

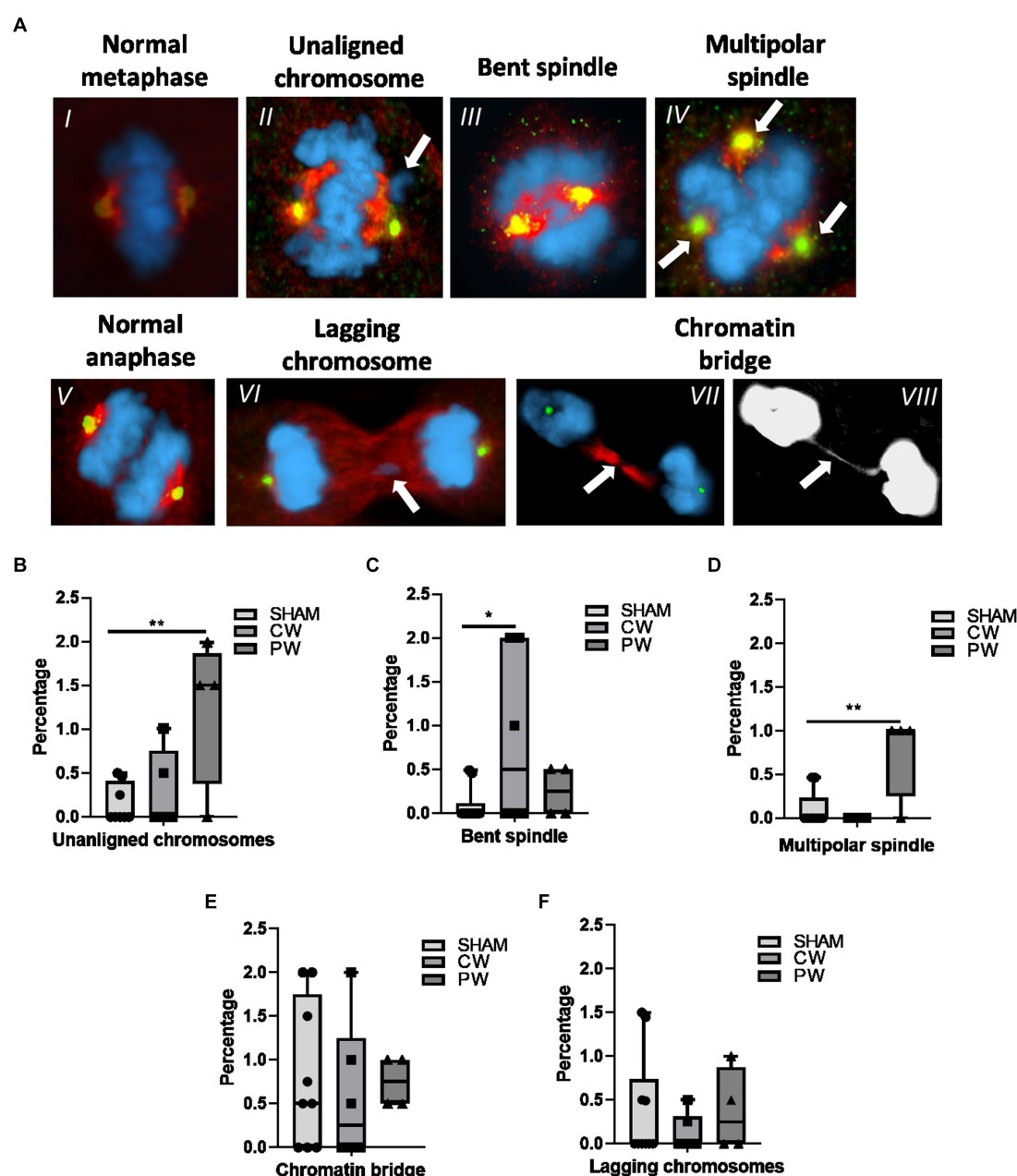


FIGURE 6

Immunofluorescence staining of γ -Tubulin (green) and α -Tubulin (red) in CW, PW 1.6 GHz RF exposed and sham HDF. (A) Representative images of a normal metaphase (I), a metaphase displaying an unaligned chromosome (II) and a bent spindle (III). Representative image of a multipolar spindle (IV) (three centrosomes as indicated by green signals). Representative images of a normal anaphase (V), an anaphase containing a lagging chromosome (VI), and an anaphase displaying a chromatin bridge (VII) in merge and gray scale (VIII). Percentage of unaligned chromosomes (B) bent spindles (C), multipolar spindles (D), chromatin bridges (E) and lagging chromosomes (F) in sham and 1.6 GHz CW and PW RF-exposed HDF cells. At least three independent experiments were conducted.

this non-ionizing radiation, particularly non-thermal effects. However, the results of the current knowledge are controversial, probably due to a lack of standardization in methodologies, homogeneity in cell/animal models and in study design.

