



Genotoxic effects of intermediate frequency magnetic fields on blood leukocytes in vitro

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ABSTRACT

The widespread presence of electromagnetic sources in daily life has initiated several studies on the effects of radiofrequency and power frequency fields. Only few investigations on the genotoxic effects of exposure to intermediate frequency magnetic fields (IF-MF) have been done so far. Therefore, the aim of this study was to evaluate possible genotoxic effects of exposure to 123.90 kHz and 250.80 kHz IF-MF on canine and human blood. Blood was exposed to IF-MF at 630 A/m (0.79 mT) and 80 A/m (0.10 mT) with exposure durations of 1–5 h (hourly), 20 and 24 h. Cylindrically divided Petri dish system was developed for *in vitro* exposures where different induced current could be achieved in the samples at the same magnetic flux density level. For the assessment of genotoxicity the alkaline comet assay was applied. We detected a statistically significant increase in DNA damage only following 20 h exposure to IF-MF.

1. 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 intermediate frequency (IF) magnetic fields (MF) are defined as a frequency range between the ELF and RF fields, from 300 Hz to 10 MHz [1] (See Fig. 1). Many different consumer and industrial devices used in the public and working environment produce intermediate frequency magnetic fields varying widely in frequency and field strength. As published by the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) Opinion document occupational exposure to IF fields in certain areas is considerably higher than exposure to the general public and is increasing among workers in e.g. security, shops, and certain industries (heating and welding) [2]. This expected increase of the occupational exposure to IF requires studies on biomarkers and health outcomes in workers which could be supplemented with experimental studies [2]. The exposure of general public to IF is obtained mostly at lower level than in industry, but the affected population could be much larger including more sensitive and vulnerable people such as elders, pregnant women and children [3]. IF-MFs are generated in household devices such as induction cookers, and 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) [4,5]. The compact fluorescent lamps (CFL) produce electric fields in the 1.20–150 kHz frequency range, much higher than any other device or appliance previously available to the general public [6]. The electric and hybrid vehicles also produce IF-MF in the range of few kHz - 1 MHz including the wireless electric vehicle charging (WEVC) systems [7–9]. The proximity Radio Frequency Identification (RFID) readers operate at 125 kHz or 13.56 MHz for remote reading of magnetic badges of personnel passing through control gates. Similar devices are the electronic article surveillance systems (EAS) or anti-theft devices, which are commonly installed in shops and libraries and operate over a wide range of frequencies (20 Hz - 2.45 GHz) [10]. 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 WPT.

As reported by the World Health Organization (WHO) there is a need for high quality research in the IF spectrum, so the following key areas were assigned for further research: epidemiological studies, exposure evaluation, animal studies, biological interaction and dosimetry [1]. According to SCENIHR there are only few studies about the effects of the IF exposure [2]. In addition, only a limited number of *in vitro* experiments were found investigating genotoxicity endpoints induced

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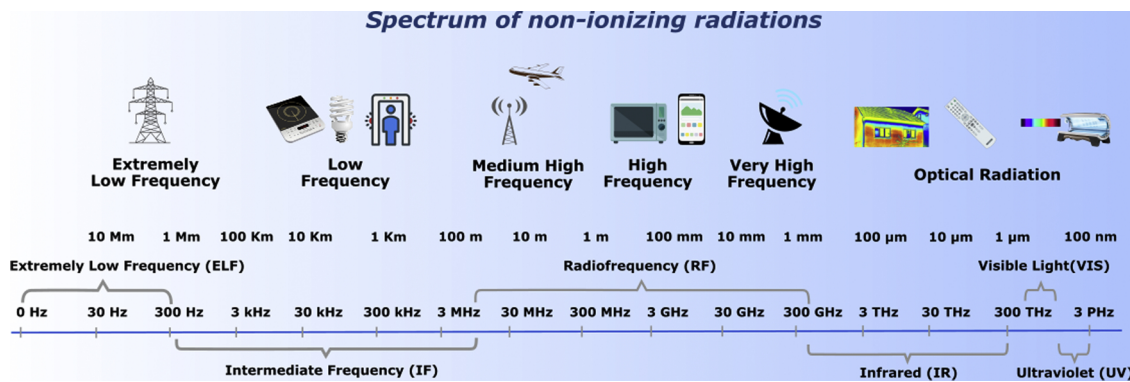


