Mutation Research - Genetic Toxicology and Environmental Mutagenesis 899 (2024) 503817



Contents lists available at ScienceDirect

Mutation Research - Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox





Investigation of genotoxicity induced by intermediate frequency magnetic field combined with ionizing radiation: In vitro study on human fibroblast cells

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ARTICLE INFO

Keywords:
Intermediate frequency
Adaptive response
In vitro
Genotoxicity
Oxidative stress
Skin

ABSTRACT

These days, exposure to electromagnetic fields has become omnipresent in modern society. Not only the extremely-low frequency and radiofrequency, but also intermediate frequency (IF) magnetic field (MF) might be absorbed in the human body resulting in an ever-growing concern about their possible health effects. Devices, such as induction cooktops, chargers, compact fluorescent lamps, touchscreens and electric vehicles emit a wide range of intermediate frequency fields. We investigated the effects of 22 kHz or 250 kHz intermediate frequency magnetic field exposure on the human skin cells. We also examined the adaptive response phenomenon; whether IF MF exposure could possibly reduce the harmful genotoxic effects of ionizing radiation. To get answers to these questions, in vitro studies were carried out on fibroblast cells to investigate the effects on oxidative stress, DNA damage and micronucleus formation. We found a decreased micronucleus formation due to the 22 kHz IF MF exposure and significantly increased oxidative stress in fibroblast cells, which were exposed only to 250 kHz IF MF. We were unable to detect the protective or co-genotoxic effects of intermediate frequency magnetic field exposure combined with ionizing radiation, thus we found no evidence for the adaptive response phenomena.

1. Introduction

The public concern about the harmful effects of human exposure to electric and magnetic fields has focused mainly on the extremely-low frequency and radiofrequency (RF) ranges. However, the usage of intermediate frequency (IF) magnetic field (MF) emitted by typical household and industrial devices has increased significantly in the last decade. According to the WHO, the IF ranges from 300 Hz to 10 MHz between the extremely-low frequency and RF electromagnetic fields. Several products of emerging technology generate IF magnetic fields, varying widely in frequency and field strength [1]. Compact fluorescent lamps and devices, which use inverter technology (e.g., refrigerators, microwave ovens and laundry machines) are commonly found in households. Induction cookers, chargers and heaters operate in the 19–70 kHz range [2,3], while the frequencies of fields from fluorescent lamps range from 1.2 to 100 kHz [4]. Electronic article surveillance systems and anti-thief gates - which are commonly installed in shops and libraries - operate in a wide range (20 Hz to 2.45 GHz) [5–8]. Exposure to IF fields has been measured both around touchscreens [9] and on electric and hybrid vehicles [10]. The smart metering program in France uses the power grid for communication by adding an electrical signal at IF frequencies between 35 and 90 kHz. Human exposure to IF fields from smart metering systems was evaluated by ANSES [11]. The proximity readers - installed in e.g., hospitals and offices, allowing remote reading of magnetic badges when a person passes through the control gates - operate at 120 kHz or 13.6 MHz. In order to safeguard the population against the possible adverse effect of non-ionizing radiation, the International Commission on Non-ionizing Radiation Protection (ICNIRP) issued guidelines and the European Commission (EC) also published recommendations for the exposure limits of electromagnetic field respectively [12,13].

The number of in vitro studies investigating the genotoxicity of IF MF exposure is limited [14] and their results vary. Most of these studies investigated the 23 kHz IF electromagnetic fields similar to those emitted by induction cooktops. Nakasono et al. [15] found that 2 kHz, 20 kHz and 60 kHz IF MFs do not have mutagenic, co-mutagenic or gene

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https://doi.org/10.1016/j.mrgentox.2024.503817
Received 15 November 2023; Received in revised form 4 July 2024; Accepted 8 August 2024
Available online 10 August 2024
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conversion potentials in microbial genotoxicity tests. IF MF was measured by Herrala et al. [16] suggesting that 7.5 kHz MF at magnetic flux densities up to 300 μT has no genotoxic or co-genotoxic effect in vitro or in vivo. On the contrary, there was some evidence that IF MF exposure might reduce the level of genetic damage. Exposure to 90 kHz magnetic fields at 93.36 μT was examined on human lens epithelial cells, indicating no detectable cellular genotoxicity [17]. Two other papers report about the 250.8 kHz frequency: Brech et al. [18] found significantly increased DNA damage in canine and human blood leukocytes, however Németh et al. [19] did not detect a genotoxic effect of 250.8 kHz IF MF exposure in human leukocytes, nor in H295R human adrenocortical carcinoma cell line.

The phenomenon of adaptive response can be observed when cells which were pre-exposed to low and non-toxic doses of a genotoxic agent (as adaptive dose) become resistant to the damage induced by subsequent exposure to a higher and toxic dose of the same, similar or another genotoxic agent (as challenge dose, CD) [20]. The adaptive response is a well-described phenomenon in the field of ionizing radiation [21–24], however there are other studies investigating different types of stressors (e.g., chemical agents), which report the protective effect [25–27]. A review summarized the in vitro and in vivo studies in which the adaptive dose was substituted with non-ionizing radiation [20]. Most of these studies, which investigated the RF irradiation combined adaptive response had contradicting results. Other studies suggest that RF pre-exposure was capable of inducing the adaptive response in cells exposed to ionizing radiation [21,28–32]. This phenomenon was described in human blood lymphocytes [33,34] and in mice also [35]. Both of them utilized 900 MHz RF radiation, which was later challenged by chemical agents. Kim et al. [36], by detecting an attenuation effect of radiofrequency irradiation, suggested that 2 MHz frequency exposure may have decreased melanin synthesis in the skin by increasing Hsp70 expression and decreasing p53 expression. In contrast, in our previous study [37], we did not detect the protective effect of RF radiation against ultraviolet irradiation in a 3D in vitro human skin model.