This *in vitro* study aims to contribute to a better understanding of the potential non-thermal biological effects of RF exposure in HDF. The frequency of 1.6 GHz RF and a SAR value of 0.4 W/kg (whole body occupational exposure limit of the current European regulation) (4) were employed.

Moreover, different signal modulations, either CW or PW, were used to investigate the different biological impact associated with signal modulation.

To evaluate genotoxic effects, γ -H2AX/53BP1 foci analysis for DSBs, CREST-stained MN and mis-segregation assays for chromosome anomalies were performed. Cellular alterations were investigated by examining cell cycle, HSP and pro-survival proteins expression, ultrastructure and mitotic spindle perturbations potentially induced by RF-EMF exposure.

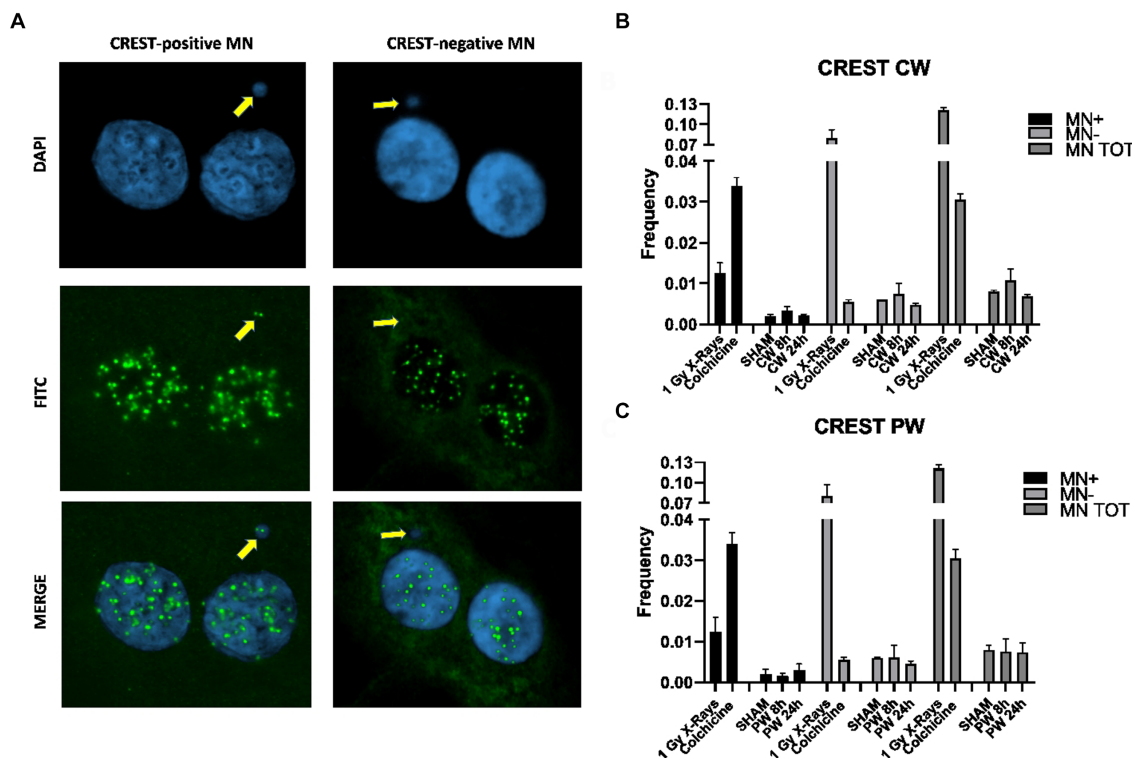


FIGURE 7

CBMN assay and CREST staining in HDF exposed to 1.6 GHz CW, PW RF. (A) Representative images of BN cells with MN CREST-negative (MN-) and MN CREST-positive (MN+) indicated by yellow arrows. (B) Bar plots of micronuclei frequencies represent the percentages of CREST-positive, -negative and total MN in HDF sham cells and CW exposure cells. Bars indicate the standard error. (C) Bar plots showing the frequencies of CREST-positive, -negative and total MN in HDF control cells exposed to PW. Bars indicate the standard error. As positive control for CREST-negative MN and CREST-positive MN, cells were either irradiated using 1Gy X-rays or treated with 10 ng/mL colchicine. At least three independent experiments were performed.