Fig. 1. Diagram of the non-ionizing radiation spectrum, showing the area of IF across the range of frequencies and wavelengths.

by IF-MF. (For review see the study of Bodewein et al. [11]). One of the most investigated frequency of the IF spectrum is that emitted from inductive cook top heaters, 23 kHz. Single exposure for 2 h (at magnetic flux density of 532 μ T and 6.05 mT) did not affect the growth of CHO-K1 cells, caused neither single nor double DNA strand breaks in comet assay, no micronucleus formation, no significant change in the mutation frequency at the hypoxanthine-guanine phosphoribosyltransferase (HPRT) locus compared to sham exposure (a control group treated as exposed samples but in absence of radiation) [12,13]. Exposure of human fetus-derived astroglia cells to an IF-MF at 23 kHz and 2 mT and 100 μ T for up to 6 h did not induce detectable changes in gene expression profile [14,15]. The same research group investigated the effects of exposure to a 23 kHz IF-MF of 2 mT for 2, 3, or 4 h on the cellular immune response (neutrophil chemotaxis and phagocytosis) using differentiated human HL-60 cells *in vitro* and no differences between the exposed groups and the sham exposed cells were detected [16]. Long term (up to 10 days) embryotoxic effects of 21 kHz IF-MF (at 3.9 mT) were investigated on murine embryonic stem cells and fibroblast cells based on the embryonic stem cell test. There was no significant difference in cell proliferation or cell differentiation [17]. The exposure to 90 kHz IF-MF generated by WPT based on magnetic resonance did not cause detectable cellular genotoxicity on cultured human lens epithelial cells [18]. For assessment of genotoxicity, cellular proliferation, cell apoptosis, alkaline comet assay and phosphorylated histone H2AX (γ H2AX) foci formation tests were used.

While a few epidemiological studies have suggested links between IF exposure and health effects [19], other studies did not find such association [20]. Results in the range of IF-MF do not indicate health risk at exposure levels below the recommended guidelines, which are established by extrapolating from the limits of the ELF and RF frequency ranges, based on dosimetric consideration and assumption about the frequency dependence of effects [4].

In this study we investigated the genotoxicity of IF-MF on blood cells *in vitro*. The frequency range of IF is too wide to screen the potential adverse health effects over all or several frequencies. This screening would be very time consuming. Therefore we selected frequencies in the middle of this frequency range at few hundred kHz, where several medical (e.g. surgical devices, nano-hyperthermia equipment), industrial (e.g. induction heating) and household devices operate. We evaluated the effects of exposure to 123.90 kHz and 250.80 kHz magnetic fields (emitted by e.g. proximity readers, inverters, and some industrial devices) at 0.79 mT and 0.10 mT magnetic flux density, respectively.

The body is “transparent” to magnetic fields because the body tissues are essentially nonmagnetic. The internal induced electric field in the tissue is the sum of the fields induced by the external electric and magnetic fields. Therefore, exposure from electric and magnetic fields can be considered separately [4]. In order to investigate the effects of induced internal electric field at a constant level of external magnetic

field in biological materials, we used specially developed cylindrically divided Petri dishes in our experiments. Since the internal induced electric field in the cell culture (sample) depends on the radius where the induced current can flow we could define three levels of induced electric fields according each consecutive cylindrical part of the Petri dish. In line with the basic restrictions of the International Commission on Non-Ionizing Radiation Protection (ICNIRP) Guidelines published in 2010 [21] the internal induced electric field strength in the human body (tissue) is the physical quantity that is used to specify the basic restriction of exposure to IF-MF.

