Do 50/60 Hz magnetic fields influence oxidative or DNA damage responses in human SH-SY5Y neuroblastoma cells?

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Summary

**Purpose:** We investigated the possible effects of 50 and 60 Hz magnetic fields (MFs) on reactive oxygen species (ROS) production, DNA damage, DNA damage repair rate, as well as gene expression related to oxidative stress and DNA damage signaling.

**Materials and methods:** Human SH-SY5Y neuroblastoma cells were sham-exposed or exposed to 100 μT RMS MFs for 24 h, then assayed or further treated with 100 μM menadione for 1 h before the assay. The levels of ROS and cytosolic superoxide anion (O$_2^*$) were assayed fluorometrically. DNA damage and gene expression were assayed by comet assay and RT-qPCR, respectively. To examine whether MFs affected DNA damage repair rate, cells were allowed to repair their DNA for 1 or 2 h after menadione treatment and then assayed for DNA damage.

**Results:** There was suggestive evidence of a general low-magnitude increase in the expression of ROS-related genes (primarily genes with antioxidant activity) when quantified immediately after MF exposure, suggesting a response to a small increase in ROS level. The possible upregulation of ROS-related genes is supported by the finding that the level of menadione-induced ROS was consistently decreased by 50 Hz MFs (not significantly by 60 Hz MFs) in several measurements 30–60 min after MF exposure. MF exposures did not affect cytosolic O$_2^*$ levels, DNA damage, or its repair rate. Changes in the expression of DNA damage-signaling genes in the MF-exposed cells did not exceed the expected rate of false-positive findings. No firm evidence was found for differential effects from 50 vs. 60 Hz MFs.

**Conclusions:** While only weak effects were found on the endpoints measured, the results are consistent with MF effects on ROS signaling.

**Introduction**

Humans are ubiquitously exposed to environmental extremely low-frequency (ELF) magnetic fields (MFs) from electricity use and transmission. Over the last decades, questions have been raised about the health consequences of exposure to these fields. Epidemiological studies have rather consistently reported an increased risk of childhood leukemia associated with residential exposure to weak MFs with magnetic flux density above 0.3 mT associated with residential exposure to weak MFs with 30–60 min after MF exposure. MF exposures did not affect cytosolic O$_2^*$ levels, DNA damage, or its repair rate. Changes in the expression of DNA damage-signaling genes in the MF-exposed cells did not exceed the expected rate of false-positive findings. No firm evidence was found for differential effects from 50 vs. 60 Hz MFs.

**Conclusions:** While only weak effects were found on the endpoints measured, the results are consistent with MF effects on ROS signaling.
such effects are likely to be secondary changes rather than a direct consequence of altered radical yield through the radical pair mechanism. A plausible sequence of events might include detecting the MF by biological molecules involving radical pairs and consequent changes in cellular signaling (Juutilainen et al. 2018). ROS have multiple roles in cell signaling, including DNA damage signaling (Shackelford et al. 2000; Sancar et al. 2004; Wang et al. 2016; Mori et al. 2017). The size of MF effect on ROS has generally been small in our previous studies (Luukkonen et al. 2014; Kesari et al. 2015, 2016; Höytö et al. 2017) and other studies reporting ELF MF effects on ROS (Mattsson and Simkó 2014; Juutilainen et al. 2018), suggesting MF effects on ROS signaling rather than induction of oxidative stress. However, these studies have not directly addressed ELF MF effects on the ROS signaling pathway.

In this study, we investigated whether ELF MFs could affect the ROS signaling pathway by assaying the expression of genes related to ROS formation, ROS metabolism, and defense against oxidative stress. We also examined the possible effects of ELF MFs on the levels of ROS and cytosolic superoxide anion radical ($O_2^{\cdot-}$) and explored whether ELF MFs could induce DNA damage or influence DNA damage repair or gene expression related to DNA damage signaling. All our previous studies were conducted using 50 Hz MFs. As frequency-specific effects could potentially help to understand different responses to ELF MFs and the (static) geomagnetic field, experiments in the present study included both 50 or 60 Hz, the two power-line frequencies used in different countries around the world. In our experiments, we used human SH-SY5Y neuroblastoma cells, a widely used in vitro model in studies investigating the biological effect of MFs (Calabrò et al. 2013; Luukkonen et al. 2014; Vergallo et al. 2014; Benassi et al. 2016; Höytö et al. 2017). The flux density of the applied MFs was 100 μTRMS, chosen based on previous studies in which this flux density modified biological responses in SH-SY5Y cells (Markkanen et al. 2008; Luukkonen et al. 2011, 2014, 2017; Kesari et al. 2015; Höytö et al. 2017). In addition to exploring the effects of exposure to sole MFs, we examined whether MFs can alter cellular responses to menadione (a chemical known to induce intracellular ROS production and cause DNA damage).