The IF irradiation might affect the human skin as the first defense of the human body. Although fibroblasts are commonly found in the connective tissue, a large number of them are present in the dermis. On these fibroblast cells, the possible effects of the non-ionizing radiation [38–40] and their capability for adaptive response to ionizing radiation [41–43] have been studied several times.

In this study, we investigated whether IF MF in itself could affect the DNA integrity, and whether it could possibly induce a protective effect against ionizing radiation by the adaptive response phenomenon. The protocol and irradiation conditions chosen for the experiments allowed us to investigate both phenomena: the direct genotoxic and co-genotoxic effects of exposure to IF MF, and the adaptive response as well. Therefore, the aim of this study was, firstly, to examine whether 22 kHz or 250 kHz IF MF ranges can cause any effects, and secondly, to determine the protective properties of the human skin cells against the harmful effects of ionizing radiation. The genotoxic or co-genotoxic effects such as the induction of DNA single-strand breaks (SSBs), double-strand breaks (DBSs), oxidative stress and micronuclei formations were assessed by in vitro experiments.

2. Materials and methods

2.1. Cell cultures

Human Dermal Fibroblast cells from adult skin (Gibco, Thermo Fisher Scientific, Waltham, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Thermo Fisher Scientific, Waltham, USA) containing 10 % fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Waltham, USA) at 37 °C in a humidified incubator (Binder, Tuttlingen, Germany) with 5 % CO_2. The medium was replaced every other day and the cells were subcultured with trypsinization at 70–80 % confluence, as recommended by the manufacturer. The cells were reseeded into 35 mm Petri dishes (Corning®, NY, USA) at 3–6 * 10^3 cells/ cm^2 density one day before starting the exposures and maintained at 37 °C in a humidified atmosphere with 5 % CO_2. The fourth passages of cell culture were used for experiments.

2.2. IF MF exposure

The exposure system consisted of a function-generator, an RF power amplifier and a solenoid coil cylinder, which was described earlier [18,19] (Fig. 1). The entire coil system was placed in a CO_2 incubator (HETO-HOLTEN A/S, Cellhouse 154, Allerød, Denmark) where the background power frequency (50 Hz) stray magnetic field was negligible, ranges between 0.5 and 1.5 μT [44]. Petri dishes were placed into the coil, using a plastic holder. The coil was operated at resonant mode and the magnetic flux density was 100 μT (~ 80 A/m) at 22 kHz or 250 kHz. The magnetic flux density in the exposure space (where Petri dishes were placed) was measured using ELT 400 Exposure Level Tester with ELT B-Field-Probe 3 cm^2 (Narda Safety Solutions GmbH, Germany). The inhomogeneity of the magnetic flux density inside the coil and within the exposure area at different levels was less than 10 %. The temperature of the sample was maintained at 37 °C by the water flow system. In this exposure system fibroblast cells were exposed to IF MF for 24 h. During sham exposure, sham samples were kept in another CO_2 incubator, in the same milieu (37 °C with 5 % CO_2) in the absence of the exposure coil.

2.3. Ionizing irradiation

Ionizing irradiation was applied as CD to investigate the adaptive response phenomenon. X-RAD 225/Xli X-ray source (Precision X-ray, North Branford, USA) was used for X-ray irradiation at a dose rate of 0.5 Gy/min or 1 Gy/min. This X-ray source is a fully automated system. It has a large internal x-ray chamber, equipped with a motorized turntable and adjusted programmable specimen shelf. Automated or fixed collimators with integrated filter recognition provide focal irradiation, while software contributes dose measurement and control. A full screen, real time specimen view and image capture are possible during exposure.

Fibroblast cells were exposed to ionizing radiation in the culture dishes, at room temperature. Each genotoxicity assay has been developed to detect different endpoints - such as SSBs, DSBs, oxidative stress or genetic aberration -, thus, they differ in their sensitivity. The appropriate dose of ionizing radiation had to be chosen according to the type and sensitivity limit of the assays. For the comet assay, a 2.5 Gy dose of ionizing radiation was used as CD and 12 Gy as a positive control. For the Cytokinesis Block Micronucleus Assay, 0.5 Gy was used as CD and 1 Gy as a positive control. 2.5 Gy dose of ionizing radiation was also used for the γH2AX Assay method as CD and 4 Gy, as a positive control.

2.4. Experimental protocols

Two series of investigations were performed; the first examined the effects of 22 kHz, and the other one examined the 250 kHz of IF MF. An investigation series consisted of three independent experiments, each of them evaluated with three different methods (comet assay, Cytokinesis Block Micronucleus Assay, γH2AX Assay).

The following 5 exposure conditions were examined:

sham and sham exposure as negative control (SH + SH),

IF MF and sham exposure (IF MF + SH),

IF MF exposure and 4 h later challenged with ionizing radiation as CD (AR),

ionizing irradiation in itself, according to the selected CDs (IR)

ionizing radiation as a positive control (PC).