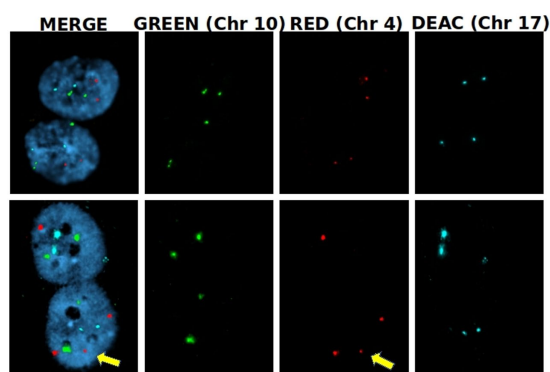


FIGURE 8

Nondisjunction frequency/percentage in HDF sham and exposed to 1.6 GHz CW RF. Representative images of two BN cells displaying a normal chromosome distribution of the three homologous chromosomes (upper row) and an unequal distribution of chromosome 4 (3 + 1) (yellow arrow).

After a 2h exposure, either PW or CW, for each time point examined, we did not find any effect on cell cycle. Also, we did not observe genotoxic effects, neither clastogenic nor aneugenic damage, according to the majority of the investigations performed on several cellular models *in vitro* exposed to different RF-EMF frequencies.

Specifically, no variation in the number of total CREST-stained MN, CREST-positive and CREST-negative MN and of γ -H2AX/53BP1 foci was found in human fibroblasts after exposure to RF-EMF as reported in a previous study (13). The absence of MN increase with both signals suggests that 1.6 GHz RF exposure did not induce any chromosomal alteration as reported by several studies (63–67). Also, no significant difference in γ -H2AX/53BP1 colocalized foci, between sham and exposed cells with both CW or PW signal was observed, indicating no induction of DSBs in agreement with the absence of CREST-negative MN.

This result is in accordance with other studies performed on various cell models indicating no increase of γ -H2AX foci number (51, 68, 69). A study in which γ -H2AX/53BP1 foci induction was evaluated in human dermal fibroblasts after PW and CW 2.54 GHz exposure also did not describe any significant differences between sham and exposed samples for both signals (13).

In addition, although our results did not evidence a significant induction of chromosome mis-segregation, it is worth noting that data were collected analyzing only three out of 23 chromosomes. The difference in non-disjunction between exposed and sham samples could have been more representative if the number of chromosomes analyzed was higher. Using a correction factor of 23/3 (the ratio between the pairs of all human chromosome and the chromosomes analyzed) we could extrapolate a percentage of non-disjunction on the whole genome comprised between 0.5 and 1% for CW and PW

TABLE 2 Non-disjunction frequencies in sham and RF exposed cells.

		Chromosome 4						Chromosome 10						Chromosome 17						4 + 10 + 17	
1.6 GHz	Sample	BN	2 + 2	3 + 1	ND%	SD	N	2 + 2	3 + 1	ND%	SD	N	2 + 2	3 + 1	ND%	SD	N	ND%			
CW	Sham	5,500	5,500	0	0.00	0.00	6	5,500	0	0.00	0.00	6	5,500	0	0.00	0.00	6	0.00			
	8 h	1,505	1,505	0	0.00	0.00	3	1,505	0	0.00	0.00	3	1,504	1	0.07	0.58	3	0.07			
	24 h	4,000	3,997	3	0.07	1.00	3	4,000	0	0.00	0.00	3	4,000	0	0.00	0.00	3	0.07			
PW	Sham	3,564	3,564	0	0.00	0.00	8	3,564	0	0.00	0.00	8	3,564	0	0.00	0.00	8	0.00			
	8 h	2,101	2,098	3	0.14	0.55	5	2,101	0	0.00	0.00	5	2,101	0	0.00	0.00	5	0.01			
	24 h	1,531	1,531	0	0.00	0.00	3	1,530	1	0.06	0.58	3	1,531	0	0.00	0.00	3	0.06			

Binucleated cells (BN), non-disjunction (ND), standard deviation (SD), and experimental replicates (N).

exposed samples, suggesting that further experiments will be needed to address the biological relevance of such events.

Mitotic spindle analysis following exposure to PW indicated a significant rise in the frequency of multipolar spindles and unaligned chromosomes, while cells exposed to CW exhibited an increase in bent spindles, as compared to sham cells.

It is well known that unaligned chromosomes may recover their alignment on the equatorial plate or display a delay in cell segregation forming lagging chromosomes (70, 71). However, our data did not evidence the presence of lagging chromosomes suggesting that the latter phenomenon did not occur in our experimental setting. On the other hand, both bent and multipolar spindles have been associated to chromosome mis-segregation (72, 73).