**Materials and methods**

**Cell culture**

We conducted the experiments using human SH-SY5Y neuroblastoma cells (obtained from Dr. Sven Påhlman, University of Uppsala, Sweden). Cells were grown in Dulbecco’s modified Eagle medium containing 4.5 g/l glucose (Gibco, Paisley, UK) and supplemented with 10% v/v heat-inactivated fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cell cultures were maintained at a density between 1 and $2.5 \times 10^5$ cells/cm² in 75 cm² EasyYFlask™ cell culture flasks (Nunclon, Roskilde, Denmark) and kept in a humidified atmosphere containing 5% CO₂ at 37°C in a Panasonic MCO-170AICUV cell culture incubator (Panasonic Healthcare Co., Japan). Cells were subcultured every 3–4 days and harvested with 0.02% w/v Ethylenediaminetetraacetic acid (EDTA) in phosphate buffer saline (PBS; 2.7 mM KCl, 1.5 mM KH₂PO₄, 137 mM NaCl, 8.1 mM Na₃HPO₄), w/o Ca²⁺ and Mg²⁺. The count and viability of the cells were determined with LUNA-II™ automated cell counter (Logos Biosystems, Gyeonggi-do, South Korea) after staining with 0.4% trypan blue.

For the experiments, cells were cultured 24 h before the exposure. In gene expression and comet assay experiments, a count of $2 \times 10^6$ cells was seeded in 5 ml media per dish (60 x 15 mm Nunclon™ Delta cell culture dish, Nunclon, Roskilde, Denmark). For assaying the levels of ROS and cytosolic $O_2^{\cdot-}$, a count of $1 \times 10^5$ cells was seeded in 0.5 ml medium per well in Costar® 48-well plates (Corning, NY, USA).

**Exposure of the cells**

A comprehensive description of the exposure system and its integrated computerized blinding system was previously reported in our earlier paper (Mustafa et al. 2021). Briefly, an identical pair of three square copper coil systems in a Merritt-like configuration generated horizontal MFs. These two coils systems were housed in two identical Panasonic MCO-170AICUV cell culture incubators where the temperature and atmosphere were monitored and autoregulated (+37°C, 5% CO₂).

Sinusoidal MF signal (at 50 or 60 Hz) was generated and amplified by BK4052 5 MHz Waveform Generator (B&K Precision, California, USA) and EP4000 Power Amplifier (Behringer, Willich, Germany), respectively. For cell exposure, the magnetic flux density was set to 100 μTRMS and monitored with TM-192 triaxial MF meter (Tennmars, Taipei, Taiwan). The flux density of the background low-frequency MFs in the incubators was <2 μTRMS. The maximum static geomagnetic field in the incubators was perpendicular to the applied MFs, and its intensity (~30 μT) was measured with GM08 Gaussmeter (Hirst, Cornwall, UK). The culture vessels were placed in the center of the coil systems to ensure a uniform MF exposure and identical condition for sham exposure. Field homogeneity in the position of culture vessels was >98%. The temperature was monitored with a 52 K/J digital thermometer (Fluke, Washington, USA), and no difference between the exposure incubators was observed during the experiments. No mechanical vibration was detected by B&K 4366 accelerometer (Bruel & Kjaer, Nærum, Denmark). In every new experiment, the computerized blinding system randomly selected one of the two coil systems to apply the current. The other coil system served as a sham exposure unit with no current flowing in the coils. The blinding system random selections were automatically recorded to be revealed after sample analyses.

**Experimental protocol**

A flowchart of the experimental procedures is presented in Figure 1. Cells were exposed to sham exposure or 50/60 Hz MFs for 24 h. For assaying the expression of 84 oxidative
stress-related genes (QIAGEN PAHS-065YA RT² Profiler PCR Array; Supplementary Table 1) and 84 DNA damage-signaling involved genes (QIAGEN PAHS-029ZA RT² Profiler PCR Array; Supplementary Table 2), cells were collected after exposure for reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) or further treated with 100 μM menadione for 1 h, then collected.

The menadione treatment parameters used in the present study were selected based on preliminary experiments and previous studies investigating co-exposure effects using menadione as DNA damaging/ROS-inducing agent. The menadione treatment parameters used in the present study were selected based on preliminary experiments and previous studies investigating co-exposure effects using menadione as DNA damaging/ROS-inducing agent (Luukkonen et al. 2011, 2017; Kesari et al. 2015, 2016).

