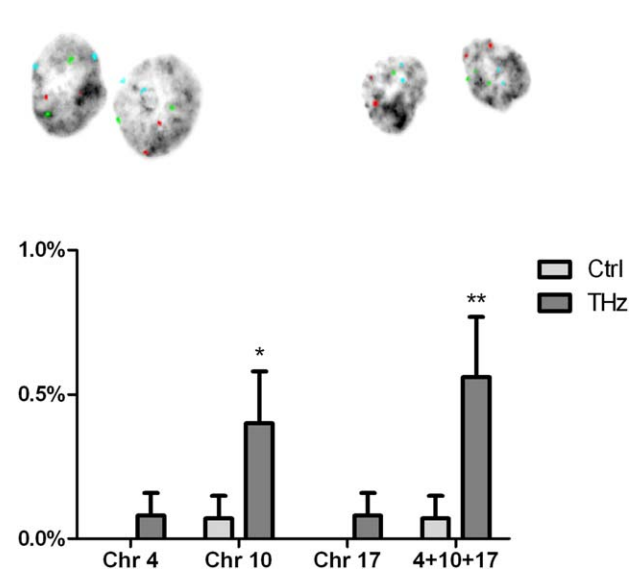


Fig. 3. Analysis of nondisjunction (ND) by centromeric-FISH of three couples of chromosomes 4, 10, and 17. Upper row, left: Example of BN with a normal chromosome distribution of the three homologous (2 + 2). Green chromosome 4, red chromosome 10, cyan chromosome 17. Upper row, right: Example of BN with unequal distribution of chromosome 4 (green) (3 + 1) and a normal distribution of chromosomes 10 and 17. Lower row: Frequencies of ND in unirradiated and irradiated cells. Bars represent percentage of BN with 3 + 1 ND (4 + 0 ND was never observed). We detected a significant increase ($P < 0.001$) of total nondisjunction (ND chr 4 + 10 + 17) events in irradiated samples respect to the control ones. For the chromosome 10 we observed a significant increase ($p < 0.01$) of ND. Significance: a: $P < 0.01$, b: $P < 0.001$, by binomial probability test.

evidenced in control and irradiated cells for each chromosome pairs analyzed.

γ -H2AX and 53BP1 Foci Analysis

DNA damage, as DSBs, was evaluated by the presence of γ H2AX and 53BP1 foci (Fig. 4A). To ensure that the assay was performed under optimal condition an X-ray irradiated sample (1 Gy) was used as positive control. As reported in Figure 4B, no significant differences between THz-irradiated and control cells were observed for each time point.

Telomere Length Analysis

Telomere length is an important feature for chromosome stability. To evaluate any variation in telomere length, we performed Q-FISH analysis before and after THz exposure (Fig. 5A). We collected cells at 24 and 48 hr post exposure. As shown in Figure 5B, no significant telomere length modulation was observed 24 and 48 hr after THz exposure in irradiated cells as compared to the control ones.

Expression of Heat Shock, Cytoskeleton, and Pro-Survival Signaling Proteins

The effect of THz exposure on the expression of heat shock proteins (HSPs), pro-survival signaling proteins,