2. Materials and methods

2.1. Reagents

The normal melting point agarose, KCl, DMSO, Triton-X, 0.25% Trypsin-EDTA, were purchased from Merck KGaA (Darmstadt, Germany), low melting point agarose from ICN Biomedicals Inc. (California, USA), glass slides and coverslips from VWR International (Pennsylvania, USA), alcohol, Sodium Hydroxide (NaOH), Sodium Chloride (NaCl), Na₂EDTA, Tris from Reanal (Budapest, Hungary), RPMI-1640 medium from Thermo Fisher Scientific (Massachusetts, USA). BD Vacutainer (lithium heparin) 10 ml tubes from Becton Dickinson (Plymouth, UK). Gel Red stain was purchased from Csertex Kft (Budapest, Hungary), Petri dishes from Corning (NY, USA).

2.2. Exposure system and dosimetry

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 2×30 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 into a CO₂ incubator (HETO-HOLTEN A/S, Cellhouse 154, Allerød, Denmark, Fig. 2B) where the background power frequency (50 Hz) stray magnetic field was the lowest (between 0.50–1.50 μ T) [22]. The Petri dishes were placed in the coil, using a plastic holder (Fig. 2C). The solenoid coil was operated at resonant mode. The magnetic flux density in the exposure space (at all levels where the 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 within the exposure area at different levels was less than 10%. The measurement of the temperature within the sample was performed by four channel non-perturbing optical temperature probes (LumaSense Technologies Inc., USA) and monitored through the exposures. The

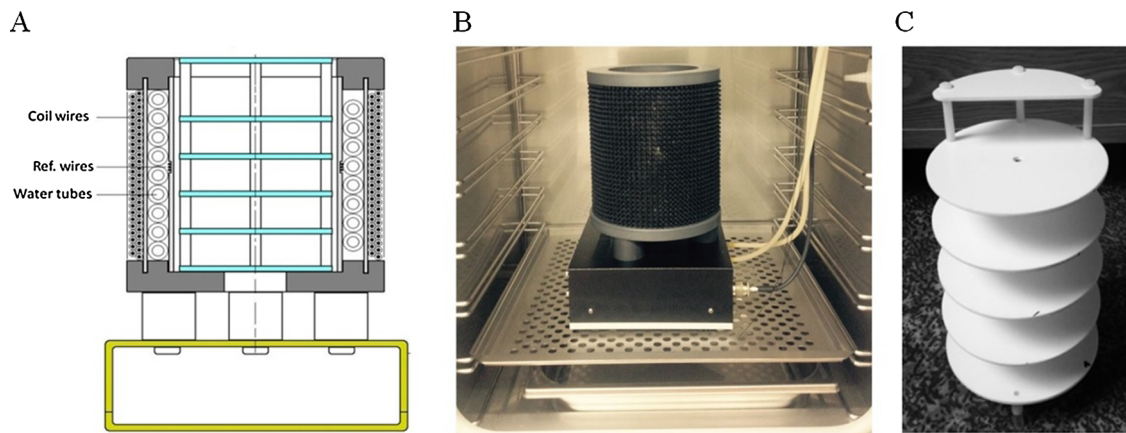


Fig. 2. The IF exposure system. A: scheme of the solenoid IF exposure system. B: the solenoid coil exposure system placed in the CO₂ incubator. C: Sample holder within the solenoid coil.

temperature of the sample was maintained at 37 °C by the water flow system. At the same time the control samples (sham exposed) were kept in another CO₂ cell incubator at 37 °C.

The cells were exposed in a 55 mm Petri dish divided cylindrically into three parts by concentric rings (Fig. 3A). The diameters of the wells starting from the center were 15 mm, 35 mm and 55 mm, respectively. At the same magnetic flux density level different induced current (induced electric fields) could be achieved in the samples within the three concentric rings of the divided Petri dish. In this study two IF frequencies were used: 123.90 kHz frequency (magnetic field strength 630 A/m) and 250.80 kHz (magnetic field strength of 80 A/m) with a magnetic flux density 0.79 mT_{rms} and 0.10 mT_{rms}, respectively.

2.3. Simulation of induced electric field

The electric field distributions induced in each annular ring of the divided Petri dish was computed through the magneto quasi-static solver as implemented by the simulation platform SIM4Life (by SPEAG, Schmid and Partner Engineering, Zurich, Switzerland, www.speag.com).

