Original article DOI: 10.1515/aiht-2017-68-2928

Electromagnetic fields at a mobile phone frequency (900 MHz) trigger the onset of general stress response along with DNA modifications in *Eisenia fetida* earthworms

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[Received in December 2016; Similarity Check in December 2016; Accepted in May 2017]

Eisenia fetida earthworms were exposed to electromagnetic field (EMF) at a mobile phone frequency (900 MHz) and at field levels ranging from 10 to 120 V m^{-1} for a period of two hours (corresponding to specific absorption rates ranging from 0.13 to 9.33 mW kg^{-1}). Potential effects of longer exposure (four hours), field modulation, and a recovery period of 24 h after two hours of exposure were addressed at the field level of 23 V m^{-1}. All exposure treatments induced significant DNA modifications as assessed by a quantitative random amplified polymorphic DNA-PCR. Even after 24 h of recovery following a two hour-exposure, the number of probe hybridisation sites displayed a significant two-fold decrease as compared to untreated control earthworms, implying a loss of hybridisation sites and a persistent genotoxic effect of EMF. Expression of genes involved in the response to general stress (HSP70 encoding the 70 kDa heat shock protein, and MEKK1 involved in signal transduction), oxidative stress (CAT, encoding catalase), and chemical and immune defence (LYS, encoding lysenin, and MYD, encoding a myeloid differentiation factor) were up-regulated after exposure to 10 and modulated 23 V m^{-1} field levels. Western blots showing an increased quantity of HSP70 and MTCO1 proteins confirmed this stress response. HSP70 and LYS genes were up-regulated after 24 h of recovery following a two hour-exposure, meaning that the effect of EMF exposure lasted for hours.

KEY WORDS: *field modulation; genotoxicity; HSP70; immune defence; RAPD; oxidative stress*

Electromagnetic fields (EMF), particularly those emitted by mobile phones, constitute a much controversial topic, which is related to their effects on living organisms. The roots of this controversy stem from the fact that the World Health Organization launched contradictory reports about EMF effects on health. Indeed, the WHO International Agency for Research on Cancer classified in 2001 non-ionising radiofrequency and extremely low frequency radiations as possible carcinogens. This is the same category as dichlorodiphenyltrichloroethane (DDT), lead, and engine exhaust gases. However, in terms of mechanisms, WHO claims that health effects attributable to RF-EMF are caused by temperature elevation. In 2005, WHO concluded that there was no scientific basis to link electro-hypersensibility symptoms to EMF exposure. Several reviews on this topic link electromagnetic hypersensitivity symptoms to psychogenic origin or psychiatric disorder. Some even say that the media warnings push people into an auto suggestive process (1). However, several epidemiological studies support a possible association between heavy mobile phone use and brain tumours (2-4). These studies are quite explicit: the risk of brain tumours or meningioma increase with long-term use of mobile phones.

In order to address the possible effects of EMF on earthworms as a model organism, we used environmentally relevant field levels. The WHO's recommended upper limit is 41 V m^{-1}. But when we consider population exposure, compact fluorescent lamps deliver 15 V m^{-1}, cell phone towers 5 V m^{-1} at a distance of 40 m, and mobile phones 100 V m^{-1} (reduced to 10 V m^{-1} with a hand-free kit). Therefore, we selected field levels ranging from 10 to 120 V m^{-1}, using a special conic device (5) to ensure that the temperature did not change between the beginning and the end of exposure, not only inside the apparatus but also in earthworm tissues - thereby ruling out any influence of hyperthermia. In addition, the applied power densities and relatively low specific absorption rate (SAR) values used in the present experiment (up to 9.33 mW kg^{-1}) do not induce thermal stress (6).

In a previous study on *E. fetida*, it was observed that EMF triggered DNA strand breaks in coelomocytes, as evidenced using the comet assay, as well as the onset of

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oxidative stress as exemplified by protein carbonylation, lipid peroxidation, increased catalase activity, and increased glutathione reductase activity (7).

The aim of the present study was to complete previous findings by evaluating whether EMF is also able to trigger more subtle DNA modifications such as single base pair substitutions, deletions, or insertions. Furthermore, this study aims at answering the following specific questions:

1/ Can applied EMF modify the expression of stress response genes?

