



## Research article

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## Intermediate frequency magnetic field at 250.8 kHz does not induce DNA damage or “Adaptive Response” *in vitro*

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### Abstract

The aim of this study was to examine whether intermediate frequency (IF) magnetic field (MF) induces genotoxic effects or play a role in the induction of adaptive response after combined exposure to IF MF and ionizing radiation in leukocytes and in adrenocortical carcinoma cell line H295R. Cells were exposed to 250.8 kHz at the magnetic field strength of 80 A/m (equivalent to 100  $\mu$ T magnetic flux density) for 20 hours, or exposed to IF MF for 20 hours and 24 hours later challenged with ionizing radiation (1.5 Gy X-ray). Evaluation of the DNA damage was performed with alkaline comet assay. Our results showed that there was no significant genotoxic effect of IF MF exposure compared to the controls in both cell types. Furthermore, results did not indicate a statistically significant change in DNA strand breaks in IF MF pre-exposed cells when they were subsequently exposed to 1.5 Gy. Consequently no adaptive response was detected.

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### Keywords

*genotoxicity, alkaline comet assay, intermediate frequency, magnetic field, adaptive response*

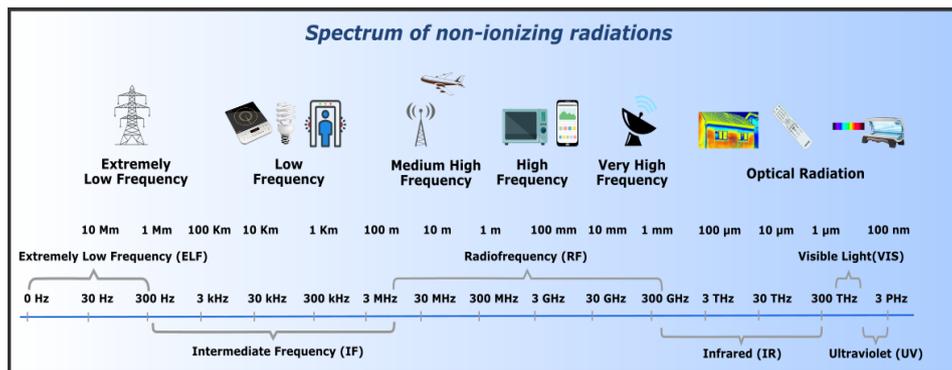
### Introduction

Electromagnetic fields (EMF), especially extremely low frequency (ELF) electric and magnetic fields (0-300 Hz) and radiofrequency (RF) fields (10 MHz –

300 GHz) have been thoroughly studied through the decades. The World Health Organization defines the intermediate frequency (IF) magnetic field (MF) as a frequency range between the ELF and RF fields, from 300 Hz to 10 MHz (WHO, 2005)(Figure 1). Many different consumer and industrial equipment used in public and working environment produce intermediate frequency magnetic fields, varying widely in frequency and strength. IF MFs are generated in household devices such as compact fluorescent lamps (CFL), induction cookers, devices

that use inverter technology to gain better control of speed or temperature at higher energy efficiency (microwave ovens, refrigerators, laundry machines, air conditioning systems)(Litvak et al. 2002, Aerts et al. 2017). Electronic article surveillance systems (EAS) or anti-theft devices are commonly installed in shops and libraries and operate over a wide range of frequencies (20 Hz - 2.45 GHz) (Roivainen et al. 2014). Electric and hybrid vehicles produce IF MF in the range of few kHz - 1 MHz (Percebon et al. 2016, Tell et al. 2014, Vassilev et al. 2015). Proximity Radio Frequency Identification (RFID) readers operate at 125 kHz or 13.50 MHz for remote reading of magnetic badges of personnel passing through control gates. Wireless power transmission (WPT) a new and rapidly developing technology is aimed to supply power to electronic equipment without wires. Household appliances, mobile phones, and even electric cars can be charged by