The geometry of the simulation (Fig. 3B.) was set according to the realistic exposed Petri dish (Fig. 3A.). The dielectric properties of the sample material were taken from literature values [23] of blood set (electrical conductivity $\sigma = 0.70$ S/m, relative permittivity $\epsilon_r = 5078$), whereas Petri dish insulator material is Perspex ($\sigma = 0$, $\epsilon_r = 2.60$). Given the measured good uniformity of magnetic field in the Petri dish, a uniform magnetic field with a direction perpendicular to the three layers was simulated. The computational domain was discretized by using a rectilinear uniform mesh with a grid discretization step equals to 0.20 mm.

The resulting electric field distribution induced over an axial slice passing through the inner sample at half height is shown in Fig. 3B and it is the same at each height of the sample volume. As expected the

induced electric field is distributed in concentric rings and its amplitude decrease from the boundary of the outer ring, to the center of the inner ring where it is null. For example, the peaks of the induced electric field in each separated blood sample at 123.90 kHz and 630 A/m were 7.97, 5.25 and 2.28 V/m for the outer, median and inner sample, respectively. The induced electric fields along the centerline of each annular ring were as follows: 5.75, 3.60 and 1.53 V/m in the outer, median and inner sample, respectively. The peaks for induced electric field in each separated annular ring with blood sample at 250.80 kHz with 80 A/m were 2.04, 1.35 and 0.59 V/m for the outer, median and inner sample, respectively. The induced electric fields along the centerline of each annular ring were as follows: 1.45, 0.92 and 0.38 V/m in the outer, median and inner sample, respectively. The results of simulation at both frequencies are shown in Fig. 4.

2.4. Experimental protocol

For the experiments two types of whole blood was used: canine blood for screening experiments and human blood in the second part of the study.

Blood samples were taken from healthy, young (1 year old) male Beagle dogs. Approximately 15 ml blood was drawn by venous puncture into sterile heparinized Vacutainer vials by authorized personnel and diluted with RPMI-1640 medium to three times of its original volume. The part of the study with animals was approved by the Local Scientific Ethical Council (No.: PE/EA/2010-1/2016).

Approximately 10 ml peripheral blood was taken from each healthy human donor with their informed consent (ages and genders: female 35 and 52 years, male 28). It was also drawn by venous puncture into sterile heparinized Vacutainer vials by authorized personnel and diluted with RPMI-1640 medium to three times of the original volume. Conducting experiments with human blood was approved by the Scientific and Research Ethics Committee of the Medical Research

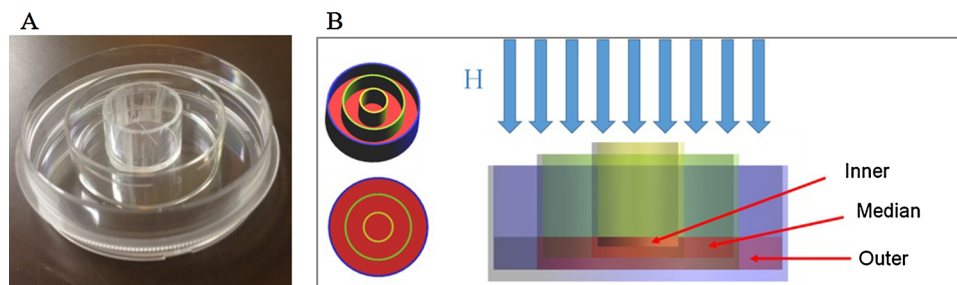


Fig. 3. A: Petri dish system divided cylindrically into three parts by concentric rings. B: Simulated uniform magnetic field (H) perpendicular to Petri dish with blood sample.