2/ Can applied EMF modify the concentration of the 70 kDa heat shock protein HSP70 chaperon in earthworm tissue?

3/ Do these effects, if any, linger on after a 24 h recovery period following a two hour-exposure to 23 V m^{-1}?

MATERIALS AND METHODS

Collection and maintenance of earthworms

Adult *E. fetida* earthworms with well-developed clitella (0.4-0.6 g of fresh weight) were obtained from an earthworm farm "Eršek" (Donja Bistra, Croatia). They were kept in the laboratory at 20±2 °C in the dark, in containers filled with soil from the farm.

Exposure of earthworms

Exposure to a homogeneous electromagnetic field was carried out in a gigahertz transversal electromagnetic (GTEM) cell as previously reported (5). Eight earthworms per treatment group were exposed for two hours to continuous radiofrequency electromagnetic fields (RF-EMFs) at 900 MHz and field levels of 10, 23, 41, and 120 V m^{-1} corresponding to the power flux densities of 0.3, 1.4, 4.2, and 38.2 W m^{-2} respectively. At the field level of 23 V m^{-1}, the effect of longer exposure (four hours), field modulation (80 % amplitude modulation, 1 kHz sinusoidal), and the longevity of effects after 24 h of recovery were investigated. The reason amplitude modulation was tested here is because almost all communication is modulated, mobile telephony included, and the most common and widely used type of modulation is amplitude modulation. An HP 8657A signal generator with a continuous wave and a 5 W minicircuits amplifier were used to generate and produce the required levels of EMF. Unexposed earthworms kept in the dark under the same conditions, but without the field, were used as a control group. The temperature inside the GTEM cell was measured (K2 K/J Thermometer, Fluka) at the beginning and at the end of exposure. To assess the influence of hyperthermia as a possible underlying cause of the effects induced by RF-EMF, temperature was monitored in earthworm tissue prior and after exposure to RF-EMF (by pinning the temperature probe into animal tissue). During the exposure to EMF, the temperature in the exposed earthworms did not increase by more than 0.1 °C, indicating that mechanisms other than hyperthermia were involved in the generation of DNA damage.

The electric field and specific absorption rate (SAR) inside earthworms were calculated by the finite element method using the Quickfield 4.2 software (Tera Analysis Ltd., Denmark). The conductivity was set at 0.943 S m^{-1}, relative permittivity at 55.03, and density at 1060 kg m^{-3}. For the applied field levels of 10, 23, 41, and 120 V m^{-1}, the calculated SAR values were 0.13, 0.35, 1.10, and 9.33 mW kg^{-1}, respectively.

Tissue sampling

Immediately after the exposure, three tissue pieces were sampled from each earthworm: one for qRAPD, another for gene expression analysis, and a third one for Western blot. For qRAPD and Western blot, tissues were placed in a cryo tube and immediately frozen in liquid nitrogen. For gene expression, a piece of tissue was first placed in a microcentrifuge tube containing 100 µL of RNAlater stabilisation reagent (Qiagen, Germany) to prevent RNA degradation and was then frozen at -80 °C.

Quantification of genotoxic damages by RAPD-PCR and analysis of the melting temperature curves of PCR products

Genotoxic effects of EMF were assessed using a random amplified polymorphic DNA (RAPD)-based methodology. This method was successfully used on zebrafish exposed to cadmium, gold, or cadmium sulphide NPs, on clams *Ruditapes philippinarum* and polychaete worms *Hediste diversicolor* exposed to a metallic blend, and on oligochaete worms *Tubifex tubifex* exposed to cadmium sulphide nanoparticles (8-13). Genomic DNA was isolated using the DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's instructions. Primers used for RAPD-PCRs were the decamer oligonucleotides OPA9 (5'-GGGTAACGCC-3') and OPB10 (5'-CTGCTGGGAC-3'). The quantification of the hybridation sites per genome unit of the selected RAPD probes was normalised using oligonucleotide probes matching the locations internal to the *E. fetida* Efpo gene (GenBank accession number: GQ385195): forward 5'-AGAGGACCACCATTGTCGC-3' and reverse 5'-AGCCCAACCTCGGTCAAAC-3'. Real time RAPD-PCRs were done with the Lightcycler apparatus (Roche) as previously described (14). Dissociation curves indicated that two PCR products were obtained with these Efpo primers. Therefore, following the same principle already described (14), from the threshold cycle (Ct) obtained with Efpo probes, it was possible to calculate the number of hybridisation sites per genome unit of a given RAPD probe: $2^{[Ct(Efpo) - Ct (selected probe) + 3]}$, in this case either with Ct(OPA9) or Ct(OPB10). Eventually, the value of each probe's relative hybridisation efficiency was determined from the mean of ten individuals for each experimental condition. The melting