They concluded that IF magnetic field for 2 hours does not cause cellular genotoxicity. A few years later the same working group (Sakurai et al. 2012, 2013) exposed human fetus-derived astroglia cell line for 2, 4 and 6 hours at 23 kHz (100  $\mu$ T and 2 mT) and found that IF MF did not induce detectable changes in gene expression profile. The other investigated intermediate frequency is the frequency of wireless power transmission. Shi et al. (2014) conducted experiments on human lens epithelial cells at 90 kHz (93.36  $\mu$ T) for 2 and 4 hours and found, that there was no effects on cell proliferation, apoptosis, comet assay and  $\gamma$ H2AX foci formation. Sun et al. (2017) exposed the retinal pigment epithelial cells to 100 kHz for 24 hours and found no statistical differences at the comet assay parameters. The term adaptive response (AR) describes the phenomenon where a relatively low dose of ionizing radiation (IR) as adaptive dose (AD) induces a kind



**Figure 1:** Diagram of non-ionizing radiation spectrum, showing intermediate frequency across the range of frequencies and wavelengths.

WPT. So far, research focus was mainly on IF field emitting sources while the number of studies concerning exposure to IF MF on biological systems is very low (SCENIHR, 2015). There are just a few *in vitro* studies investigating genotoxic endpoints (Bodewein et al., 2019). One of the most investigated frequencies within the spectrum of intermediate frequency is that emitted from inductive cook top heaters, 23 kHz. Miyakoshi et al. (2007) and Sakurai et al. (2009) exposed CHO-K1 cells at 23 kHz for 2 hours with the magnetic flux density of 532  $\mu$ T and 6.05 mT, respectively and investigated the cell growth, comet assay, micronucleus formation and hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene mutation.

of adaptation (sometimes referred to as resistance) to genetic damage induced by a subsequently higher dose of IR (challenge dose, CD). Adaptive response induced by the low dose ionizing radiation depends on several factors, e.g. cell types, but also the cell metabolic state. Jiang et al. (2008) studied the adaptive response in different non-cancer (healthy) and cancer cell lines and showed that adaptive response was induced only in normal human fibroblast, but not in lung carcinoma cell line, glioma cell line, erythroleukemia cell line, or acute promyelocytic leukemia cell line. It seems that cancer cells show less sensitivity to AR than healthy cells. AR is not only inducible via the same agent, but there is a kind of "cross-resistance" to similar

genotoxic agents (Vijayalaxmi et al. 2014). Combined exposures to different agents such as non-ionizing radiation (as AD) and chemicals/pharmaceuticals or IR (as CD) induce the AR phenomenon also. For example, Sannino et al. (2013) studied the AR in human lymphocytes *in vitro* after exposure to RF at 1950 MHz as AD and later to 1.0 or 1.5 Gy as CD. It was shown that the IR induced significant decrease of micronuclei incidence rate when the lymphocytes were pre-exposed to RF-EMF. In earlier studies of Sannino and colleagues (2009; 2011), AR was also detected in human lymphocytes using 900 MHz RF EMF and the chemical mutagen mitomycin C.

Accordingly, the question arises: do other frequencies of the electromagnetic field spectrum induce the AR as well? Since adaptive response has not yet been studied after IF exposure, we investigated if IF MF could cause an adaptive response against ionizing radiation and if IF MF by itself could induce genotoxic effect. We selected one frequency in the middle of the IF frequency range at few hundred kHz, where several medical, industrial and household devices operate (e.g. proximity readers, inverter technology). We used two different cell types, leukocytes from healthy human blood and a human cancer cell line to test whether these two cell types act differently in the adaptive response protocol.