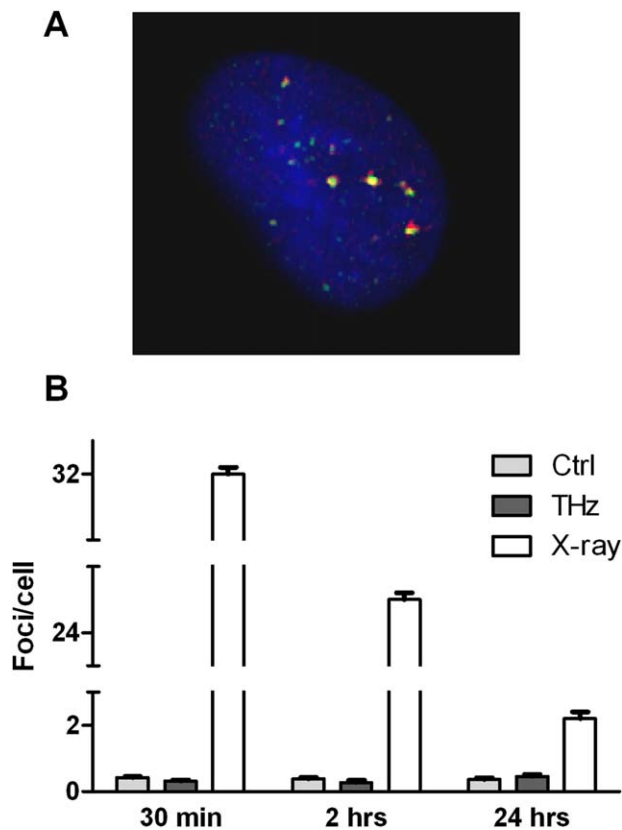


Fig. 4. (A) Representative immunofluorescent image of colocalized γ -H2AX, 53BP1 in a HDF interphase nucleus DAPI counterstained. (B) Bar-plot describes the mean number of colocalised γ -H2AX, 53BP1 foci per cell in untreated, THz-exposed and positive control (X-ray irradiated sample): no differences were observed 30', 2 and 24 hr after exposure to THz radiation respect to control cells.

and cytoskeleton proteins such as actin, was evaluated in HDF 24 hr after THz irradiation by western blotting analysis. Exposure of HDF culture to THz radiation did not determine modulation of the HSPs expression as compared to control cells (Fig. 6A).

There were also no differences in the expression of actin, NF- κ B, ERK 1/2 and AKT between THz irradiated and control cells, neither activation of ERK (Fig. 6B).

Ultrastructural Analysis

HDF cells were observed by inverted microscope before ultrastructural analysis. No morphological differences (nuclei/cytoplasm ratio, cellular shape, presence of vacuoles in the cytoplasm or cellular swelling) were found between control and THz irradiated cells (Fig. 7A).

At the ultrastructural analysis, control HDF cells displayed an elongated appearance with elongated centrally located nuclei, essentially formed by euchromatin with poor heterochromatin and well organized nucleoli. Condensed or occasionally dilated mitochondria, rough endoplasmic reticulum, primary and secondary lysosomes

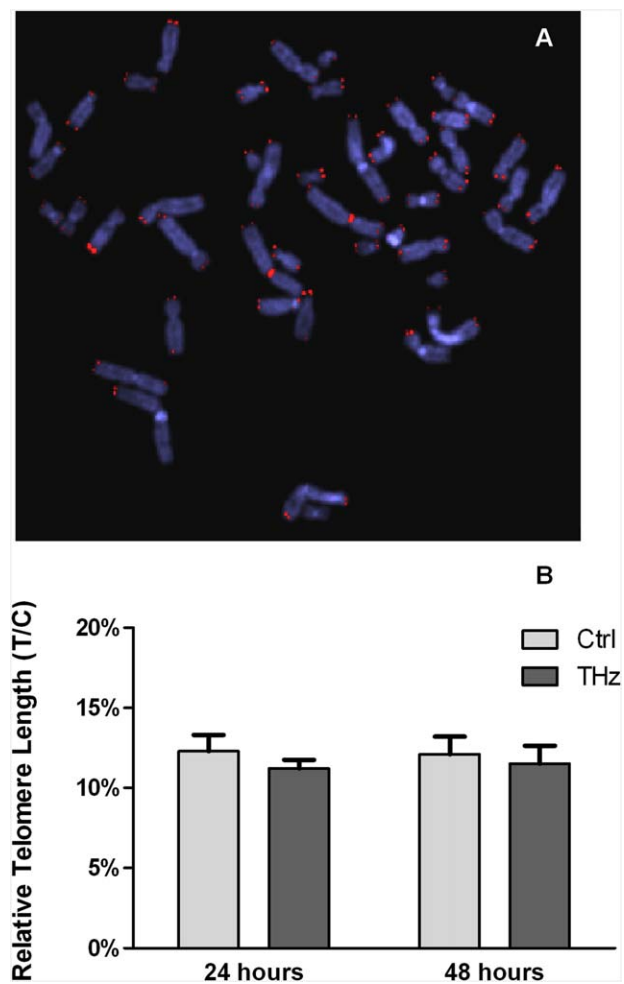


Fig. 5. (A) Representative metaphase staining by FISH for telomere analysis, showing chromosomes (DAPI stained, blue), telomeric sequences (red) and centromeres of chromosome 2 (red). (B) Data obtained by Q-FISH analysis. Results showed no significant differences in telomere length (T/C%) between irradiated (0.15 THz) and control samples. Bars indicate standard error.

were observed. Under the plasma membrane, some pinocytotic vesicles and polymerized thin and intermediate filaments were observed (Fig. 7B).