The fluorometric probes 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Invitrogen, California, USA) and Dihydroethidium (DHE; Sigma, Buchs, Switzerland) were used to assay the production of ROS and cytosolic O$_2^{-*}$, respectively. In these experiments, the exposed cells were treated with menadione for 30, 40, 50 min, or 1 h, then assayed. According to the fluorescent probe manufacturers, cells should be incubated with the probes for at least 30 min before the measurement. Thus, loading the cells with the probes and the chemical treatment started simultaneously.

To explore the effect of MFs on DNA damage level, the exposed cells were assayed immediately via comet assay or further incubated in menadione-free or menadione-containing media for 30 min or 1 h, then assayed. For investigating whether MFs modify the repair rate of DNA damage induced by menadione, cells were incubated in fresh media for 1 or 2 h to allow for DNA repair after chemical treatment, then assayed for DNA damage.

The menadione treatment parameters used in the present study were selected based on preliminary experiments and previous studies investigating co-exposure effects using menadione as DNA damaging/ROS-inducing agent (Luukkonen et al. 2011, 2017; Kesari et al. 2015, 2016).

**RT-qPCR**

Cells were collected by scraping in PBS w/o Ca$^{2+}$ or Mg$^{2+}$, centrifuged, snap-frozen in liquid nitrogen, and stored at −80°C for the later RNA extraction. As in our earlier study (Mustafa et al. 2021), RNA was extracted using RNeasy® Mini Kit (QIAGEN, Hilden, Germany) and assayed for purity and quantity with NanoDrop® ND-1000 ultraviolet-visible spectrophotometer (ThermoFisher Scientific, Waltham, USA). The extracted RNA was reverse transcribed into Complementary DNA (cDNA) using RT² First Strand Kit (QIAGEN, Maryland, USA). The synthesized cDNA was then used as a template for SYBR-Green I fluorescence-based qPCR reactions. The reactions were run as instructed by RT² Profiler™ PCR Array Handbook in LightCycler® 480 II (Roche, Basel, Switzerland). At the end of each run, LightCycler® software determined a quantification cycle (C_q) value for every assayed gene. These values were normalized to the average expression of five reference genes: ACTB, B2M, GAPDH, HPRT1, and RPLP0. The $2^{-ΔΔC_q}$ method (as originally described by Livak and Schmittgen 2001) was used to calculate gene expression fold changes (MF/sham).

**Fluorometric assay of ROS and cytosolic O$_2^{-*}$**

After sham or MF exposure, cells were washed and then loaded with the fluorescent probes in the absence or the presence of menadione. DCFH-DA (40 μM) was used as a fluorescent probe to measure ROS level at 485/535 nm excitation/emission wavelengths. DHE (10 μM) was used as a fluorescent probe to measure the levels of cytosolic O$_2^{-*}$ at 492/595 nm. Of notice, DCFH-DA is poor at detecting O$_2^{-*}$; thus, DHE was used as a specific probe for cytosolic O$_2^{-*}$. The fluorometric measurements were done with Infinite® 200 PRO microplate reader (Tecan, Männedorf, Switzerland). The measured fluorescence intensity was expressed in relative fluorescence units (RFUs) by Magellan™ data analysis software (Tecan, Männedorf, Switzerland).

**Comet assay**

Comet assay or single cell gel electrophoresis was performed to evaluate DNA damage and its repair rate. A detailed description of the procedures and reagents used in the assay was previously reported in our earlier paper (Mustafa et al. 2021). Briefly, cells were scraped, centrifuged, and resuspended in PBS w/o Ca$^{2+}$ and Mg$^{2+}$ at a final concentration of $1 \times 10^6$ cells/ml. From each sample suspension, 15 μl (≈1.5 × 10⁴ cells) were mixed with 75 μl 0.5% low melting point agarose. These mixtures were spread using coverslips on coded microscope slides precoated with a thin layer of 1% normal melting point agarose and left on ice for at least 5 min to solidify. After that, coverslips were removed, and...
slides were immersed in Triton x-100 lysis buffer for 1 h at 4°C in dark conditions. Thereafter, DNA was allowed to unwind by placing the slides in the NaOH-based electrophoresis buffer at room temperature for 15 min. The electrophoresis was run at 380 mA and 24 V (0.6 V/cm) for another 15 min using Horizon 20.25 Electrophoresis System (Life Technologies, Baltimore, USA). The electrophoresis occurred in an alkaline condition (pH > 13) to assay DNA single- and double-strand breaks (Singh and Lai 1998). Subsequently, slides were neutralized in trisaminomethane buffer (pH 7.5, 3 × 5 min) and then fixed by immersion in 96% ethanol for 1 min. For blinded analysis of the coded slides, they were stained with 20 μg/ml ethidium bromide fluorescent dye. AxioImager A1 microscope (Carl Zeiss, Göttingen, Germany) was used. The microscope was connected to Comet assay IV image analysis software (Perceptive Instruments, Haverhill, UK). Olive tail moment (OTM), a measure of tail length × a measure of DNA in the tail, was used to indicate the extent of DNA damage.