It is worth noting that multipolar spindle development can be caused by different cellular events. For instance, centriole overduplication, fragmentation of the pericentriolar material (PCM) or cytokinesis failure in human cells can lead to the formation of multipolar spindles, accompanied by centrosome amplification during mitosis (74, 75). A number of researchers have hypothesized that also non-ionizing radiation may affect the normal formation of spindle poles (63, 76, 77). Specifically, components of the electric field in radiofrequency could disturb the moment of the electric dipole of tubulin, generating mitotic spindle abnormalities as previously proposed (25). It is worth noting that multipolar spindles may contribute to non-disjunction events, as suggested by Thompson and Compton in 2011 (72). Despite this, it is known that multipolar spindles observed during mitosis frequently evolve into normal bipolar spindles, primarily due to the clustering of centrosomes, facilitating progression through normal mitosis (78). However, the clustering of centrosomes can have a side effect in the mis-segregation, resulting from a stronger attachment of two segregating chromosomes to the same pole of the spindle (merotelic attachment). Equi-merotelic attachment (those with approximately equal numbers of microtubules orientated toward the correct and wrong poles) evolves in lagging chromosome in anaphase that end up as micronuclei. Instead, multi-merotelic attachments (those with many microtubules oriented toward the wrong pole) make a large contribution to mis-segregation without displaying lagging in anaphase (72). This phenomenon may offer a partial explanation for those rare non-disjunction events observed in RF exposed cells, suggesting a mechanism that could correlate the presence of multipolar spindle with non-disjunction events and, as a consequence, the absence of lagging chromosomes and CREST-positive micronuclei.

Besides on mitotic spindle, significant effects were found on cell ultrastructure and protein expression after 2 h CW or PW exposure. Morphological observation employing transmission electron microscope showed an increased deposition of filaments below the plasma membrane, similar to what was previously reported in the same cell line for other EMF frequencies (25, 79). The expression of actin, as detected in western blotting assays, was similar in 1.6 GHz RF exposed and sham cells, thus suggesting that the increase of deposition of filaments below the plasma membrane might be due to a different polymerization of filaments rather than to an increase of the synthesis of filamentous proteins. Several double membrane-surrounded lysosomes were observed 24 h after PW exposure, suggesting the formation of autophagosomes and activation of autophagy as pro-survival cell signaling. Activation of autophagy mediated by ROS generation was previously observed in mouse spermatocyte-derived

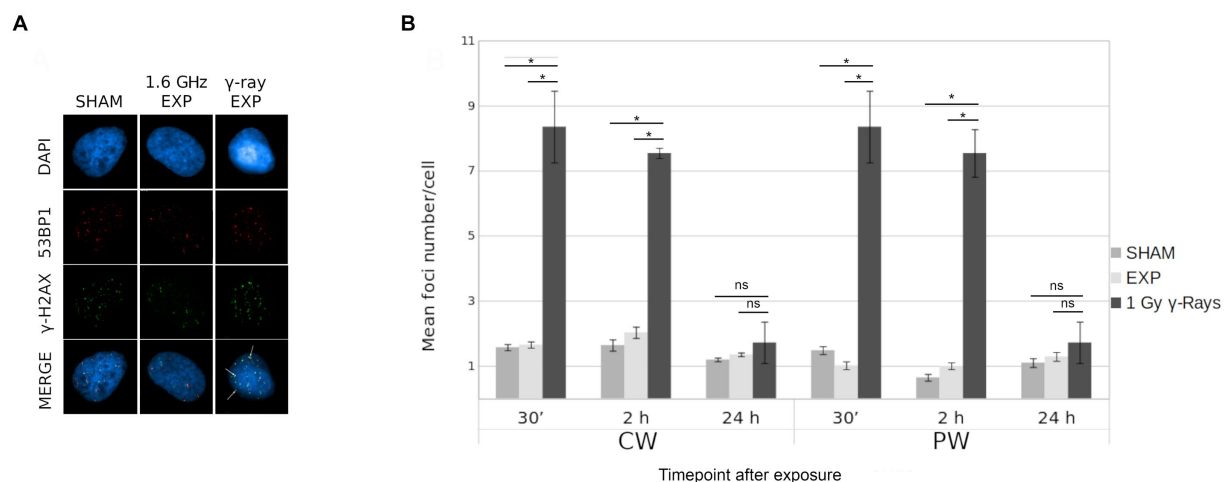


FIGURE 9

γ-H2AX/53BP1 colocalization foci in in CW, PW 1.6 GHz RF exposed and sham HDF. (A) Representative images of γ-H2AX/53BP1 foci acquisition: DAPI (blue), γ-H2AX foci (green), 53BP1 foci (red) and co-localized foci (yellow) in sham cell, 1.6 GHz RF exposed cell and γ-ray exposed cells. Colocalized foci are indicated by white arrows. (B) Bar plot of mean H2AX/53BP1 foci/cell in 1.6 GHz CW, PW and in γ-rays exposed and sham cells at 30 min, 2 h, and 24 h after exposure. Bars indicate the standard error. As positive control cells were irradiated at 1 Gy γ-ray. Three independent experiments were performed. (* $p \leq 0.05$; ns, $p > 0.05$).