Cells showed an elongated morphology with centrally located elongated nuclei one hour after THz irradiation. The rough endoplasmic reticulum was well preserved but often appeared dilated with the formation of cisterns containing finely granulo-fibrillar material. In addition, some vacuoles and primary and secondary lysosomes were observed. A slight increase of pinocytotic vesicles below the plasma membrane and a substantial increase of the polymerization of thin and intermediate filaments, below the plasma membrane and in the cytoplasm were seen (Fig. 7B). Only a slight increase of polymerization of cytoskeletal filaments was observed 48 hr after THz exposure. No other differences between THz irradiated and control cells were observed, thus indicating that the

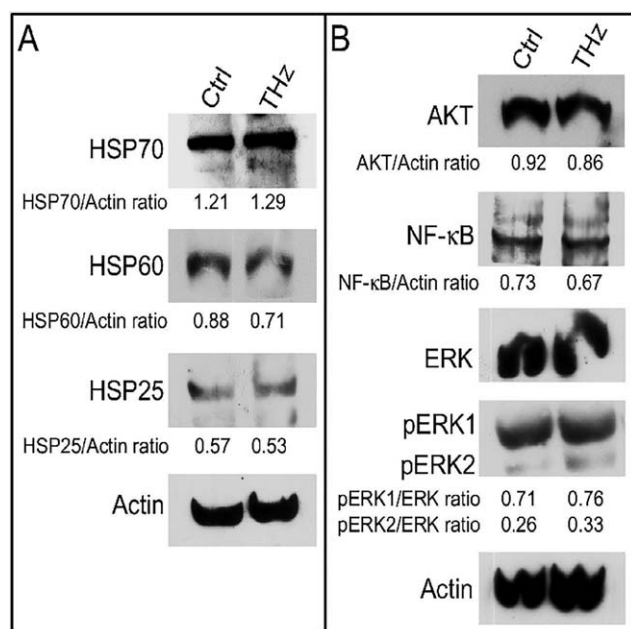


Fig. 6. Effect of THz radiation on heat shock proteins (HSP 70, 60, 25–27), pro-survival signaling proteins (NF-κB, ERK and phospho- ERK, AKT) and actin in adult human fibroblasts. Panel A: Effect of THz exposure on expression of HSPs. Western blotting was performed on control and irradiated cells 24 hr after exposure. Panel B: AKT, NF-κB, ERK and actin expression, ERK1/ERK2 phosphorylation status in control and irradiated cells 24 hr after THz exposure. Actin was used as an internal control. The intensity of the bands obtained in two independent experiments was quantified using ImageJ software after blot scanning, and the densitometric ratios between HSP70/60/25 and actin, between AKT and actin and between NF-κB and actin are reported. The levels of pERK1 and pERK2 proteins were compared with the total ERK protein levels.

morphological changes revealed immediately after exposure were transitory (Fig. 7B).

DISCUSSION

The present study investigated the effects of 0.1–0.15 THz radiation exposure on HDF genome integrity. As genomic instability is a hallmark of cancer [Dürbaum and Storchová, 2016], the analysis of the genotoxic properties of ultra-high frequency electromagnetic fields from the microwaves to the THz range is important for human safety and protection. In a previous report, employing human fetal fibroblasts, we found the induction of aneuploidy and cell cycle perturbations after THz exposure [De Amicis et al., 2015]. However, the exposure of human fetuses to THz radiation is extremely improbable. Therefore, we chose to investigate the effects of THz on adult cells and to compare the present results with those observed in fetal fibroblasts. Although the investigation on THz radiation biological effects is a relatively recent field of research, the studies performed so far suggest which effects THz electromagnetic fields exert on genome, cell cycle and response of HSPs (Table III). Our

results indicated that HSPs protein expression (24 hr post-exposure) and cell cycle distribution (1 and 24 hr post-exposure) were not modified after irradiation. These results are in agreement with other reports where the expression of HSPs appears to be unaffected [Bogomazova et al., 2015; De Amicis et al., 2015], with the exception of one study [Wilmsink et al., 2011]. However, in that work temperature of the cell culture reached 40°C (37°C at the beginning of the experiment +3°C due to THz exposure). Thus, the HSP response was reasonably due to the thermal effect of the radiation. The cell cycle seems to be unaffected in all studies but one [De Amicis et al., 2015]: in that study, the observed effect was probably due to the greater sensitivity of fetal cells compared to the adult ones.