temperature curve analyses were done using the LightCycler Software 3.5 (Roche) as described (14).

Gene expression analysis in the earthworm E. fetida

Total RNA was extracted using an Absolutely RNA RT-PCR Miniprep kit (Stratagene, Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's recommendations with the following modification: to 100 µL of the lysis buffer (containing guanidine thiocyanate and 0.7 µL β-mercaptoethanol) a piece of earthworm tissue was added and after the homogenisation step an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added. After this extraction step, further manufacturer's recommendations were followed. The elution volume was 30 µL and the concentration of RNA was quantified using a nanodrop spectrometer (Epoch, Biotek). RNA purity was checked and it met the following requirements: $A_{260}/A_{280} > 1.7$ and $A_{260}/A_{230} > 1.5$. The integrity of the 18 and 26S ribosomal bands was checked on a 1 % agarose-formaldehyde gel. First-strand cDNA was synthesised from 5 µg of total RNA using the Affinity Script Multiple Temperature cDNA Synthesis kit (Stratagene, Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's recommendations. The cDNA mixture was conserved at -20 °C until used in a real-time PCR reaction. Real-time PCR amplifications were performed in a qPCR MX3000P thermal cycler (Stratagene, Agilent Technologies, Santa Clara, CA, USA) using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Stratagene, Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's recommendations. For each treatment condition, five cDNA samples were analysed (10 for the control samples). Each 20 µL reaction contained 17 µL of the master mix, 2 µL of a 2 µM primer pair mix, and 1 µL of the reverse-transcribed product template. After 10 min at 95 °C, the products were amplified through 45 cycles under the following conditions: denaturation for 30 s at 95 °C, annealing for 30 s at 52 °C, and extension for 45 s at 72 °C. After a final elongation step for 1 min at 95 °C, the reaction specificity was determined for each reaction from the dissociation curve of the PCR product by following the SyberGreen fluorescence level during gradual heating of the PCR products from 60 to 95 °C. Melting curves were examined to verify that only one target was amplified and to assure that no genomic contamination was present in the RNA samples. The expression of nine genes involved in different cell activities related to detoxification processes was monitored in the earthworms: MDR1 or Pgp transporter homologue (ABCB1), catalase (CAT), coactosin-like protein (COA), 70 kDa heat shock protein (HSP70), lysenin (LYS), MEK kinase 1 (MEKK1 or MAP3K1), metallothionein (MT), myeloid differentiation factor 88 (MYD), protein kinase C1 (PKC1). The primer pairs used for the amplification of the analysed genes were adopted from the literature (15-17). Their sequences, accession numbers, and reference sources are shown (Table 1). The amplification efficiency of each primer pair was determined (Table 1) and differential gene expression quantification was calculated using the Pfaffl formula. Briefly, for each sample, the level of expression of the target gene (*targ*) was compared to the expression of the constitutively expressed β-actin (act) gene. The relative expression of the target gene (REX) was calculated according to the formula $REX = (E_{act})^{Cact}/(E_{targ})^{Ctarg}$, where E_{act} and E_{targ} stand for the PCR efficiency of the act gene and of the target gene, respectively, and C*targ* and C*act* stand for the PCR cycle threshold (C*t*) of the target gene and of the β-actin gene, respectively. The differential expression of a gene DE is the ratio of its relative expression under an exposed condition over that under the control condition, DE = REX(exposed)/REX(control), which is just another way to write the Pfaffl equation. The β-actin was selected as a housekeeping gene to normalise the expression levels of target genes after its invariable expression ($P>0.05$, determined by one way ANOVA) had been proven in EMF exposed earthworms. The recorded β-actin gene relative expressions were not significantly different from the control value and were as follows (mean±SD): 1.0±0.5 for the control exposure; 0.9±0.1 for the 10 V m^{-1} exposure; 1.4±2.3 for the 23 V m^{-1} exposure (two hours); 1.9±0.9 for the 23 V m^{-1} exposure (four hours); 1.0 ± 0.5 for the modulated 23 V m^{-1} exposure (two hours); 1.3 ± 0.5 for the 41 V m^{-1} exposure; 1.7 ± 0.7 for the 120 V m^{-1} exposure; 0.8±0.4 for the 23 V m^{-1} exposure (two hours) after a 24 h recovery period. The β-actin gene has been successfully used as a reference gene in many experiments dedicated to the ecotoxicology of *E. fetida* earthworms (15, 18-26).