Sham exposed samples received all of the environmental conditions that other samples had at the time, but in the absence of the actual exposure. All Petri dishes were kept in the same milieu and handled

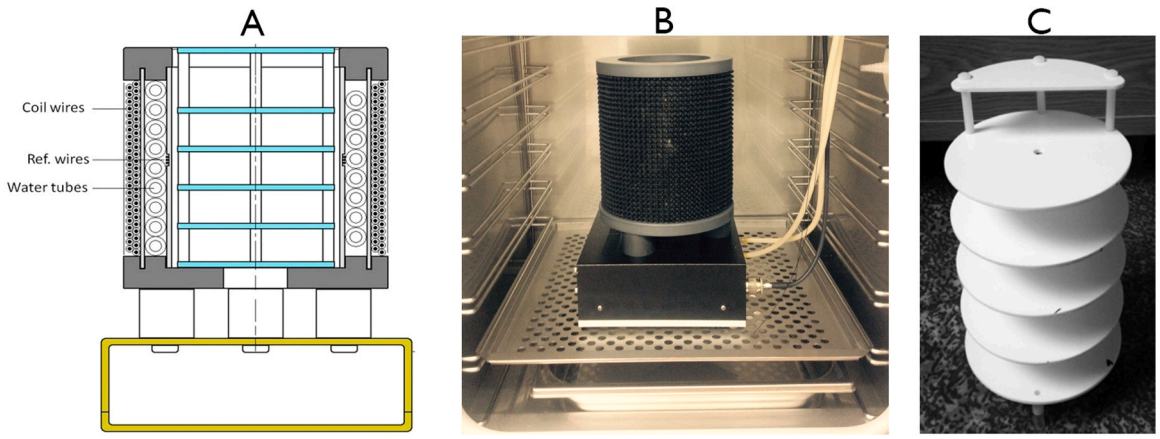


Fig. 1. Intermediate frequency (IF) magnetic field (MF) exposure system. (A): The scheme of the solenoid IF MF exposure system. (B): The solenoid coil exposure system was placed in a CO_2 incubator. (C): The sample holder within the solenoid coil.

simultaneously.

The Alkaline Comet Assay protocol (with FPG modification) is presented in Fig. 2. (A). Since the oxidative stress and SSBs of the DNA are significantly regenerated within 4 h by DNA repair, the FPG-modified Alkaline Comet Assay protocol was completed with two additional exposure condition ('SH' and 'IF MF' condition) to examine the possible genotoxic effect of IF MF independently. In this case, cells were exposed for 24 h to sham or IF MF and immediately processed for analysis with the comet assay. Fig. 2 also shows the protocol of the Cytokinesis Block Micronucleus Assay (B) and the γH2AX Assay (C). Two Petri dishes were used in parallel in each exposure condition. Moreover, in order to minimize individual biases, all experiments for each endpoint were assessed in a blind manner.

2.5. Assay procedures

2.5.1. FPG-modified alkaline comet assay

The comet assay (a single-cell gel electrophoresis technique) was used as a method to detect the SSBs of DNA damage [45]. The FPG-modified alkaline comet assay protocol introduced by Dušinská [46] was applied to detect 8-oxoguanine in DNA. The formamidopyrimidine DNA glycosylase (FPG) from *E. coli* releases damaged purines, including 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) and 8-oxo-7,8-dihydroguanine (8oxoG), generating an AP site. The enzyme cleaves the AP sites, creating nucleotides that appear in the Comet Assay additionally to SSBs, allowing the measurement of oxidative stress as well.

The cells were trypsinized with 0,05 % Trypsin-EDTA solution after the radiations. Cell suspension from a Petri dish was mixed with 1 % low-melting point agarose and pipetted into eight slides pre-coated with 1 % normal-melting point agarose. This suspension was immediately covered with coverslip and kept at 4 °C until the agarose solidified. After 5 min the cover glass was gently removed, and all slides were immersed in freshly prepared cold lysis buffer (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, pH; 1 % Triton X-100) and lysed at 4 °C (Lysis condition). Six slides were used to measure oxidative stress. These slides (Buffer condition and FPG enzyme condition) were washed three times with enzyme reaction buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM Na2EDTAH2O, 0.2 mg/ml BSA, KOH, pH 8.0), 5 min each, then were transferred into an ice-cold metal surface. 50 μl enzyme reaction buffer or FPG solution (1:1000 enzyme dilutions) was added to the gels and covered with a coverslip. Slides were transferred into the incubator at 37 °C for 30 min. All slides, from the incubator or lysis buffer were rinsed in cold electrophoresis solution (1 mM Na2EDTA, 300 mM NaOH, pH 13) and placed in a horizontal gel electrophoresis tank (APELEX, Maxigel Eco2, France), which was filled with cold, fresh electrophoresis solution. The duration of DNA unwinding was 20 min and electrophoresis lasted 22 min. The voltage and the running time were set up by the following: V/cm * min = 20. After electrophoresis, the slides were washed once with PBS, once with distilled water. Slides were dried at room temperature and stored until analysis. Staining was performed with Sybr Gold (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) and slides were examined at a 20 × magnification objective using a Zeiss AxioPlan fluorescence microscope (Oberkochen, Germany). The pictures of the cells were taken by a CCD camera and were evaluated using the Comet Assay IV software (Instem, UK). 100 comets were examined per slide and the tail DNA % parameter was calculated to describe each comet.

2.5.2. Cytokinesis block micronucleus assay

The cytokinesis-block MN assay was carried out according to standard procedures [47,48]. Fibroblast cells were cultured on 20 * 20 mm coverslips in 35 mm Petri dishes. After exposure, 1 μg/ml cytochalasin-B (Sigma-Aldrich, St. Louis, MO USA) was added to the culture medium and fibroblasts were incubated for 72 h further. Hypotonic potassium chloride (0.075 M) solution was added for 5 min and fixation was performed by adding methanol. Fixed cells were stained with 10 μg/ml Acridine Orange (Sigma-Aldrich, St. Louis, Missouri, USA) in PBS and examined at 63 × magnification objective using a Zeiss AxioPlan fluorescence microscope (Oberkochen, Germany). According to Fenech et al. [47], scoring was carried out manually. 100 cells in each sample were measured for Nuclear Division Index (NDI) calculation and cytotoxicity scoring. Micronuclei formations (MNi) were evaluated in 500 binucleated (BN) cells. The frequency of BN cells containing MNi (BN with MNi) and the frequency of MNi in BN cells (MNi in BN) were counted.