## Materials and methods

### *Chemicals and reagents*

The normal melting point agarose, KCl, DMSO, Triton-X, 0.25% Trypsin-EDTA, Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 HAM were purchased from Sigma (St. Louis, USA), low melting point agarose from ICN Biomedicals Inc. (Ohio, USA), glass slides and coverslips from VWR International (Pennsylvania, USA), alcohol, Sodium Hydroxide (NaOH), Sodium Chloride (NaCl), Na<sub>2</sub>EDTA, Tris from Reanal (Budapest, Hungary), RPMI-1640 medium from Thermo Fisher Scientific (Massachusetts, USA), ITS+Premix, Nu-Serum from BD Bioscience. Gel Red stain was purchased from Csertex Kft (Budapest, Hungary), T25 flasks from Nunk (Denmark), Petri dishes from Corning (NY, USA).

### *Exposure system*

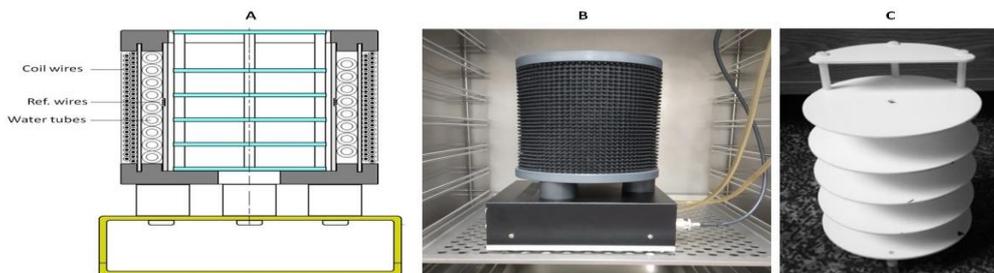
The exposure system consisted of a solenoid coil cylinder (inner diameter 12 cm, height 19 cm), a function-generator, and an RF power amplifier. The solenoid coil cylinder was constructed by double wrapped coils made from electrically isolated copper wires of 2x30 loops (outer layer), and was cooled by water tubes (inner layer) (Fig. 2A). Between the water tubes and the inner wall of the cylinder a referent coil loops were located in order to record the magnetic field continuously. The temperature of the inner wall of the coil was controlled and cooled by water flow. The entire coil system was placed in a CO<sub>2</sub> incubator (HETO-HOLTEN A/S, Cellhouse 154, Allerød, Denmark) where the background power frequency (50 Hz) stray magnetic field was the lowest, between 0.5-1.5  $\mu$ T (Gresits et al. 2015)(Fig. 2B). The cell flasks or the Petri dishes were placed in the coil, using a plastic holder (Fig. 2C). The coil was operated at resonant mode. The magnetic field strength was 80 A/m (equivalent to 100  $\mu$ T magnetic flux density) at 250.8 kHz. The magnetic flux density in the exposure space (at all levels where the flask or Petri dishes were placed) was measured using EM Field Analyzer EFA-3 (Wandel & Goltermann, Germany). The inhomogeneity of the magnetic flux density inside the coil and within the exposure area at different levels was less than 10%. The measurement of the temperature in samples was performed by four channel non-perturbing optical temperature probes (Luma Sense Technologies Inc., USA) and monitored during exposures. The temperature of the sample was maintained at 37°C by the water flow system. At the same time the control samples (negative control) were kept in another CO<sub>2</sub> cell incubator at 37°C.