Statistical analyses

As earlier (Mustafa et al. 2021), data were analyzed in IBM® SPSS® data analysis software using two-way and three-way ANOVA. The fixed factors were MF exposure, menadione treatment duration, and DNA damage repair duration. The dependent variables were normalized Cq values, RFUs, and normalized OTMs. The replicate effect was considered a random factor. Data were from 3 to 4 independent experiments. p-Value <.05 was considered statistically significant. In gene expression analyses, multiple testing correction of the p-values was not used because the purpose of our study was exploratory and hypothesis-generating (Streiner and Norman 2011), aiming at identifying potential MF-responsive genes for further studies.

Results

Oxidative stress-related genes

Clear responses to menadione were observed (Figure 2, Supplementary Table 3). Four of the oxidative stress-related genes studied showed either a statistically significant up-regulation (DUSP1, HSPA1A, SQSTM1) or down-regulation (FHL2) to menadione in all exposure conditions (with or without MFs). Most of the genes that were statistically significantly up- or down-regulated in only some of the conditions showed small fold changes (<2.0), so the differences in statistical significance are most likely due to chance (false

Figure 2. Oxidative stress-related genes showing statistically significant changes in response to menadione treatment with or without MF exposure. The asterisk (*) indicates changes (up- or down-regulation) of >2-fold changes.
positive or false negative findings in some of the conditions. A suggestive modification of the menadione effect by MF exposure was seen in two cases. The expression of ALOX12 was increased in both sham exposures (fold changes with SEM: 2.09 ± 0.16 and 3.98 ± 0.71), but not significantly in the MF-exposed groups. HMOX1 was upregulated after sham exposures (2.95 ± 0.28 and 3.606 ± 0.430) and in the cells exposed to 60 Hz MFs (3.849 ± 0.854), but not significantly in the 50 Hz MF group. However, examination of the data revealed that the fold changes were of similar direction and magnitude in all treatment groups, so the lack of significance in the MF groups was apparently due to chance.

Another way to evaluate possible MF effects on gene expression is to compare the MF-exposed groups to corresponding sham-exposed groups (Table 1). All statistically significant differences observed immediately after MF exposure (before menadione treatment) were upregulations, both for the 50 and 60 Hz MF exposures. This pattern may be worth noting, as the expected number of false positives is two upregulations and two downregulations in each experimental condition (84 genes are measured, so there will be, on the average, 4.1 ‘statistically significant’ findings just by chance when $p = .05$ is used as the limit for significance; it can be assumed that half of these false positives will be upregulations and the other half downregulations). Only one of these upregulations (i.e. GCLC) was seen in both the 50 and 60 Hz MF groups. However, the fold changes were generally small; none of the increases showed a fold change $>2$, so the chance is a plausible explanation for false positive or false negative findings in one of the MF groups.

Table 1. Oxidative stress-related genes showing statistically significant changes in response to MFs. Human SH-SYSY neuroblastoma cells were sham-exposed or exposed to 100 μM menadione at 50 or 60 Hz for 24 h. After exposure, cells were either immediately assayed for gene expression (0 min) or further incubated in menadione-containing or menadione-free media for 1 h, then assayed. Fold changes (MF exposure/sham exposure ± standard error of the mean) are from three independent experiments, $n = 3$. *$p < .05$, **$p < .01$.