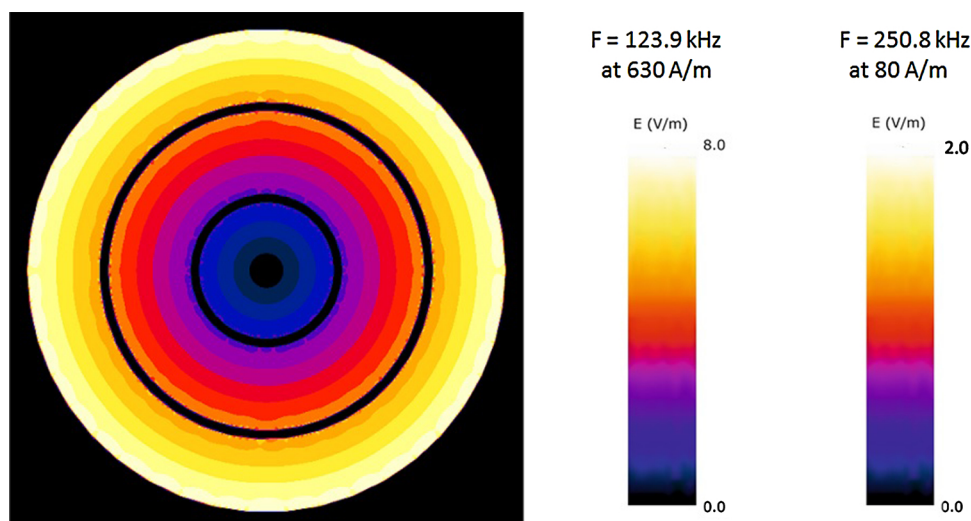


Fig. 4. Distribution of induced electric field (V/m) within the sample of each separated annular ring at 123.9 kHz and 250.8 kHz frequencies under exposure to 630 A/m and 80 A/m magnetic field, respectively. The maximum induced electric field (E) in samples were 7.97 V/m at 123.9 kHz and 2.04 V/m at 250.8 kHz at the edge of outer ring, respectively. F represents frequency in kHz.

Council (No.: 7478-1/2012/EKU).

The blood was distributed in the Petri dish system according to the following: 0.30 ml diluted blood to the 15 mm diameter Petri dish (inner), 2.50 ml to the 35 mm (median) and 6 ml to the 55 mm (outer) ring, thus the blood level in the three dishes were the same. At the first set of screening experiments on canine blood, the magnetic field strength was applied at 123.90 kHz frequency (630 A/m), with exposure durations of 1, 2, 3, 4, 5, 20 and 24 h. In the second set of experiments the canine blood was treated for 20 h at 250.80 kHz (80 A/m). The third set included the experiments with human blood on both frequencies (123.90 kHz and 250.80 kHz) with exposure duration of 20 h.

For positive control 4 Gy X-ray ionizing irradiation at a dose rate of 1.23 Gy/min (200 kV, 20 mA and 1 mm Cu filter with a RTG THX-250 device) was used.

2.5. Comet assay

The DNA damage in white blood cells was determined by the slightly modified alkaline Comet assay (alkaline single cell gel electrophoresis method) of Singh et al. [24]. It is a standard method for assessing DNA damage in genotoxicity testing [25]. The Comet assay is a rapid and sensitive technique for accurately analyzing DNA damage and repair in individual cells. The alkaline (pH > 13) version of the comet assay is susceptible of detecting DNA single-strand breaks (SSB), alkali-labile sites (ALS), DNA-DNA/DNA-protein cross-linking, and SSB associated with incomplete excision repair sites [26]. Fifteen μ l of exposed or sham exposed samples taken immediately after exposure were mixed with 65 μ l of 0.50% low melting point agarose solution. This solution was dropped onto slide coated previously by 1% agarose, covered with coverslip and allowed to solidify on ice. After the removal of coverslip the cells were lysed in cold lysis buffer (2.50 M NaCl, 100 mM Na₂-EDTA, 10 mM Tris in stock solution and 1% Triton X-100, 10% DMSO added fresh before the experiment) at 4 °C overnight. The slides were incubated in cold alkaline (pH > 13) electrophoresis solution (1 mM Na₂-EDTA, 300 mM NaOH, pH 13) for 30 min in a horizontal gel electrophoresis unit (Apelex PS 608, France) to allow unwinding of DNA. The electrophoresis was conducted for 20 min at 25 V, 0.83 V/cm (based on the set up voltage and the size of the electrophoresis tank) at 4 °C. The EPT value (Electric Potential * Time) for our setup was 16.67 V/cm*min [27]. The slides were neutralized three times in neutralization buffer (0.40 M Tris, pH 7.5) for 5 min and immersed for 5 min in ethanol, air dried and stored at room temperature until analysis. To prevent additional DNA damage all steps were conducted in the dark. The DNA strand breaks were examined at 40x