### *Cell cultures and experimental protocol*

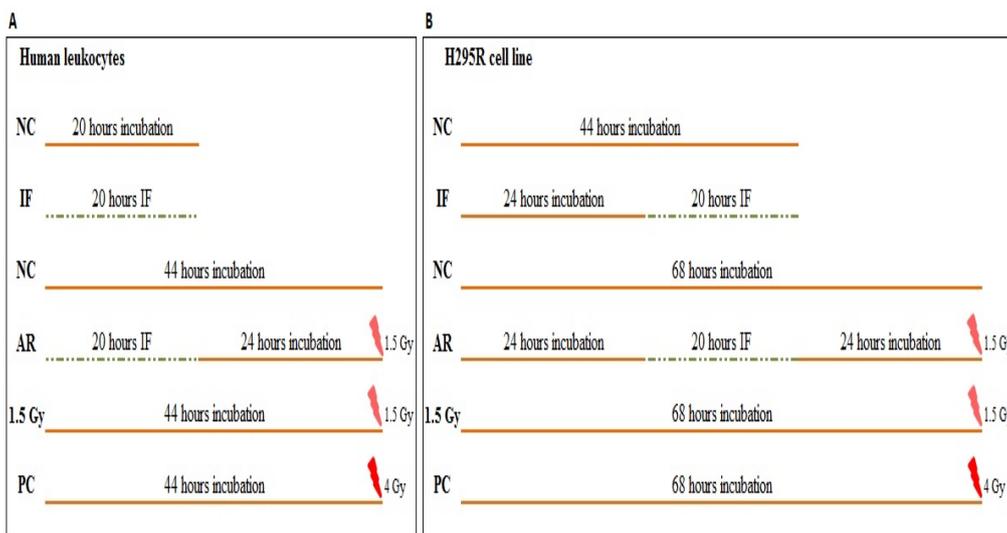
Two cell types were used in this study: leukocytes from whole blood and H295R human adrenocortical carcinoma cell line. Blood samples were taken from three healthy human donors, one for each experiment. The volunteers had not been exposed to pharmaceuticals, drugs and ionizing radiation three months prior to venous puncture and gave informed consent previous to experiments. Permission for the

experimental work on human blood tissues of volunteers was received from the Hungarian Scientific and Research Ethics Committee of the National Scientific and Medical Council (7478-1/2012/EKU 110/PI/12.). Approximately 10 ml blood was drawn by venous puncture into sterile heparinised Vacutainer vials by authorized personnel and on the same day diluted with RPMI-1640 medium to three times of its original volume. The diluted blood was distributed in 35 mm Petri dishes (2 ml/Petri-dish) for the exposure treatments. H295R cells (ATCC® CRL-2128™) were cultured in DMEM/F-12 supplemented with 1 % ITS+ Premix, 2.5 % Nu-Serum and 0.5 % penicillin-streptomycin at 37°C in a humidified atmosphere at 5 % CO<sub>2</sub> and 95 % air. Cells were routinely maintained as monolayers and sub-cultured twice a week by trypsinization when the confluency of the cells reached 70%. For our experiments, cells

were used between the fifth and tenth passage and seeded in T25 flasks for the exposure treatments. Diluted blood samples in Petri dishes were exposed either to IF MF (250.8 kHz, 80 A/m, 100 μT) or to control conditions (incubation in CO<sub>2</sub> thermostat at 37°C) for 20 hours. For the AR experiments blood cells were exposed to IF MF as an adaptive dose (250.8 kHz, 80 A/m) for 20 hours followed by 24 hour incubation period before it was exposed to challenge dose of 1.5 Gy ionizing radiation (X-rays) (Fig. 3A). The control samples were sham exposed cells that were held in same conditions as exposed ones except for the exposures: during IF MF they were incubated in CO<sub>2</sub> thermostat at 37°C and during X-ray irradiation were taken to the irradiation facility but were not irradiated. For positive control 4 Gy X-ray ionizing irradiation was used at a dose rate of 1.23 Gy/min (200 kV, 20 mA and 1 mm Cu filter with a RTG THX-250 device). The same protocol was used for both cell types except



**Figure 2:** The intermediate frequency exposure system. A: scheme of the solenoid IF exposure system. B: the solenoid coil exposure system placed in the CO<sub>2</sub> incubator. C: sample holder within the solenoid coil.



**Figure 3:** Scheme of experimental protocol to investigate the genotoxicity and the adaptive response of IF MF exposure in leucocytes (A) and H295R cells (B)

that H295R cells were pre-incubated for 24 hours before exposures to allow cells to attach to the flasks bottom (Fig. 3B). Immediately after the end of exposure protocol they were collected with trypsinization and centrifugation (30 - 40 min procedure) for the comet assay testing. There were three independent experiments for both cell types.