| MF     | Time | Menadione | Upregulated genes | Downregulated genes |
|--------|------|-----------|-------------------|---------------------|
| 50 Hz  | 0 min| No        |                   |                     |
|        |      | FOXM1 1.059 ± 0.014* |                   |                     |
|        |      | GCLC 1.049 ± 0.010* |                   |                     |
|        |      | HMOX1 1.667 ± 0.144* |                   |                     |
|        |      | HSP90A1 1.028 ± 0.006* |                   |                     |
|        |      | PRNP 1.974 ± 0.241* |                   |                     |
|        |      | RNFL 1.079 ± 0.015* |                   |                     |
|        | 1 h  | Yes       |                   |                     |
|        |      | DUSP7 1.028 ± 0.005* |                   |                     |
|        |      | APOE 1.501 ± 0.038** |                   |                     |
|        |      | CAT 0.943 ± 0.010* |                   |                     |
|        |      | DHCR24 0.941 ± 0.012** |                 |                     |
|        |      | NCOA7 0.937 ± 0.011* |                   |                     |
|        |      | SIRT2 0.945 ± 0.011* |                   |                     |
| 60 Hz  | 0 min| No        |                   |                     |
|        |      | ALOX12 1.367 ± 0.089* |                   |                     |
|        |      | FHL2 1.220 ± 0.039* |                   |                     |
|        |      | GCLC 1.117 ± 0.017* |                   |                     |
|        |      | PRDX3 1.143 ± 0.032* |                   |                     |
|        |      | TXN 1.234 ± 0.044* |                   |                     |
|        |      | UCP2 1.166 ± 0.013** |                   |                     |
|        | 1 h  | No        |                   |                     |
|        |      | FOXM1 0.932 ± 0.012* |                   |                     |
|        |      | HMOX1 1.769 ± 0.034* |                   |                     |
|        |      | AOX1 1.646 ± 0.030* |                   |                     |
|        |      | BAG2 0.863 ± 0.022* |                   |                     |
|        |      | CCS 0.859 ± 0.030* |                   |                     |
|        |      | NCF1 0.743 ± 0.049* |                   |                     |

Two gene expression differences showed a fold change $>1.5$ in the 50 Hz MF group (HMOX1 $1.667 ± 0.144$, PRNP $1.974 ± 0.241$) (Table 1). Although the expression of HMOX1 was not statistically significantly altered in the 60 Hz MF groups, the direction of the fold change was similar (1.212 ± 0.528). PRNP showed a potentially different response to 50 vs. 60 Hz MFs; it was significantly upregulated in the 50 Hz MF group but showed a fold change value close to 1.0 in the 60 Hz group (0.959 ± 0.308).

The statistically significant differences observed at 1 h after MF exposure (with or without menadione exposure) were generally small (fold changes $<1.5$), and the number of statistically significant differences did not clearly differ from the expected number of chance findings.

DNA damage signaling-related genes

Clear responses to menadione were observed (Figure 3, Supplementary Table 4). Seven of the DNA damage signaling-related genes studied showed either a statistically significant up-regulation (DDIT3, GADD45G, H2AFX, PPM1D, PPP1R15A, RNFL8) or down-regulation (SMC1A) to menadione in all exposure conditions (with or without MFs). Most of the genes that were statistically significantly up- or down-regulated in only some of the conditions showed small fold changes ($<2.0$), so the differences in statistical significance are most likely due to chance.

Comparison of the MF-exposed groups to the corresponding sham-exposed groups (with or without menadione) showed only small fold changes and no marked deviations from the expected number of false-positive findings (Table 2), indicating that the MFs used did not affect DNA damage signaling or alter the responses to menadione.

ROS and cytosolic O$_2$$^\ast$−

Compared to sham exposure, MFs reduced the levels of menadione-induced ROS over all the assayed time points (Figure 4(A,B)). This overall effect was statistically significant in the case of 50 Hz exposure ($p = .037$, Figure 4(A)) but not significant in the case of 60 Hz exposure ($p = .165$, Figure 4(B)). Concerning individual time points, 50 Hz MFs significantly reduced the levels of menadione-induced ROS after menadione treatment durations of 40 min (13% reduction, $p = .036$), 50 min (12% reduction, $p = .034$), and 1 h (11% reduction, $p = .036$). The reduction after the 30-min menadione treatment (14%) was not statistically significant ($p = .052$, Figure 4(A)).

No effects on menadione-induced cytosolic O$_2$$^\ast$− levels were observed; the differences between MF-exposed and sham-exposed cells were not statistically significant, and the direction of the differences was not consistent (Figure 4(C,D)). Moreover, exposure to sole 50 or 60 Hz MFs did not affect ROS or cytosolic O$_2$$^\ast$− levels (Figure 4(A–D)).
Compared to sham exposure, MFs alone (50 or 60 Hz) did not change DNA damage levels assayed immediately after the exposure; neither did they significantly modify the DNA damage induced by 30-min or 1-h menadione treatments (Figure 5(A,B)). In cells exposed to 50 Hz MFs, the OTM value was slightly higher in the MF\textsubscript{+}Menadione group than in the Sham\textsubscript{+}Menadione group after both 30-min and 1-h menadione treatments (Figure 5(A)); however, this difference was not statistically significant (\(p = .126\)). Moreover, MF exposure did not affect the repair rate of the DNA damage induced by 1-h menadione treatment assessed immediately after the treatment (0 min), or 1 or 2 h after it (Figure 5(C,D); also see Figure 1(C)).