cells (GC-2) and in spiral ganglion neurons (SGN) exposed to 1800 MHz (GSM talk signal) for 24h, as showed by the formation of autophagosomes, increased levels of LC3-II, Beclin1 and decreased levels of the protein p62 (50, 51). AKT has a key role in autophagy induction. Indeed, in response to the increase in ROS levels under nutrient-rich conditions, AKT inhibits the initiation of autophagy (80). We observed a decrease in activation by phosphorylation of the AKT kinase after exposure to 1.6 GHz RF-EMF.

During stress response, the highly conserved proteins called heat shock or stress proteins (HSPs, categorized according to their size in families HSP-70, HSP-27, HSP-60, HSP-90, and HSP-100), are rapidly produced to protect cells from stressful conditions (heat shock, nutrient deprivation, ultraviolet exposure, and others), mainly through their ability to prevent misfolding of other proteins and to accelerate their refolding and renaturation (81). No uniform data on the expression of HSPs after RF exposure are reported in the literature. This is probably due to the cell type employed, exposure modality or endpoint chosen for the experiments in each study. Gerner et al. reported that mitochondrial HSP-60, HSP-70, and HSP-90 were found to increase, among several more proteins, in cultured human fibroblasts exposed to 1800 MHz (exposure time of 8 h with intermittence pattern 5 min. ON /10 min OFF) (82). Conversely, Sanchez et al. reported that 900 MHz RF had no effects on HSP-27 or HSP-70 expression after 48 h of exposure of human primary dermal fibroblasts (83). Similarly, IMR-90 human fibroblasts exposure to 2.1425 GHz CW for up to 28 h did not vary the expression of HSP-27 and HSP-70 (84). In human peripheral blood mononuclear cell, exposed to 1.8 GHz RF-EMF for up to 44 h, the HSP-70 level, as analyzed by flow cytometry, was not modified (85). In our experiment, we found an increased expression of only HSP-60 and HSP-25 in HDF after 2 and 24 h of 1.6 GHz PW RF exposure, suggesting an activation of these proteins. In contrast, we observed a decreased expression of HSPs 24 h after a CW RF exposure.

In this controversial scientific scenario, our results are in agreement with most *in vitro* findings on different cell models about the lack of genotoxic damage induced by RF, either

clastogenic or aneugenic. Despite this, we find alterations of the mitotic spindle, with a significant increase in multipolar spindles following PW exposure and we also observed a tendency of RF to induce non-disjunction events with both signals. However, our study reveals an increase in these spindle abnormalities without a concomitant increase in MN-positive CREST, suggesting no aneuploidy effect probably due to spindle abnormalities reversion, as proposed by other authors (78). Nonetheless, this apparent discordant result requires further investigations.

The reported observations indeed highlight the complexity of cellular response to RF and emphasize the need for further investigations to clarify the overall biological effects of 1.6 GHz PW and CW RF, given the widespread and constant public RF-EMF exposure, mostly due to mobile communication devices.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

LuM: Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. SDS: Conceptualization, Data curation, Methodology, Project administration, Supervision, Writing

– original draft, Writing – review & editing. VF: Data curation, Formal analysis, Investigation, Writing – review & editing, Writing – original draft. ELR: Conceptualization, Data curation, Formal analysis, Investigation, Writing – review & editing, Writing – original draft. GA: Investigation, Writing – review & editing, Data curation. CF: Writing – review & editing, Formal analysis. MB: Writing – original draft, Writing – review & editing, Data curation, Formal analysis, Investigation, Validation, Visualization. LC: Writing – review & editing. AS: Conceptualization, Data curation, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. FrB: Writing – review & editing, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft. JM: Writing – review & editing, Conceptualization, Data curation, Formal analysis, Investigation. FeB: Writing – review & editing, Formal analysis, Investigation. ErR: Writing – review & editing, Formal analysis, Investigation. DN: Writing – review & editing, Formal analysis, Investigation. LaM: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. RB: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing. FL: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpubh.2024.1419525/full#supplementary-material>

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