#### *Single cell gel electrophoresis assay*

The comet assay (a single-cell gel electrophoresis technique) was used as a method to detect the DNA damage. A slightly modified alkaline comet assay protocol of Singh et al. (1988) was used. Cell suspension was mixed with 0.5 % low-melting point agarose (37°C) and pipetted to 1 % normal-melting point agarose pre-coated slides. This suspension was immediately covered with cover glass, and kept at 4°C until agarose solidified. After 5 minutes the cover glass was gently removed, and the slides were immersed in freshly prepared cold lysis buffer (2.5 M NaCl, 100 mM Na<sub>2</sub>EDTA, 10 mM Tris, pH; 1% Triton X-100, 10% DMSO added freshly) and lysed overnight at 4°C. Slides were rinsed in cold electrophoresis solution (1mM Na<sub>2</sub>EDTA, 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 to a level approximately 0.5 cm above the slides. Duration for DNA unwinding was 30 minutes, and for electrophoresis 20 minutes. The voltage was constant at 25V, 0.83 V/cm (300 mA). After electrophoresis, the slides were washed 3 times with Tris buffer (0.4M Tris, pH 7.5), once with ethanol, air dried and stored at room temperature until analysis. Staining was performed with GelRed and slides were examined at 40x magnification objective using a Zeiss AxioPlan fluorescence microscope (Oberkochen, Germany). Microphotographs of comets were taken by CCD camera and evaluated using the CaspLab image analysing software (University of Wroclaw, Poland). For each exposure condition 100 comets were examined per slide and the tail DNA % parameter was calculated. Three independent experiments were performed (n=3). Data analysis was done using R statistical software version 1.1.463 (2009-2018 RStudio, Inc). Effects of the treatment on tail

DNA% was investigated by linear mixed-effects models (LMMs). Post-hoc Tukey test was used for treatment comparison. Results for each exposure condition are presented as mean ( $\pm$ SD).