2.5.3. γH2AX assay

The cells were cultured on 20 * 20 mm coverslips in the 35 mm Petri dishes. Based on our preliminary studies, a 24-h incubation period after the ionizing radiation was necessary to detect a manageable number of γH2AX foci. After this incubation, the cells were washed three times with PBS at room temperature with shaking. Fixation was performed by adding freshly prepared ice-cold methanol/acetone solution (1:1) at 4 °C for 10 min. This was followed by three more washes with PBS at room temperature with shaking. Blocking was performed with 5 % BSA in PBS for 30 min at room temperature with shaking. Primary γH2AX antibody (Phospho-histone H2A.X, Ser139, 20E3, Rabbit, mAb, 1:4000, Leiden, Netherland) were added to the cells and incubated overnight at 4 °C. The cells were washed three times with PBS, then reacted with goat–conjugated secondary antibodies (Alexa Fluor 488 goat anti-rabbit IgG (H+L), Invitrogen®, Thermo Fisher Scientific, Waltham, MA, USA) and incubated for further 2 h at 4 °C. It was followed by three more

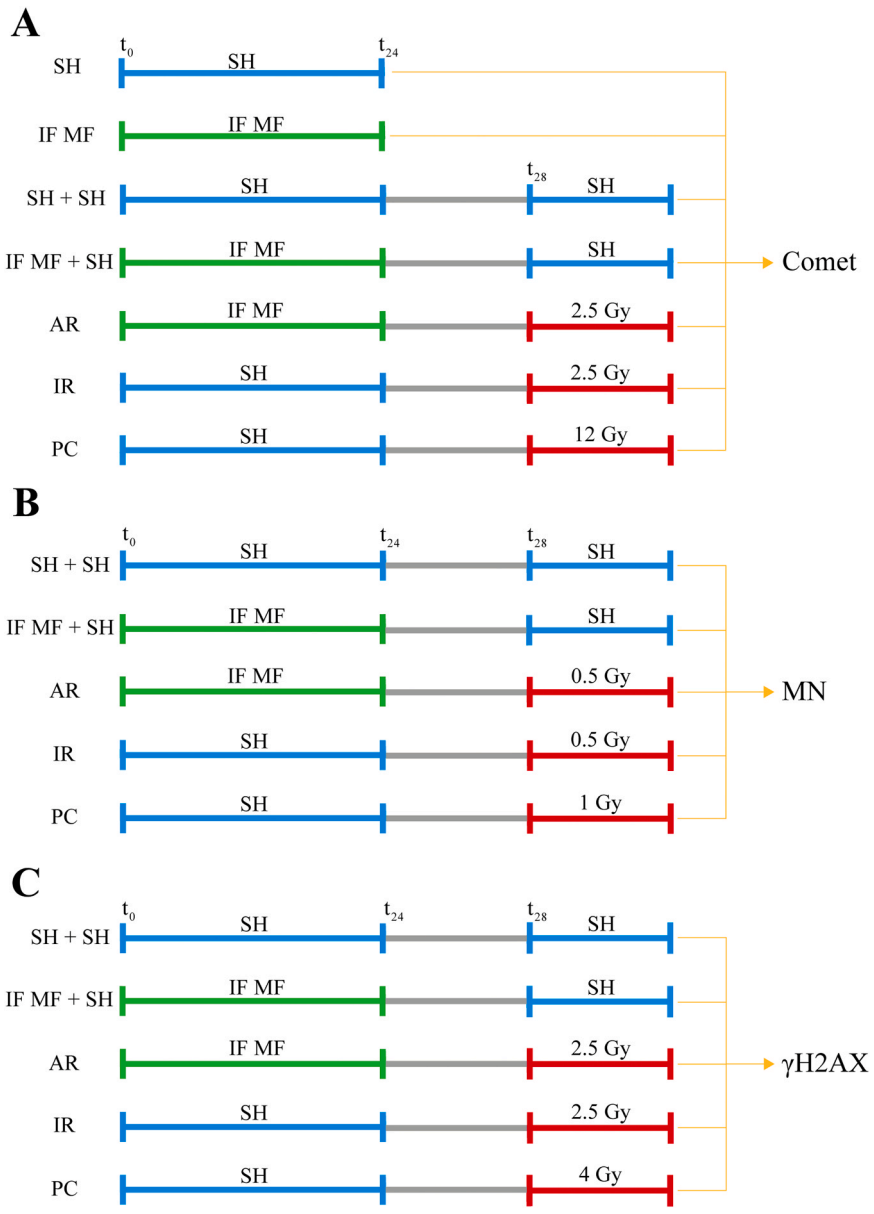


Fig. 2. Schematic representation of the experimental protocols. The protocol and irradiation conditions allowed us to investigate the direct genotoxic effects of exposure to IF MF and the adaptive response (AR) respectively. Human dermal fibroblast cells were exposed to 22 kHz or 250 kHz IF MF for 24 h (at t_0 - t_{24}) and/or four hours later were exposed to ionizing irradiation (at t_{28}). The time of ionizing irradiation was maximum of 15 min. (A): The protocol and irradiation conditions used for FPG-modified Alkaline Comet Assay. SH: 24 h of sham exposure, IF MF: 24 h of IF MF exposure, SH + SH: sham and sham exposure, IF MF + SH: IF MF and sham exposure, AR: IF MF exposure and 4 h later challenged with 2.5 Gy ionizing radiation as CD, IR: 2.5 Gy ionizing irradiation only, PC: 12 Gy as a positive control. (B): The protocol and irradiation conditions used for Cytokinesis Block Micronucleus Assay. SH + SH: sham and sham exposure as a negative control, IF MF + SH: IF MF and sham exposure, AR: IF MF exposure and 4 h later challenged with 0.5 Gy ionizing radiation as CD, IR: 0.5 Gy ionizing irradiation only, PC: 1 Gy as a positive control. (C): The protocol and irradiation conditions were used for the γH2AX Assay. SH + SH: sham and sham exposure as a negative control, IF MF + SH: IF MF and sham exposure, AR: IF MF exposure and 4 h later challenged with 2.5 Gy ionizing radiation as CD, IR: 2.5 Gy ionizing irradiation only, PC: 4 Gy as a positive control.

washes with PBS at room temperature with shaking. The cells were stained and coverslips were stuck onto glass slides with Fluoroshield Mounting Medium containing DAPI (abcam® TM, Cambridge, UK). 200 cells from each slide were examined at 63–100 × magnification objective by using a Zeiss AxioPlan fluorescence microscope (Oberkochen, Germany). Foci containing cells and the foci number in these cells were counted.

2.6. Statistical analysis

Analyses were carried out with R Studio version 1.4.1106 software (Software Foundation Inc., Boston, MA, USA). Statistical significance was considered for the p-value < 0.05. The following comparisons were tested: SH + SH vs IF MF + SH, AR vs IR, SH + SH vs IR. A further comparison of SH vs IF MF was relevant for comet assay method.

For FPG-modified Alkaline Comet Assay, the median of Tail DNA% was calculated for each slide for three experiments and analyzed by Linear Mixed-Effects Model.