**Discussion**

Clear responses to menadione were seen in all endpoints measured, indicating that the assays worked as expected.

**Table 2.** DNA damage signaling-related genes showing statistically significant changes in response to MFs. Human SH-SYSY neuroblastoma cells were sham-exposed or exposed to 100 \(\mu\text{T}_{\text{rms}}\) MFs at 50 or 60 Hz for 24 h. After exposure, cells were either immediately assayed for gene expression (0 min) or further incubated in menadione-containing or menadione-free media for 1 h, then assayed. Fold changes (MF exposure/sham exposure ± standard error of the mean) are from three independent experiments, \(n = 3\). *\(p < .05\), **\(p < .01\).

| MF | Time | Menadione | Upregulated genes | Downregulated genes |
|----|------|-----------|-------------------|---------------------|
| 50 Hz | 0 min | No | – | – |
| 1 h | No | BBC3 1.130 ± 0.024* | EXO1 0.966 ± 0.006* |
| | | CDC25A 1.155 ± 0.036* | OGG1 0.916 ± 0.017* |
| | Yes | CDC25A 1.155 ± 0.036* | EXO1 0.966 ± 0.006* |
| | | TOPBP1 1.084 ± 0.013* | OGG1 0.916 ± 0.017* |
| 60 Hz | 0 min | No | FANCG 1.091 ± 0.019* | MRE11A 1.037 ± 0.008* |
| 1 h | No | PNKP 1.077 ± 0.004* | SMC1A 0.904 ± 0.019* |
| | Yes | PNKP 1.077 ± 0.004* | SMC1A 0.904 ± 0.019* |
| | | CRY1 0.894 ± 0.022* | GADD45G 0.881 ± 0.003* |
| | | PMS1 0.876 ± 0.018* | TOPBP1 0.945 ± 0.007* |

**Figure 3.** DNA damage signaling-related genes showing statistically significant changes in response to menadione treatment with or without MF exposure. The asterisk (*) indicates changes (up- or down-regulation) of \(>2\)-fold changes.
The most obvious MF effect was the reduction of menadione-induced ROS levels in cells exposed to 50 Hz fields, observed systematically with all menadione treatment durations.

No obvious candidates for MF-responsive genes were identified in the gene expression experiments. However, several oxidative stress-related genes were found to be upregulated when quantified immediately at the end of MF exposure and before any menadione treatment. The upregulated genes were not consistently the same in the 50 and 60 Hz experiments—only GCLC was statistically significantly increased by both 50 and 60 Hz exposures, but its fold changes (1.049, 1.117) were very close to 1.0. As the number of statistically significant upregulations was higher than the expected number of false-positive findings in both 50 and 60 Hz experiments, these upregulations may nevertheless be worth noting. All the upregulated genes are found to be involved in responses to oxidative stress (Gelain et al. 2009; Raghunath et al. 2018). A possible interpretation of this upregulation is that the MF exposure caused a small increase in ROS level, resulting in a general low-magnitude increase in the expression of several oxidative stress-inducible genes. In this interpretation, the differences between 50 and 60 Hz MFs may not be meaningful, as chance may determine which low-magnitude upregulations become statistically significant in each experiment. With respect to the change in the expression of individual genes, our findings on HMOX1 and ALOX12 are in line with previous studies reporting that MF exposure increased the expression of these genes (Akbarnejad et al. 2017; Costantini et al. 2019; Zhou et al. 2019). It is also worth noting that our results show a lack of effects on CRY1 (the gene that encodes cryptochrome circadian regulator-1 protein). The single significant but small deviation from 1.0 in CRY1 expression observed 1 h after 60 Hz exposure (Table 2) is most likely a chance finding. Cryptochromes are magnetosensitive flavoproteins, and are of particular interest, as several research groups have suggested that cryptochromes are key molecules in responses to MFs (Lagroye et al. 2011; Vanderstraeten et al. 2012; Juutilainen et al. 2018). The null findings of the present study on CRY expression support the results from our previous study (Mustafa et al. 2021) and those from Lundberg et al. (2019) but are inconsistent with the findings from Manzella et al. (2015). This discrepancy may be related to differences in the biological models and experimental protocols used. Anyway, lack of effect on CRY expression in the present study shows that altered CRY expression (measured immediately after MF exposure) is not required to reduce

Figure 4. Levels of ROS (A,B) and cytosolic $\ce{O_2^-}$ (C,D) measured 30, 40, 50 min, or 1 h after sham exposure or 100 $\mu$T$_{max}$ MF exposure at 50 or 60 Hz for 24 h, with or without menadione treatment. Relative fluorescence units (RFU) are the means of fluorometric measurements. Error bars represent the standard errors of the means. $n=3-4$. $^*p<.05$. 