## **Results and Discussion**

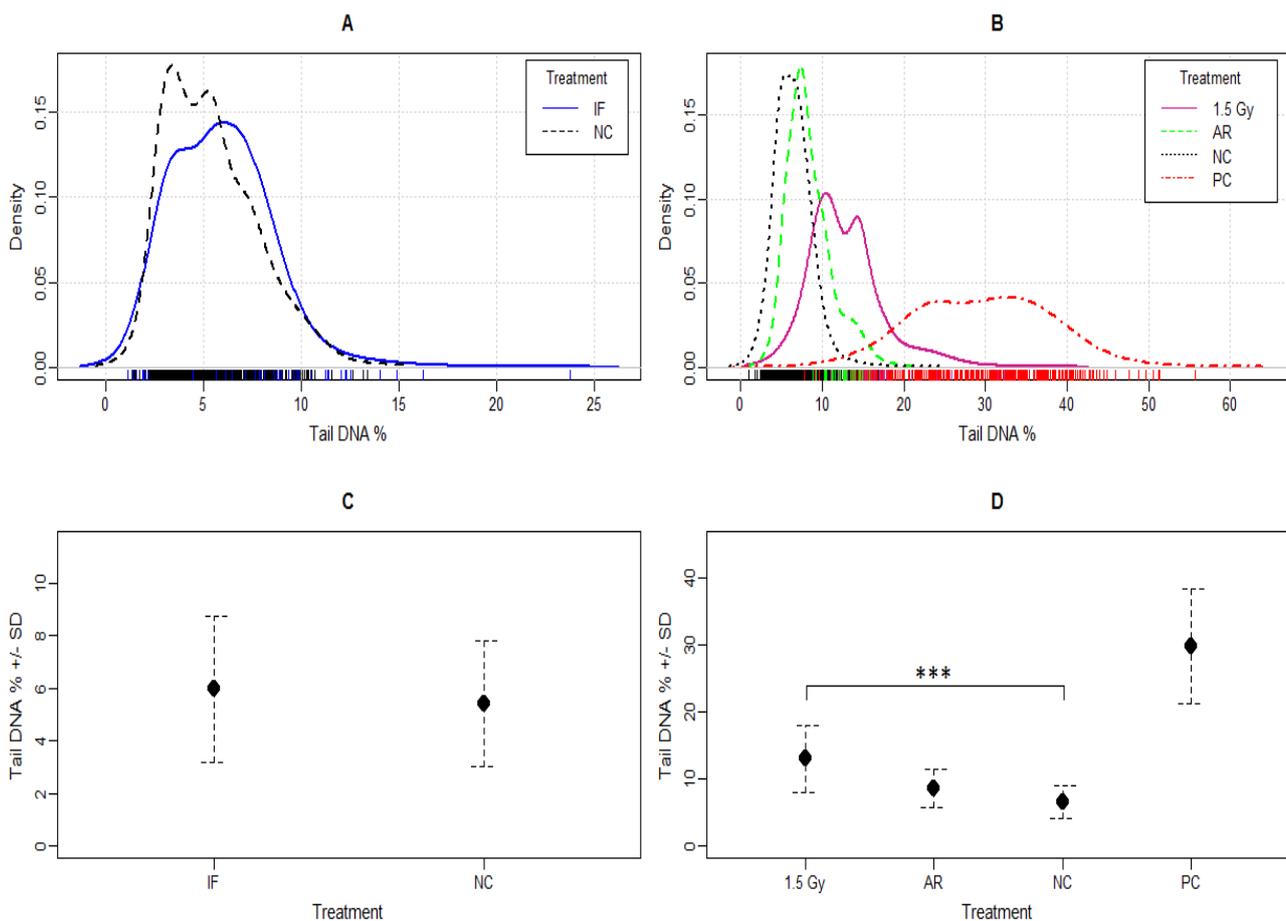
The aim of this study was to evaluate the possible genotoxic effects of intermediate frequency magnetic field exposure (250.8 kHz) in leukocytes and in adrenocortical carcinoma cell line, and to examine if IF MF as a physical agent could induce adaptive response.

The results of 20 hour 250.8 kHz exposure alone on leukocytes and in H295R cells are presented as tail DNA % in Figure 4A, 4C and 5A, 5C, respectively. The 20 hours exposure to IF MF did not alter the level of DNA damage significantly, compared to the control samples in both cell types. The mean of tail DNA % of IF exposed blood samples was  $5.97 \pm 2.8$ , while for control samples  $5.43 \pm 2.9$  (Fig. 4/C). Similar result was for the adrenocortical carcinoma cell line: in IF exposed cells  $5.02 \pm 3.57$  and for negative control cells  $4.47 \pm 2.21$  (Fig. 5/C). Our result supports the findings that intermediate frequency do not induce genotoxicity *in vitro* (Miyakoshi et al. 2007, Sakurai et al. 2009, 2012, and 2013). Although these results referred to a short term exposure for only few hours, the same was stated for the 24 hours exposure by Sun et al. (2017). The other objective of this study was related to adaptive response. To provoke the adaptive response with non-ionising radiation, mainly radiofrequency radiation (on which mobile phones operate) was used as AD. Ji et al. (2016) and He et al. (2017) applied 900 MHz for few days on mouse bone-marrow stromal cells (BMSC) *in vitro* and introduced 1.5 Gy as the challenge dose. A consistently and significantly decreased DNA damage was detected in cells exposed to RF + 1.5 Gy accompanied by a faster DNA repair kinetics. Sannino et al. (2013) exposed human blood lymphocytes to 1950 MHz RF for 20 hours and used 1.0 or 1.5 Gy X-ray as CD. The authors reported a significantly decreased incidence of micronuclei in RF pre-exposed cells when they were subsequently exposed to IR. In our study we used the intermediate frequency (250 kHz) for investigating the adaptive