The NDI data were analyzed with ANOVA. The number of binucleated cells with micronuclei (BN with MNi) and the number of micronuclei in binucleated cells (MNi in BN) were analyzed by fitting a negative binomial model.

The average number of foci was calculated for the statistical analysis of the γH2AX Assay. Statistical comparisons were carried out by fitting a

linear model using Generalized Least Squares.

3. Results

3.1. Single-strand breaks (SSBs) and oxidative stress by FPG-modified alkaline comet assay

We have investigated SSBs with Alkaline Comet Assay and oxidative stress with the FPG-modified form. The results are shown in Fig. 3 and Fig. 4. Overall, the enzyme buffer (Buffer) did not differ from Lysis, however, FPG enzyme treatment caused significantly higher Tail DNA% values. In both frequencies (22 and 250 kHz), FPG enzyme treatment showed no significant differences between SH + SH vs IF MF + SH, AR vs IR (2.5 Gy), however caused significantly increased Tail DNA% ($p < 0.001$) in IR (2.5 Gy) exposed cells compared to SH + SH exposed cells (Fig. 3.).

Regarding to the additional two conditions that determined after 24 h of exposure, 22 kHz IF MF condition did not differ from SH exposure (Fig. 4/A.). On the other hand, 250 kHz IF MF exposure showed significantly increased Tail NDA% ($p = 0.033$) from SH exposure (Fig. 4/B.). The ΔTail DNA% values derived from the difference between FPG and Buffer Tail DNA% and were shown in the secondary Y-axis in Fig. 3–4. ΔTail DNA% represents the amount of 8-oxoguanine as oxidative stress in the tail.

3.2. Micronuclei analysis

Table 1 shows the results of 22 kHz IF MF protocol. Nuclear Division Index (NDI) was significantly different ($p < 0.001$) between SH + SH and IR groups. The number of binucleated cells with micronuclei (BN with MNi) significantly decreased in IF MF + SH group ($p = 0.035$), furthermore significantly increased in IR group ($p < 0.001$) compared to SH + SH group. The difference in the number of micronuclei in binucleated cells (MNi in BN) was marginally significant between SH + SH vs IF MF + SH ($p = 0.069$) and highly significant between SH + SH vs IR groups ($p < 0.001$).

The results of 250 kHz IF MF protocol showed in Table 2. Nuclear Division Index (NDI) was significantly different ($p < 0.001$) between SH + SH and IR groups. The number of binucleated cells with micronuclei (BN with MNi) significantly increased between SH + SH and IR exposed samples ($p < 0.001$). The difference in the number of micronuclei in binucleated cells (MNi in BN) was highly significant between SH + SH vs IR groups ($p < 0.001$).

3.3. γH2AX phosphorylation

Fig. 5 shows the results of the γH2AX assay. The 22 kHz IF MF irradiation experiments (Fig. 5. (A)) and 250 kHz IF MF irradiation experiments (Fig. 5. (B)) show similarities. IF MF + SH group showed no significant changes from the SH + SH group. The difference between AR and IR groups was not statistically significant.

4. Discussion

In today's world, wireless chargers and earphones, induction cooktops, etc., have become everyday tools. Devices operating at IF are present not just in households, but on public transport as well (e.g. electric car, bus). Due to the rapid technological development, more and more devices will operate on the IF MF range, which will increase human exposure to this type of radiation. Unfortunately, this topic is underrepresented in the scientific literature. In this paper, we investigated the potential genotoxic effects of 22 kHz (represents the frequency of induction cooktops) and 250 kHz (frequency range of anti-thief gates) IF MF on human fibroblast skin cells.