The most obvious MF effect was the reduction of menadione-induced ROS levels in cells exposed to 50 Hz fields, observed systematically with all menadione treatment durations.

No obvious candidates for MF-responsive genes were identified in the gene expression experiments. However, several oxidative stress-related genes were found to be upregulated when quantified immediately at the end of MF exposure and before any menadione treatment. The upregulated genes were not consistently the same in the 50 and 60 Hz experiments—only GCLC was statistically significantly increased by both 50 and 60 Hz exposures, but its fold changes (1.049, 1.117) were very close to 1.0. As the number of statistically significant upregulations was higher than the expected number of false-positive findings in both 50 and 60 Hz experiments, these upregulations may nevertheless be worth noting. All the upregulated genes are found to be involved in responses to oxidative stress (Gelain et al. 2009; Raghunath et al. 2018). A possible interpretation of this upregulation is that the MF exposure caused a small increase in ROS level, resulting in a general low-magnitude increase in the expression of several oxidative stress-inducible genes. In this interpretation, the differences between 50 and 60 Hz MFs may not be meaningful, as chance may determine which low-magnitude upregulations become statistically significant in each experiment. With respect to the change in the expression of individual genes, our findings on HMOX1 and ALOX12 are in line with previous studies reporting that MF exposure increased the expression of these genes (Akbarnejad et al. 2017; Costantini et al. 2019; Zhou et al. 2019). It is also worth noting that our results show a lack of effects on CRY1 (the gene that encodes cryptochrome circadian regulator-1 protein). The single significant but small deviation from 1.0 in CRY1 expression observed 1 h after 60 Hz exposure (Table 2) is most likely a chance finding. Cryptochromes are magnetosensitive flavoproteins, and are of particular interest, as several research groups have suggested that cryptochromes are key molecules in responses to MFs (Lagroye et al. 2011; Vanderstraeten et al. 2012; Juutilainen et al. 2018). The null findings of the present study on CRY expression support the results from our previous study (Mustafa et al. 2021) and those from Lundberg et al. (2019) but are inconsistent with the findings from Manzella et al. (2015). This discrepancy may be related to differences in the biological models and experimental protocols used. Anyway, lack of effect on CRY expression in the present study shows that altered CRY expression (measured immediately after MF exposure) is not required to reduce

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the level of menadione-induced ROS, observed consistently in measurements performed 30 min to 1 h later. This suggests the existence of MF responses independent of altered CRY expression. It has been pointed out that studies reporting MF-induced changes in CRY expression cannot distinguish between cryptochromes as magnetic detectors vs. downstream components in magnetic signal transduction (Juutilainen et al. 2018).

The low-magnitude upregulation of oxidative stress-inducible genes observed immediately at the end of MF exposure might explain the small reduction in the level of menadione-induced ROS seen 30–60 min after MF exposure. The upregulated genes were found to be antioxidant genes, and the majority of them have been reported to be involved in mitigating the levels of induced ROS production (Yoshida et al. 2001; Cortes-Wanstreet et al. 2009; Mailloux and Harper 2011; Bertuchi et al. 2012; Hou et al. 2015; Park 2020).

In the present study, no statistically significant effect was observed in 29 out of 32 of ROS/O$_2^•$ measurements, while a small but consistent decrease was observed in menadione-induced ROS (statistically significant only for the 50 Hz MF exposure). These findings are in contrast to the majority of previous studies, including those from our group, that reported increased ROS/O$_2^•$ levels in cells exposed to ELF MFs (Luukkonen et al. 2014; Kesari et al. 2015, 2016; Höytö et al. 2017; also see comprehensive reviews of MF effects on oxidative stress parameters: Wang and Zhang 2017; Schuermann and Mevissen 2021). However, there are some reports of ROS/O$_2^•$ reduction in response to MF exposure (Zmyslony et al. 2004; Patruno et al. 2010; Song et al. 2018). The contrasting findings from different research groups may be related to differences in biological models, experimental protocols, MF exposure duration and intensity, and the time points at which ROS/O$_2^•$ levels were assayed. However, it is more difficult to explain the differences between the present study and our previous studies using similar (but not identical) experimental designs and biological models. The most influential difference between this and our earlier studies may be the different incubation times between MF exposure and measurement. Measurements of ROS and O$_2^•$ were performed in the present study 30–60 min after MF exposure, but generally after a 3-h incubation (with or without menadione) in our previous studies. In measurements done without the 3-h incubation, no MF effects on ROS, reduced glutathione, or lipid peroxidation were observed (Luukkonen et al. 2014). Other possible explanations may include different pre-experiment physiological conditions of the cells and minor differences in the geomagnetic field, which may be an essential variable in biological responses to ELF MFs (Naarala et al. 2017).