response. Human leukocytes were pre-exposed to IF MF for 20 hours and challenged with 1.5 Gy X-ray radiation (treatment named AR) after 24 hour time interval (Fig. 4B, 4D). There was a noticeable tendency of decrease in DNA damage, but no significant difference ( $P=0.086$ ) was detected compared to the samples that were only irradiated with 1.5 Gy X-rays (treatment named 1.5 Gy) (Fig. 4D). However, the samples irradiated with 1.5 Gy X-ray contained significantly more DNA damage ( $P<0.001$ ) than the sham exposed control samples (treatment named NC). The results of positive control (4 Gy) showed statistically significant increase in DNA damage in leukocytes. Although not indicated on the Figure 4D, values of the 4 Gy treatment group were significantly higher than the NC, AR and 1.5 Gy group values ( $P < 0.001$ ). Regarding the H295R cell line the combined

exposure of IF and 1.5 Gy (AR group) did not alter the level of DNA strand breaks significantly ( $P=0.901$ ) compared to its respective control (Fig. 5B, 5D). In experiments with H295R cell line the positive control value was lower than expected but still differed significantly from the AR ( $P=0.037$ ) and NC groups ( $P=0.012$ ).

We got such low values because after the end of the exposure protocols 30-40 minutes have passed (collection of attached cells with trypsinization and centrifugation procedure) and by that time the repair of DNA damage partially developed. This is the difficulty and the challenging task when using attached cells for comet assay in exploring the effects of non-ionising radiation. We presume, that if exposure to non-ionizing radiation would have had any effect, it would probably be insignificant, and even smaller after cell collection due to DNA repair.