One of the most investigated effects of THz radiation is the clastogenic potential (i.e., to induce DNA breakage). Negative results were obtained in almost all studies, which used the Comet assay [Zeni et al., 2007; Hintzsche et al., 2012; Hintzsche et al., 2013; Angeluts et al., 2014; De Amicis et al., 2015], the analysis of phosphorylation of H2AX histone [Bogomazova et al., 2015; De Amicis et al., 2015], chromosomal aberrations [Bogomazova et al., 2015], and the frequencies of CREST-negative micronuclei [De Amicis et al., 2015]. The only evidence of clastogenic properties of THz radiation was found by Titova et al. [2013], but in that case the H2AX phosphorylation was qualitatively and not quantitatively evaluated. Moreover, the power density was 57 mW/cm² and temperature was not measured, but calculated.

In our work, the exposure of HDF to THz radiation resulted in genome damage, as evidenced by the significant increase of total MN frequency. Moreover, the significant increase of CREST-positive MN frequency (containing a whole chromosome) indicated the induction of aneuploidy. The frequency of CREST-negative MN (containing a chromosome fragment) and the results from γ-H2AX analysis, indicating no increase of DNA breakage after THz irradiation (Table IV), clearly evidenced that the genomic damage was not due to clastogenesis. This result was indirectly supported also by the absence of an increased expression of proteins associated with DNA damage sensing and repair (AKT, NF-κB, ERK). The aneuploid effect of THz radiation was corroborated by the significant increase of nondisjunction events (Fig. 3). Aneuploidy is a THz radiation effect studied by many authors (Table III). Indeed, the possibility that THz exposure below the current safety limits could induce aneuploidy would have potential implications for safety and protection of professional and nonprofessional categories. It is noteworthy that all the studies on THz-induced aneuploidy performed so far, although not numerous, gave positive results. The aneuploid potential of THz radiation was evidenced by fluorescent *in situ* hybridization [Korenstein-Ilan et al., 2008], analysis of mitotic figures [Hintzsche et al., 2011] and CREST-positive micronuclei [De Amicis