Few studies have investigated the biological effects of IF MF. Sakurai et al. [49] measured the 23 kHz MF induced gene expression on human fetus-derived astroglia cells, but they did not find detectable changes. Another paper by Nishimura et al. [50] examined the carcinogenicity of 20 kHz MF using a transgenic rasH2 mouse model. Their results indicated the lack of carcinogenicity in that model. In genotoxicity studies by Herrala et al. [16,51], rat primary astrocytes were exposed to 7.5 kHz MF for 24 h in combination with menadione. The experimental design was similar to our study and the comet assay as well as the micronucleus assay scoring was performed to evaluate genetic damage. No genotoxic or co-genotoxic effects were observed after exposure to MF alone or in combination with menadione. Similar results were observed in our study, we could not detect the possible harmful effect of IF MF at any frequency. Our genotoxicity measurements of SSBs showed, that the IF MF + SH exposed cells were not different from SH cells. Compared to the Alkaline Comet Assay, techniques linked with enzymes (e.g., endonuclease III, FPG, T4 endonuclease V) create more efficient and sensible methods, giving the opportunity to detect not only breaks, but specific lesions in DNA. FPG-modified Comet Assay is a common method for detecting oxidative stress in vitro [52]. Another study of Purschke et al. [53] aimed to compare the induction and repair of radiation-induced DNA damage in human (e.g. HSF1 fibroblast cell line) and rodent cell lines. They found that FPG-modified comet assay is a very sensitive

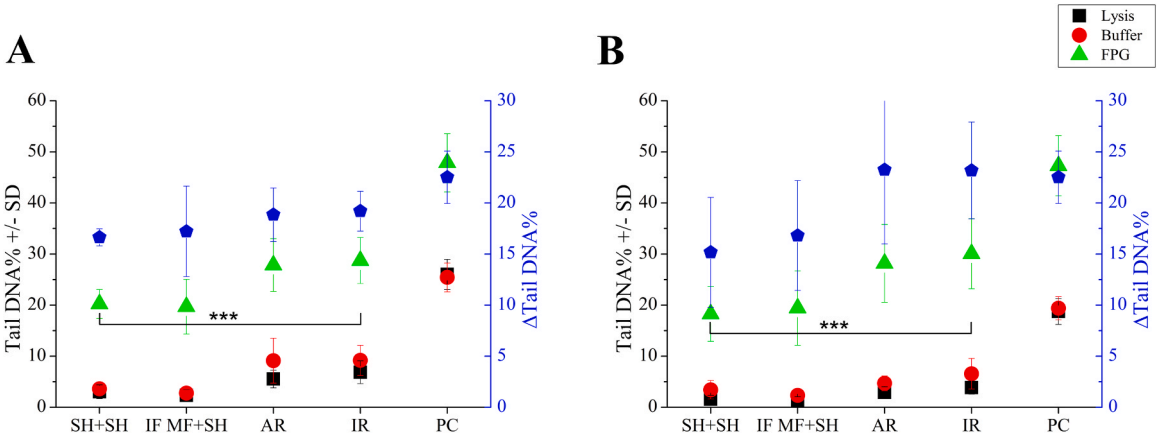


Fig. 3. Results of the FPG-modified Comet Assay. Data represent three independent experimental replicates at each experimental point. (A): The mean of the median Tail DNA% value of 22 kHz IF MF exposed cells ± standard deviation (SD). (B): The mean of the median Tail DNA% value of 250 kHz IF MF exposed cells ± SD. The ΔTail DNA% values (on secondary right Y-axis) derived from the difference between FPG and buffer Tail DNA% representing the amount of 8-oxoguanine as oxidative stress level. SH + SH: sham and sham exposure, IF MF + SH: IF MF and sham exposure, AR: IF MF and 2.5 Gy of ionizing radiation, IR: 2.5 Gy of ionizing radiation, PC: 12 Gy of ionizing radiation. ***: $p < 0.001$.

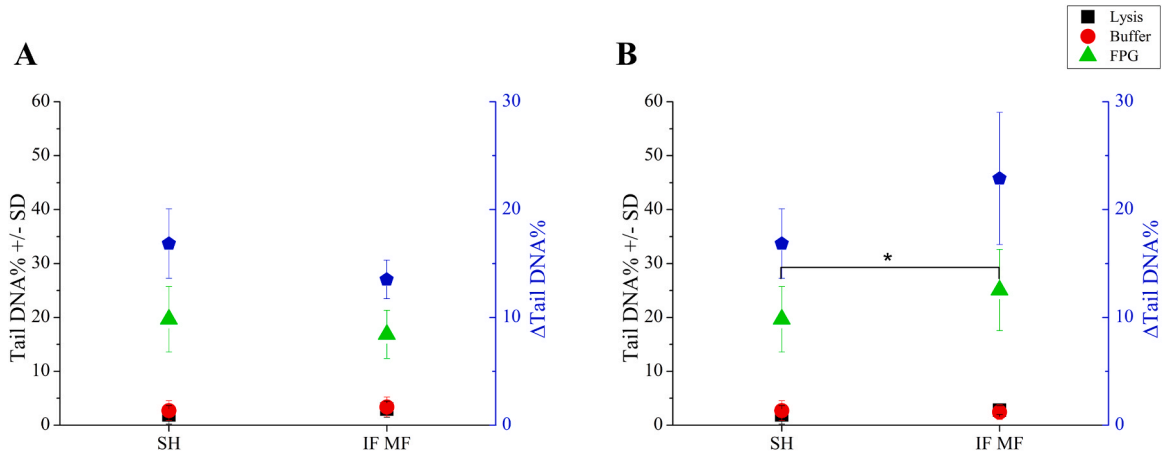


Fig. 4. Results of the FPG-modified Comet Assay. Data represent three independent experimental replicates at each experimental point. (A): The mean of the median Tail DNA% value of 22 kHz IF MF exposed cells ± standard deviation (SD). (B): The mean of the median Tail DNA% value of 250 kHz IF MF exposed cells ± SD. The ΔTail DNA% values (on secondary right Y-axis) derived from the difference between FPG and buffer Tail DNA% representing the amount of 8-oxoguanine as oxidative stress level. SH: 24 h of sham exposure, IF MF: 24 h of IF MF exposure, *: $p < 0.05$.