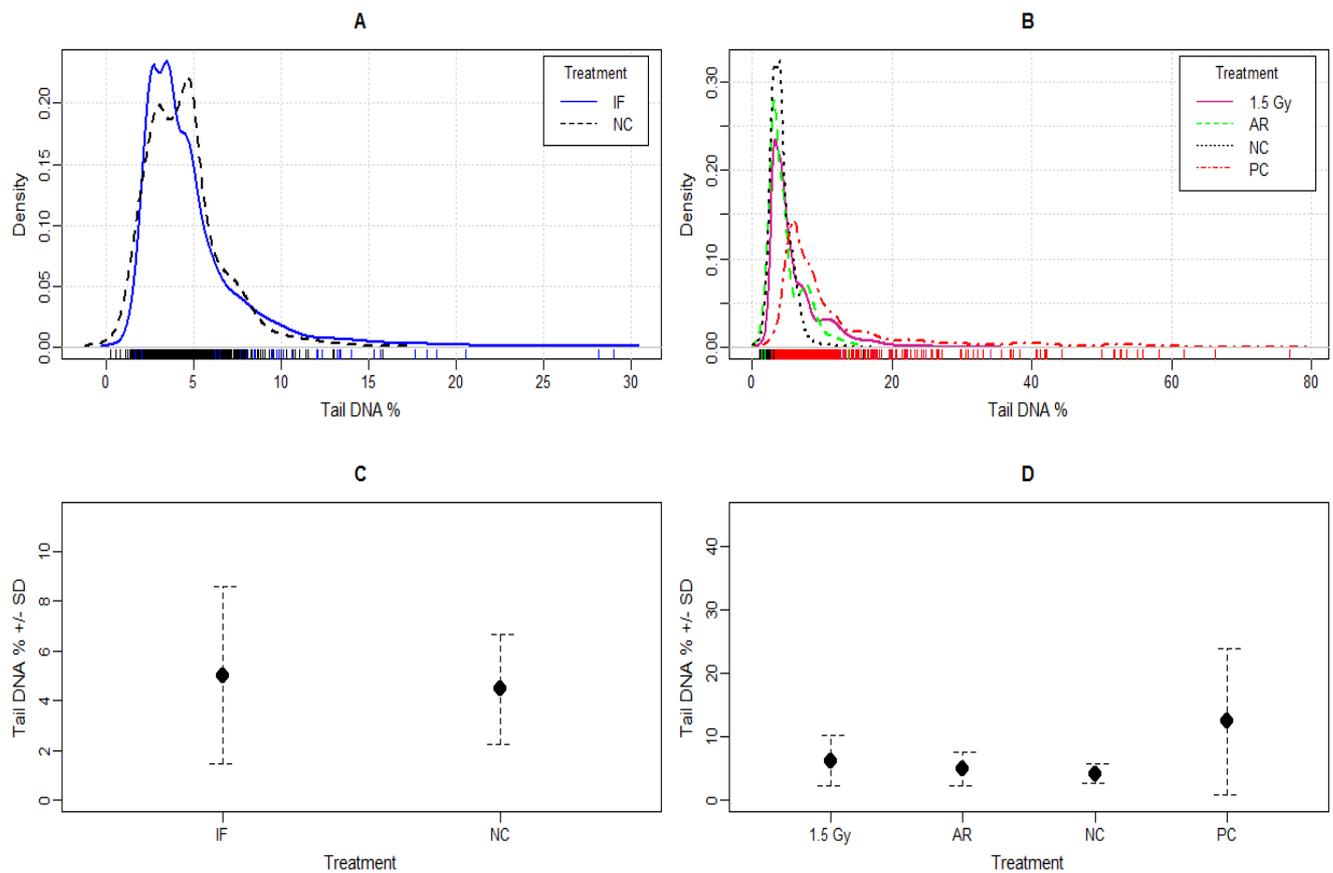
**Figure 4:** Density distribution of tail DNA % of human blood cells in IF exposed genotoxic (A) and adaptive response (B) experiments. Plot of mean values of human blood cells in IF exposed genotoxic (C) and adaptive response (D) experiments. Dots represent the mean ± SD of three independent experiments. The asterisk indicates that  $P < 0.001$ .

What might be the reason that in our experimental setup the AR was not detected? One of the reasons could be that the 250 kHz intermediate frequency is not effective in this manner. To our knowledge this frequency was not used in genotoxicity testing so far, so no results are available for comparison. The other explanation is related to work of Sannino et al. (2009). The authors discussed the presence of the heterogeneity in the induction of the adaptive response between individuals exposed to RF radiation, and that there was variability between the donors in RF-induced AR. Some individuals are “responders” and others “non-responders” when challenged to elicit the adaptive response. According to the literature the adaptive response induced by EMF were presumed to occur only in non-cancer cells (Jiang et al. 2008). To test this theory we used leukocytes from whole blood as healthy cells and the H295R adrenocortical carcinoma cell line to compare if there is difference regarding the IF MF exposure or the AR. There were no significant differences in DNA damage

(tail DNA %) of IF MF exposed groups when compared to the respective control group. Thus, our results did not indicate any significant adaptive response.

### Conclusions

Our results show that the applied IF MF alone is not inducing any genotoxic effects in any of the two cell types (human leukocytes from whole blood and H295R adrenocortical carcinoma) and has not modified the effects of X-ray irradiation in terms of AR. It should be mentioned here, that in this study we used only one biological assay to analyze the DNA damage and the number of donors were limited. In further studies we should cover other frequency ranges (like inductive cook top heater frequencies), different combined exposure protocols using other assays and endpoints to detect potential effects of IF MF.



**Figure 5:** Density distribution of tail DNA % of adrenocortical carcinoma cells in IF exposed genotoxic (A) and adaptive response (B) experiments. Plot of means of adrenocortical carcinoma cells in IF exposed genotoxic (C) and adaptive response (D) experiments. Dots represent the mean ± SD of three independent experiments.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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