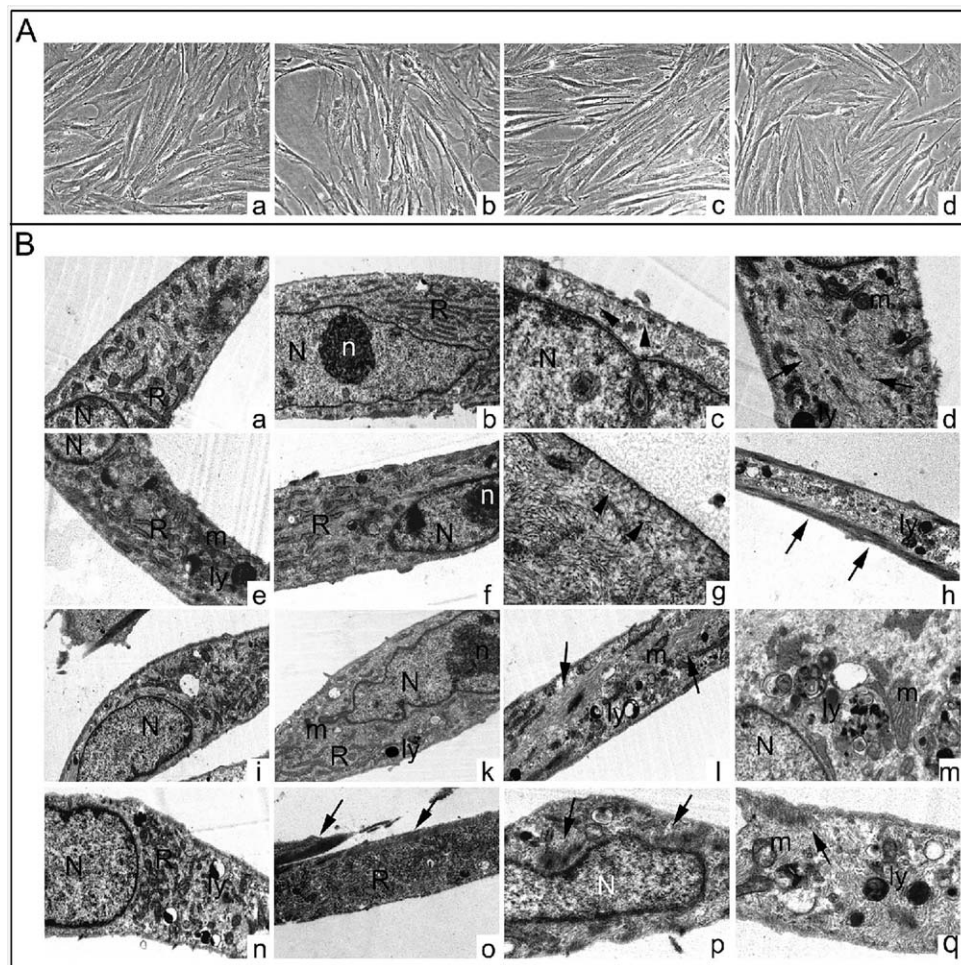


Fig. 7. Morphological analysis of THz irradiated HDF fibroblasts. Panel A: Morphological analysis of control cells (a, c) and 1 hr (b) and 48 hr (d) after THz irradiation using an inverted microscope. No morphological differences were found between control and irradiated cells. Panel B: Ultrastructural analysis of irradiated HDF 1 and 48 hr after exposure to THz radiation. Control HDF: a–d (1 hr) and i–m (48 hr): cells display an elongated appearance with condensed or occasionally dilated mitochondria (m), rough endoplasmic reticulum (R), primary and secondary lysosomes (ly), thin and intermediate filaments (arrows) and pynocytic

vesicles (arrowheads). (N: nucleus, n: nucleolus), original magnification $\times 7,000$ (a,b,d), $\times 11,000$ (c). e–h. HDF 1 hr after THz exposure. Dilated rough endoplasmic reticulum (R) and a substantial increase of the polymerization of thin and intermediate filaments (arrows) and a slight increase of pinocytotic vesicles (arrowheads) were seen. Original magnification $\times 7,000$ (e,f,h), $\times 11,000$ (g). i–q. HDF 48 hr after THz exposure: no morphological differences between THz exposed HDF and control cells were observed, only an increase of the polymerization of thin and intermediate filaments (arrows) was seen. Original magnification $\times 7,000$.

et al., 2015] (observed also in the present study). However, there is need for confirmatory studies of the few works performed so far and to elucidate mechanisms of interaction between THz radiation and the mitotic spindle, since aneuploidy is the result of disturbance to the latter. In any case, it is interesting to note that aneuploid effects of electromagnetic fields have been detected not only in the THz band (100 GHz–10 THz), but also with radiofrequencies ranging between 0.8 and 2.45 GHz [Mashevich et al., 2003; Schmid and Schrader, 2007; Mazor et al., 2008; Tkalec et al., 2009; Ballardin et al., 2011] and 50 Hz magnetic fields [Udroiu et al., 2006]. Therefore, one might speculate that the possible interactions between electromagnetic fields and the mitotic spindle are largely frequency-independent.

The mitotic spindle can be affected by interactions taking place at the macromolecular and/or ultrastructural level. The first are well known by studies on the so-called “spindle poisons”, which—disturbing the polymerization dynamics of tubulin—leads to disruption of the mitotic spindle [Matson and Stukenberg, 2011]. Therefore, it can be hypothesized that an external electromagnetic field could interact with the dipole moment of tubulins involved in the formation of the mitotic apparatus, resulting in a disorder of the latter [Ballardin et al., 2011]. However, this speculation has not been experimentally proved. To give a contribution to the comprehension of aneuploidy induced by THz radiation, we have also performed the ultrastructural analysis of irradiated cells. Interestingly, a temporarily increase of deposition of