magnification objective on randomly selected 100 cells/slide using Zeiss AxioPlan fluorescent microscope (Oberkochen, Germany). In the first set of experiments for staining of slides ethidium bromide fluorescent dye was used and the cells were evaluated by Komet 4.0 image analysing software (Kinetic Imaging, Ltd, UK). In the second and third set of experiments the slides were stained with GelRed stain and examined with the CaspLab software (University of Wroclaw, Poland).

2.6. Statistical analysis

Three independent experiments were performed (n = 3) in a blinded manner for every set of experiments. For every exposure condition we examined at least 200 comets (100 comets/slide) and used the Tail DNA % parameter which gives a clear indication of comets appearance and is linearly related to the DNA break frequency over a wide range of levels of damage. Statistical analysis of data was performed according to the recommendation of [28] using One Way analysis of variance (ANOVA) with Microsoft® Office Excel 2017 (Microsoft, USA).

3. Results

3.1. First set of experiments – screening of the exposure duration

3.1.1. Effect of 123.90 kHz (630 A/m) IF (with exposure durations of 1–5 h (hourly), 20 and 24 h) on canine blood

The effects of the exposure to 123.90 kHz (0.79 mT, 630 A/m) in Tail DNA % in case of different induced electric fields (inner 1.53 V/m, median 3.60 V/m and outer 5.75 V/m part of the Petri dish, along the centerlines) are shown in Fig. 5. Statistically significant increase in Tail DNA % parameter was found only at 20 h exposure time compared to the sham control samples taken from the inner and the outer part of the dishes (Fig. 5).

3.2. Second set of experiments

3.2.1. Effect of 250.80 kHz (80 A/m) IF (with exposure duration of 20 h) on canine blood

Since in the first set of screening experiments there was a statistically significant difference at 20 h of exposure time, this exposure duration was applied in the second set but with different IF settings: 250.80 kHz, 0.10 mT (80 A/m). As a result, a statistically significant difference was found in samples taken from the outer part of the Petri dish system (induced electric field 1.45 V/m, along the centerline) compared to sham-exposed sample (Fig. 6). The Tail DNA % parameter

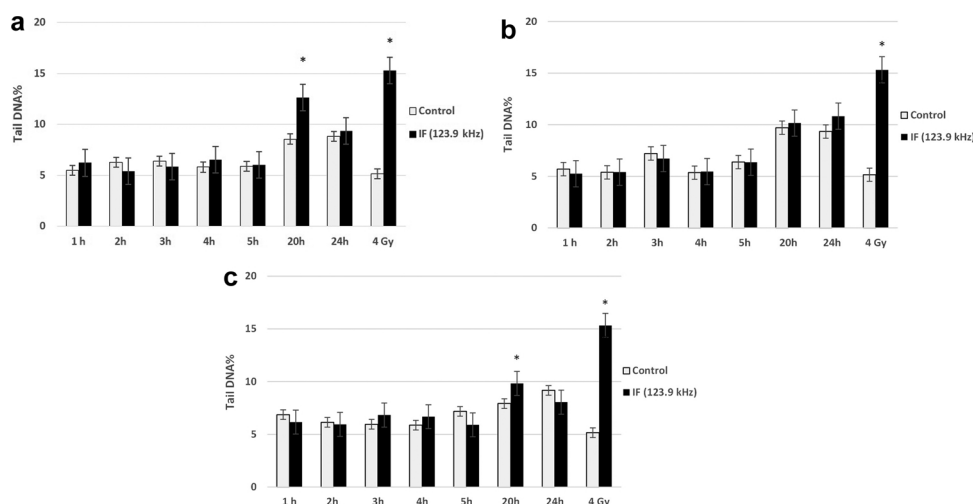


Fig. 5. DNA strand breaks presented as Tail DNA % after exposure to 123.9 kHz (630 A/m) IF (with exposure durations of 1, 2, 3, 4, 5, 20 and 24 h) on canine blood. A: inner part of the Petri dish system, B: median part, C: outer part. The columns represent the mean of three independent experiments (error bars represent the standard errors). * $p < 0.05$ in comparison to the sham exposed group.