Table 1
Results of cytokinesis block micronucleus assay at 22 kHz frequency. These data show the average Nuclear Division Index (NDI), the number of binucleated cells with micronuclei (BN with MNi) and the number of micronuclei in binucleated cells (MNi in BN) ± SD. Data represent three independent experimental replicates at each experimental condition.

	Per 100 cells	Per 500 BN cells	
Treatment	Number ofNDI ± SD	Number ofBN with MNi ± SD	Number ofMNi in BN ± SD
SH + SH	1.51 ± 0.04	10.17 ± 5.34	10.67 ± 5.79
IF MF (22 kHz) + SH	1.48 ± 0.03	5.67 ± 1.75*	6.33 ± 2.14[a]
AR	1.27 ± 0.05	35.83 ± 7.25	39.83 ± 10.34
IR	1.27 ± 0.06***	41.00 ± 10.47***	47.50 ± 11.06***
PC	1.18 ± 0.03	86.67 ± 14.08	108.83 ± 20.41

SH + SH: sham and sham exposure, IF MF + SH: IF MF (22 kHz) and sham exposure, AR: IF MF (22 kHz) and 0.5 Gy of ionizing radiation, IR: 0.5 Gy of ionizing radiation, PC: 1 Gy of ionizing radiation.
* Significantly different from SH (p-value < 0.05).
*** Significantly different from SH (p-value < 0.001).
[a] Marginally significantly different from SH (p-value = 0.0691).

Table 2
Results of cytokinesis block micronucleus assay at 250 kHz frequency. These data show the average Nuclear Division Index (NDI), the number of binucleated cells with micronuclei (BN with MNi) and the number of micronuclei in binucleated cells (MNi in BN) ± SD. Data represent three independent experimental replicates at each experimental condition.

	Per 100 cells	Per 500 BN cells	
Treatment	Number ofNDI ± SD	Number ofBN with MNi ± SD	Number ofMNi in BN ± SD
SH + SH	1.51 ± 0.02	8.40 ± 2.88	9.20 ± 3.11
IF MF (22 kHz) + SH	1.51 ± 0.08	8.50 ± 5.43	9.67 ± 6.47
AR	1.27 ± 0.06	41. 67 ± 9.22	49.83 ± 12.42
IR	1.30 ± 0.09***	46.50 ± 15.66***	57.17 ± 20.47***
PC	1.18 ± 0.03	86.67 ± 14.08	108.83 ± 20.41

SH + SH: sham and sham exposure, IF MF + SH: IF MF (250 kHz) and sham exposure, AR: IF MF (250 kHz) and 0.5 Gy of ionizing radiation, IR: 0.5 Gy of ionizing radiation, PC: 1 Gy of ionizing radiation.
*** Significantly different from SH (p-value < 0.001).

method and could detected FPG-sites even at low dose of 0.3 Gy. However, the detection of FPG-sites by this technique is limited up to 4 Gy of X-ray, which is equivalent to a maximal 55 % DNA in the tail. Our results confirm this finding since 12 Gy ionizing radiation caused about 50 % DNA in the tail. Although, this study aimed to examine the adaptive response phenomenon, the experimental protocol of the FPG-modified comet assay gave the opportunity to examine the effect of 24 h IF MF exposure. Our measurements showed that 22 kHz IF MF + SH irradiation did not cause any changes on oxidative stress. Contrarily, we found that the 24 h of 250 kHz IF MF irradiation caused significantly increased oxidative stress level in fibroblast cells. Due to the lack of further oxidative stress endpoints, this result needs to be handled with caution. Further comprehensive investigations with other methods detecting 8-oxoguanine are necessary to understand the findings.

According to micronucleus formations, we found that the cell division (NDI value) was significantly decreased, the number of binucleated cells with micronuclei (BN with MNi) and the number of micronuclei in binucleated cells (MNi in BN) were significantly increased as an effect of ionizing radiation. These results concur to the study of Litvinchuk et al. [54], which reports a linear increase in binuclear cells containing micronuclei for absorbed doses from 1 to 5 Gy. Our finding regarding to sham exposure was similar to Elbakrawy et al. [55] and Yoshioka et al. [56]. However, our results also showed that 22 kHz IF MF exposure slightly and marginally decreased the number of binucleated cells with micronuclei (BN with MNi) and number of micronuclei in binucleated cells (MNi in BN). 250 kHz IF MF exposure caused no changes in micronucleus formations, which is comparable to the studies of Miyakoshi et al. [57] and Sakurai et al. at 23 kHz MF. Even if the original cytokinesis block micronucleus assay protocol [47] suggests 1000–2000 CB cells for micronucleus counting, based on the limitation of our irradiation system we just partially implemented the protocol. In our irradiation system, the irradiation parameters and dosimetry are determined by the maximum size of the Petri dish. We used 35 mm Petri dishes and inside it the cells were cultured on the largest possible, 20 * 20 mm coverslip. The fibroblast cells divide at least once during 72 h of cytochalasin-B treatment and grow twofold by mitosis. Optimal cell counting physically limits the maximum number of cells that can be grafted onto the coverslip. Due to the limitations of these conditions, maximum of 500 CB cells could be counted. Although no changes were observed in micronucleus formation after exposure to IF MF, these results should be interpreted with caution as the number of scored cells was lower than recommended. We are aware of the difficulty of the evaluation and we admit it as a limitation of this study. In order to minimize the individual biases, all experiments for each endpoint were