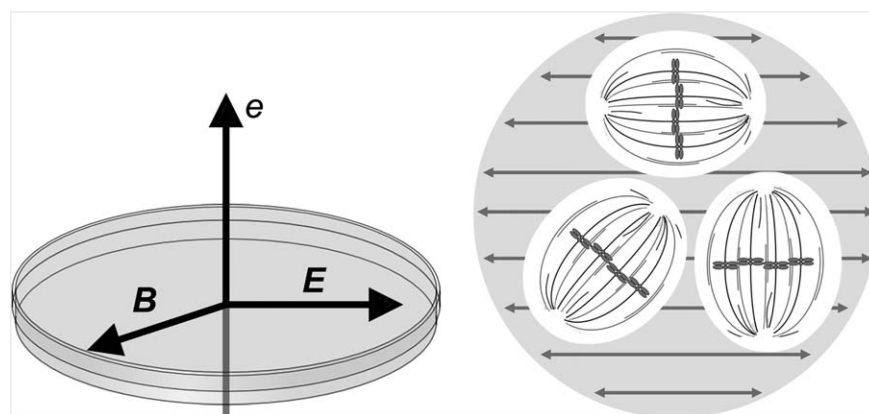


Fig. 8. Proposed interaction of THz radiation and mitotic spindle. Left: Field vectors in the irradiated petri dish; e = THz wave, B = magnetic field vector, E = electric field vector. Right: Possible alignment with the electric field of randomly orientated cells: the effect of the field on spindle microtubules is null if they are parallel (cell on top) and maximum if they are perpendicular (cell on bottom, right).

filaments below the plasma membrane was observed by transmission electron microscopy. Since the expression of actin was similar in THz irradiated and control cells, this increased deposition might be due to a different polymerization of filaments rather than to an increase of the synthesis of filamentous proteins. The different polymerization of actin could contribute to the induction of the aneuploidy observed, considering the role of this protein in spindle formation and thus in accurate chromosome segregation [Sandquist et al., 2011].

It is also interesting to consider another possible mechanism of interaction at the ultrastructural level, i.e., an effect of the electromagnetic fields on the microtubules. Zhao et al. [1999] reported that cells exposed to a 150 V/m Direct Current electric field divide with a cleavage plane oriented perpendicular to the applied field, and this is in accordance with the property of microtubules to align with electric fields [Kim et al., 2008]. This phenomenon has seen an application with the so-called “Tumor-Treating Fields”: 100 kHz, 200 V/m, Alternating Current electric fields are used to provoke mitotic disruption and arrest tumor growth [Kirson et al., 2007]. Therefore, there is increasing evidence of the effects of electric fields on the mitotic spindle. Concerning electromagnetic radiation, a study by Schrader et al. [2011], using a 900 MHz, 90 V/m electromagnetic field, indicated that the electric field component (and not the magnetic one) induced spindle disturbances in A_L cells. We can presume, therefore, that the aneugenic effects of THz radiation, which we and other authors evidenced, could be due to the alteration in spindle formation by changing tubulin polymerization and/or to the influence of the electrical component exerted on the mitotic spindle. Since this electric field could induce spindle microtubules to align parallel to its direction, the disturbance is maximum for those cells whose metaphase plate is parallel to the field, while there should be virtually

no damage to those cells where the plate is perpendicular to the field (Fig. 7). This means that in a cell culture, where cells are randomly orientated, there could be different degrees of spindle disturbance during exposure.

Another objective of our work was to compare the results of genomic damage due to aneuploidy events on adult fibroblasts to those observed on fetal fibroblasts [De Amicis et al., 2015]. Interestingly, the level of THz-induced CREST-positive MN was twice higher in fetal cells compared to the adult ones, thus indicating a higher sensitivity of the former. Leaving apart extrinsic factors (such as microenvironments), which may play a role in vivo but are not present in vitro, the higher sensitivity of fetal cells is thought to be due to less efficient DNA repair mechanisms [Udroiu and Sgura, 2016]. However, this is not a plausible explanation for the higher increase of CREST-positive MN in fetal fibroblasts compared to the adult ones. In fact, differences due to different efficiency in DNA repair implies that the observed effect is due to DNA breakage, but—as evidenced by both the present and previous work [De Amicis et al., 2015]—DNA breakage is absent and the genomic damage is due to aneuploidy induction. Therefore, the difference observed in the induction of CREST-positive MN is probably due to the fact that, during THz exposure, a greater fraction of fetal fibroblasts was undergoing mitosis compared to the adult ones (cells in G2/M: 26% and 8%, respectively). In fact, since the effects on the mitotic spindle are exerted only during mitosis, the higher percentage of cells that go into mitosis during exposure in fetal fibroblasts could explain the differences observed. Further investigations, specifically on the effects of THz radiation on the mitotic spindle and the whole cytoskeleton, could help to better understand this phenomenon.