showed higher DNA strand breaks in the exposed samples than the sham-exposed blood cells.

3.3. Third set of experiments

3.3.1. Effect of 123.90 kHz (630 A/m) and 250.80 kHz (80 A/m) IF (with exposure duration of 20 h) on human blood

The effect of IF-MF (123.90 kHz and 250.80 kHz) on DNA damage in human leukocytes from diluted whole blood is presented as Tail DNA % in Fig. 7. The 20 h exposure to both frequencies altered the level of DNA damage in samples taken from the inner parts of the Petri dish system (the induced electric fields along the centerlines were 1.53 V/m and 0.38 V/m for 123.90 kHz and 250.80 kHz, respectively) compared to sham exposed sample and it was a statistically significant increase.

Treatment with ionizing radiation significantly elevated the DNA strand breaks compared to respective sham exposed samples that were maintained in the same conditions as exposed except the irradiation.

4. Discussion

The aim of this study was to investigate whether the IF-MF could cause genotoxic effects on blood *in vitro*. For this purpose in the first part of the study we exposed canine whole blood for 1–5 h (hourly), 20 and 24 h at 123.90 kHz (0.79 mT). Since we found significantly higher results at the 20 h exposed group, this exposure duration was used in the next part of the study but with different exposure conditions (250.80 kHz, 0.10 mT) in order to test the genotoxicity of another IF. We found that at 20 h exposure both 123.90 kHz IF MF (0.79 mT) and 250.80 kHz IF-MF (0.10 mT) significantly increased the DNA damage of canine and human whole blood. So far as we know, no genotoxicity

effect was observed related to IF-MF exposure *in vitro*, until now [12,13,18]. The exposure durations which were used in these studies were short, 2 and 4 h. At those exposure duration we found the same result in our present study (no significant differences between the exposed and sham exposed cells after 2 and 4 h). Moreover we conducted experiments for longer time periods (up to 24 h) and found increased levels of DNA breaks at 20 h of IF-MF exposure detected by the comet assay. This supports our previous results where we observed significant effect (increased Tail DNA %) of exposure at 250 kHz frequency (1.26 mT) magnetic field on human leukocytes after 20 h exposure (data not shown).

The other objective of this study related to ICNIRP guidelines [21] was to investigate if the internal induced electric field is the causing agent of DNA damage rather than magnetic field at exposures to IF-MF. For this purpose we designed a special Petri dish system with three separate concentric compartments. The magnetic field strength was the same across the Petri dish, but it induced electric field that was different in various parts of the Petri dish: no induced electric field in the very center, while induced electric field increased towards the edge of the Petri dish. One might expect that there could be a dose related response regarding internal induced electric field but our findings were inconsistent regarding this matter. In the experiments with canine blood at 123.90 kHz there was a significant increase of the DNA damage in the inner (center ring) and the outer ring, while after 250.80 kHz exposure only in the outer ring. For human blood at both investigated frequencies the significant difference was found in the inner part of the Petri dish. Similar question about the induced electric field was defined in the study of Focke et al [29]. The fibroblast cells were grown on 18 mm coverslips in a 15 cm Petri dish and after exposures the cells were harvested from coverslips from central or peripheral areas of the dish.