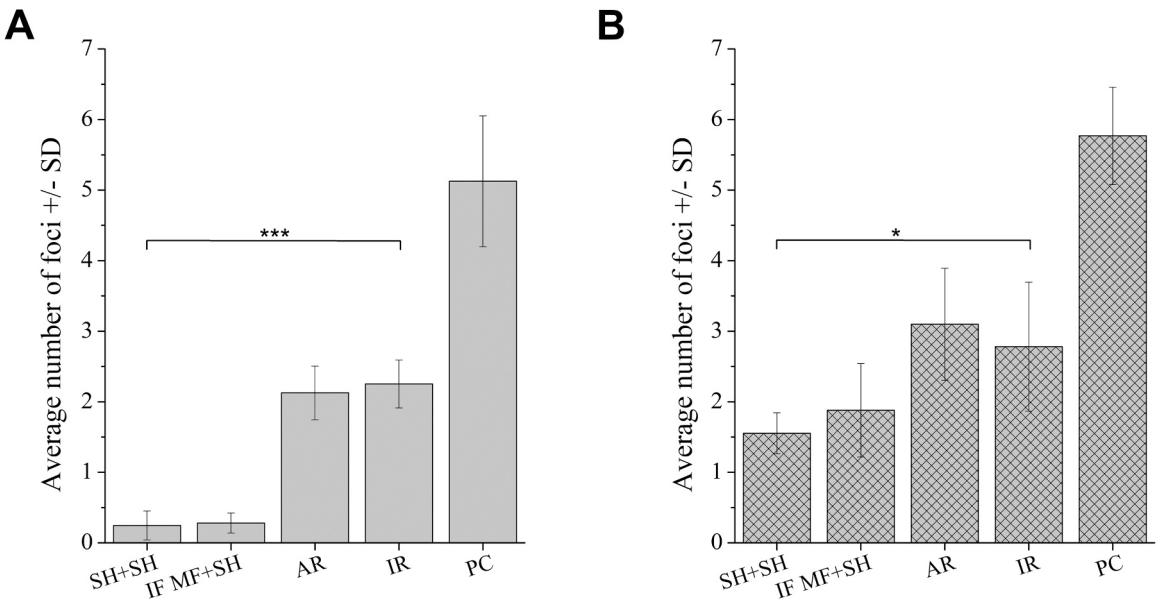


Fig. 5. The average number of foci ± standard deviation. Data represent three independent experimental replicates at each experimental condition. (A): Results of 22 kHz IF MF exposed cells ± SD. (B): Results of 250 kHz IF MF exposed cells ± SD. SH + SH: sham and sham exposure, IF MF + SH: IF MF irradiation and sham exposure, AR: IF MF irradiation and 2.5 Gy of ionizing radiation, IR: 2.5 Gy of ionizing radiation, PC: 4 Gy of ionizing radiation. ***: $p < 0.001$.

assessed in a blind manner; furthermore, the samples were coded and evaluated randomly. Based on our controversial result – oxidative stress increased due to 250 kHz IF MF and micronucleus formation decreased due to 22 kHz IF MF –, these findings should be interpreted with caution and further studies are needed.

Furthermore, to investigate the phenomenon of adaptive response, we examined whether IF MF exposure has any effect when it is combined with ionizing radiation. Based on other studies, which detected the adaptive response after the exposure to ionizing or non-ionizing radiations [30,58,59], we hypothesized that the exposure to low level of IF MF will reduce the harmful damage of ionizing radiation. Although the adaptive response is a described and published phenomenon in the field of non-ionizing radiation [30,59], similar to other investigations on carcinoma cell line H295R or 3D Reconstructed Human Skin tissue [19,37], we could not observe the protective effect of IF MF exposure against ionizing radiation on skin fibroblast cells. As a result, we were unable to detect the adaptive response under the exposure conditions of the present study. Our study is basically designed to investigate the adaptive response phenomena, but also it could be relevant to investigate co-genotoxicity. In this perspective, our findings show not just the lack of adaptive response, but also the lack of co-genotoxicity.

5. Conclusions

Here, we investigate the effects of 22 kHz or 250 kHz IF MF exposure and the possible adaptive response phenomenon in combination with ionizing radiation. Overall, in our laboratory environment during a 28-hour exposure protocol we could detect a decreased number of micronucleus formations in 22 kHz exposed fibroblast cells (IF MF + SH), although we found an increased oxidative stress upon 24 h of 250 kHz IF MF exposure alone. Furthermore, we could not observe an IF MF induced adaptive response or co-genotoxic effect on ionizing irradiated fibroblast cells. These results are in contrast with some previously reported studies, thus further investigation on IF MF induced genotoxicity is needed.

Funding

This work was supported by the French Agency for Food, Environmental and Occupational Health & Safety [Grant EST-2017 RF-08].

CRediT authorship contribution statement

Bertalan Pintér: Visualization, Validation, Methodology, Investigation. **Zsófia Szilágyi:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Erika Szabó:** Writing – original draft, Methodology, Investigation. **Yves Le Drean:** Writing – original draft, Supervision, Resources, Project administration, Conceptualization. **Györgyi Kubinyi:** Validation, Methodology, Investigation. **György Thuróczy:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Gyorgy Thuroczy reports financial support was provided by National Agency for Food Environmental and Occupational Health and Safety. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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