Differences in DNA damage-signaling gene expression were marginal and unpatterned, and the number of statistically significant differences between experimental conditions did not show marked deviations from the expected number of false-positive findings. This apparent lack of effects is consistent with the finding that no significant effects were found on DNA damage level or DNA damage repair rate. In a previous study, we found that 24-h exposure to 50 Hz, 200 µT MFs reduced the repair rate of DNA damage induced by

Figure 5. Effects of 50 and 60 Hz MFs on DNA damage (A,B, respectively) and DNA damage repair rate (C,D, respectively). (A,B) DNA damage levels were assayed immediately after sham/MF exposure (‘0 min’), or 30 min or 1 h after the exposure with or without menadione treatment. (C,D) DNA damage repair rate assayed 1 h after the exposure, with or without menadione treatment (i.e. 0 h of DNA repair duration), and then again 1 and 2 h later. OTMs are the mean of 300 nuclei, and error bars represent the standard errors of the means from three independent experiments, n = 3. Sham/MF exposures were for 24 h, and the flux density was 100 µT RMS.
1-h treatment of murine FDC-P1 cells with 20 µ/ml bleomycin, leading to an elevated DNA damage level in the MF-exposed cells at the end of the time (2 h) allowed for repair (Mustafa et al. 2021). In an earlier study from our group, 24-h exposure of human SH-SY5Y to 50 Hz, 100 µT MFs increased the level of DNA damage immediately after a 3-h treatment with 20 µM menadione, but also increased repair rate during early (measured at 7.5 and 15 min) DNA repair, and impaired the fidelity of repair, as indicated by increased post-repair micronucleus level in the MF-exposed cells (Luukkonen et al. 2011). Although the present study did not confirm disruption of DNA repair, the findings of this and the earlier studies are not necessarily contradictory; the divergent results may be related to the different cell lines, different flux densities, and different chemical agents used by Mustafa et al. (2021) and the different experimental protocol used by Luukkonen et al. (2011).

One of the main purposes of the present work was to examine possible differences in cellular responses to 50 and 60 Hz MFs. Identifying frequency-specific effects would help to develop hypotheses of plausible mechanisms that could explain the purported effects of weak ELF MFs in the presence of the geomagnetic field (Juutilainen et al. 2018). In the present study, no firm evidence was found for distinct frequency-dependent effects. Although the decrease in menadione-induced ROS was statistically significant only in cells exposed to the 50 Hz MFs, a small but consistent difference in the same direction was also seen after exposure to 60 Hz MFs. The possible non-specific, low-magnitude increase in the expression of antioxidant genes immediately after exposure was associated with both 50 and 60 Hz exposures. The genes showing statistically significant changes were different for the 50 and 60 Hz experiments, but chance, as discussed above, is a plausible explanation for this observation. The only gene that showed a potential difference in responses to 50 and 60 Hz fields was PRNP, which showed a nearly 2-fold increase after the 50 Hz exposure but no change after the 60 Hz exposure.

The effect size of all statistically significant differences observed in this study was small. This is consistent with many previous studies that have reported effects on ROS-related endpoints and supports the hypothesis that MF exposure affects ROS signaling rather than causes oxidative stress (Juutilainen et al. 2018). Real-time monitoring of ROS levels would be valuable in future studies to increase understanding of what happens during MF exposure. It would also be worth looking at frequencies other than 50 and 60 Hz (but around them) to further evaluate possible frequency-dependent effects. Although no good candidates for magnetoresponsive genes were identified, it may be informative to carry out further testing of HMOX1 as a possible MF-responsive gene and PRNP as a gene that might respond in a frequency-specific manner.

**Conclusion**

Magnetic field exposure was found to cause at most small, if any, changes in gene expression. The tendency toward general low-magnitude upregulation of ROS-inducible genes immediately after MF exposure, however, may indicate adaptation to a small increase in ROS level. Such an upregulation would fit with the observed reduction of menadione-induced ROS assayed 30–60 min after MF exposure. MF exposures were not found to affect cytosolic O$_2$^•– levels, DNA damage or its repair rate, or gene expression related to DNA damage signaling. No firm evidence was found for differential effects from 50 and 60 Hz MFs, but further studies on frequency-dependence of ELF MF effects are definitely needed. Overall, the size of any MF effects was small, suggesting effects on ROS signaling rather than induction of oxidative stress.

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**Disclosure statement**

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