Finally, although they are a less sensitive model than fetal fibroblasts, adult fibroblasts evidenced induction of

TABLE III. Overview of Studies on THz Radiation Effects on Heat Shock Response, Cell Cycle Perturbations, and Genome Integrity

f (THz)	H (mW/cm ²)	Pulse rate	Time	T (°C)	ΔT	Cells	HSP	Cycle perturbations	Clastogenesis	Aneuploidy	Ref.
0.1	0.031	CW	24 hr	37	0.3	Lymphocytes	n/d	n/d	n/d	Yes	Korenstein-Ilan et al., 2008
0.1–2.0	0.008–0.2	1–82,000 kHz	20'	n/d	1.0	Leukocytes	n/d	n/d	No	n/d	Angeluts et al., 2014
0.1–2.0	57	1 kHz	10'	21	0.7*	3D skin	n/d	n/d	Yes	n/d	Titova et al., 2013
0.1–0.15	0.23	2.5 Hz	20'	RT	0	Lymphocytes	n/d	No	No	n/d	Zeni et al., 2007
0.1–0.15	0.4	2.5 Hz	20'	RT	<0.2	HFFF2	No	Yes	No	Yes	De Amicis et al., 2015
0.1–0.15	0.4	2.5 Hz	20'	RT	<0.2	HDF	No	No	No	Yes	present work
0.106	0.043–4.3	CW	30'	36	1.4	AL	n/d	n/d	n/d	Yes	Hintzsche et al., 2011
0.106	1.96	CW	24 hr	n/d	n/d	AL	n/d	n/d	No	n/d	Kleine-Ostmann et al., 2012
0.106	0.04–0.88	CW	2–24 hr	37	<0.3	HDF, HaCaT	n/d	No	No	n/d	Hintzsche et al., 2012
0.38, 2.52	0.03–0.9	CW	2, 8 hr	37	<0.3	HDF, HaCaT	n/d	No	No	n/d	Hintzsche et al., 2013
2.3	110	CW?	60'	24	13.0	hESC	No	n/d	No	n/d	Bogomazova et al., 2015
2.52	84.8	CW	80'	37	3.0	HDF	Yes	n/d	n/d	n/d	Wilmink et al., 2011

f : frequency; H : irradiance; Time: exposure duration; T: starting temperature; ΔT : temperature increase during exposure; HSP: Heat Shock Proteins increase; CW: continuous wave; HFFF2: fetal fibroblasts; HDF: adult fibroblast; AL: human-hamster hybrid; HaCaT: keratinocytes; hESC: human embryonic stem cells; *Calculated; RT: room temperature; n/d: not determined.

TABLE IV. Summary of the Results Obtained in this Work

Biological effect	Endpoint studied	THz effect
Cell cycle perturbations	Analysis of DNA content	No effect
Heat shock response	HSPs	No effect
Genome damage	Total MN	Increase
Clastogenic genome damage	CREST-negative MN	No effect
	γ -H2AX	No effect
	AKT, NF-kB, ERK	No effect
Aneugenic genome damage	CREST-positive MN	Increase
	Nondisjunction	Increase

aneuploidy. This result should be taken into account for the risk evaluation of the human exposure to THz radiation, also because the incident power density used (0.4 mW/cm²) was below the current safety limit of 1 mW/cm² [IEEE, 2006].

CONCLUSION

Our study confirms the increasing evidences that THz radiation exposure in vitro causes nonthermal effects on the genome. Since the aneugenic properties of THz electromagnetic fields have been detected at exposure conditions below current safety limits, further studies for human protection are needed. Moreover, although there is full agreement between the studies conducted so far on THz-induced aneuploidy, the explanation of the biophysical mechanisms leading to it remains speculative and not experimentally proven. Therefore, mechanistic studies on the interactions between electromagnetic fields and the mitotic apparatus would be very useful.

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AUTHOR CONTRIBUTIONS

S.D.S., R.B., F.L., and A.S. conceived the experiments and designed the study. A.D., E.G., and G.P.G. designed and realized the exposure system and conducted the THz irradiation. R.B. performed cell cycle analysis and proteins expression. A.D.A. and E.R. performed the H2AX phosphorylation assay. V.F., J.M., and E.C. performed the Q-FISH telomere analysis, CREST-micronuclues test and nondisjunction analysis. M.B. and L.M. performed the ultrastructural morphology studies. I.U. wrote the manuscript. All the authors reviewed the manuscript.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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