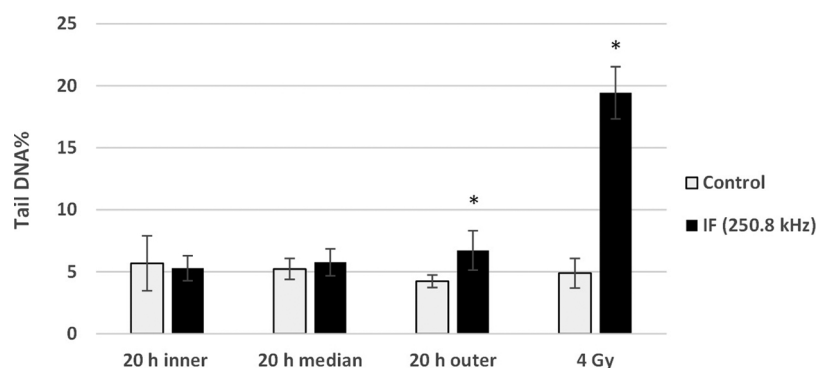


Fig. 6. DNA strand breaks presented as Tail DNA % after exposure to 250.8 kHz (80 A/m) IF (with exposure duration 20 h) on canine blood. The columns represent the mean of three independent experiments (error bars represent the standard errors). * $p < 0.05$ in comparison to the sham exposed group.

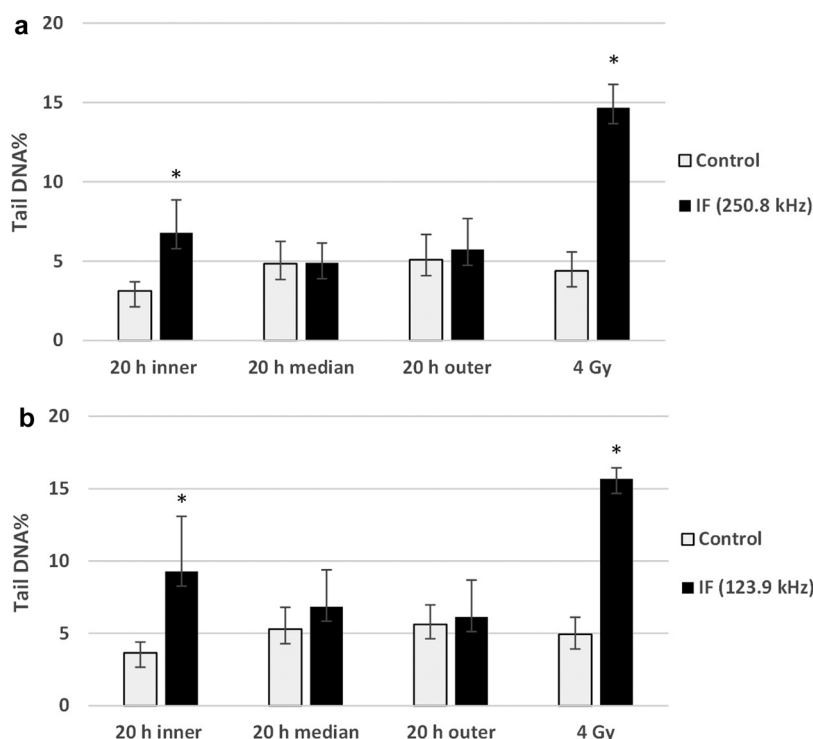


Fig. 7. DNA strand breaks presented as Tail DNA % after exposure to IF (with exposure duration 20 h) on human blood. A: experiments with 250.8 kHz (80 A/m) IF, B: experiments with 123.9 kHz (630 A/m) IF. The columns represent the mean of three independent experiments (error bars represent the standard errors). * $p < 0.05$ in comparison to the sham exposed group.

They concluded that the observed genotoxic effects of the extremely low frequency range were caused rather by the magnetic than the electric field.

In conclusion, using the comet assay method we detected genotoxicity induced by exposure for 20 h to 123.90 kHz and 250.80 kHz IF-MF on canine and human blood. This effect is rather caused by the magnetic flux density than the internal induced electric field. Further studies would be needed applying the 20 h exposure duration, using different cell types (e.g. fibroblast, keratinocytes) and different exposure setups (frequencies and magnetic field strength) including cellular repair assay or micronucleus test to further enlighten the effects of IF-MF.

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