Western blot analysis

Frozen tissues were cut into small pieces. For 100 mg of tissue, 0.5 mL of ice-cold cell lysis buffer was added (50 mmol L^{-1} Tris-HCl pH 7.6, 1 mmol L^{-1} EDTA, 1 mmol L^{-1} β-mercaptoethanol, 1 mmol L^{-1} phenylmethylsulfonyl fluoride, 150 mmol L^{-1} NaCl, 1 % Nonidet-P40, 1 % sodium deoxycholate, and 0.1 % sodium dodecyl sulfate). Between 10 and 15 glass beads (0.5 mm of diameter) were added and samples were passed through a homogenising mill for 5 min (apparatus Beadblaster® 24, Dominique Dutscher, Brumath, France). After centrifugation (5000 x *g*, 10 min, 4 °C), supernatants containing soluble proteins were recovered. The protein concentration was determined by the Bradford method using a protein assay reagent from BioRad (reference #5000205) and bovine serum albumin as a standard protein. Protein homogenates were then diluted with a cell lysis buffer to a final concentration of 1 mg mL^{-1}. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12 % polyacrylamide gels. Proteins were electro-transferred onto polyvinylidene difluoride (PVDF) membranes (Immun-Blot® PVDF Membrane, BioRad, reference #1620177) using the Trans-Blot® SD semi-dry transfer cell (BioRad,

Table 1 *Sequences of primers used to analyse* E. fetida *gene expression.*

Genes	GenBank accession number	Primers (5'-3') [a]	Primers' efficiency (E)	Reference
b-actin	DQ286722	GTACGATGAGTCCGGG GCATGTGTGTGGTGTC	1.99	15
Pgp/ABCB1	Not deposited	GCGGCTGTGGGAAGAGCAC TGTTGTCTCCGTAGGCAATGTT	1.97	16
Catalase (CAT)	DQ286713	AGAATTTGACGGGTGCTGAG TGGTCCACGAAGGGTAGTTT	2.00	17
Coactosin-like protein	EU296921	TGCTCGTTAAGGTGGTC AACGCAAACATGGAGT	1.96	15
Heat shock 70kDa protein (HSP70)	DQ286711	GGTGTGCTGATCCAGGTCTT CCAGTCAGCTCGAACTTTCC	1.99	17
Lysenin	EY892971	CGGCAACAAACGTCTAC GTGAAATACAGGCAGAAGC	1.97	15
MEK kinase 1 (MEKK1)	EH672240	CAAGGAACGATCCCATTCAT GTATCATGGTGCAACCAACG	1.98	17
Metallothionein (MT)	DQ286714	CGCAAGAGAGGGATCAACTT CTATGCAAAGTCAAACTGTC	2.00	15
Myeloid differentiation factor 88 (MyD88)	EH670202	CAGGTGCCAAGGAGAAGAAG CGTGCAGATGTGGTTTAGGA	1.94	17
PCS	EF433776	TCATGGTCCTGAACACG GAGTTTCGGCAACTTGTG	1.98	15
Protein kinase C1 (PKC1)	DQ286716	GCCAGAAAGTTTGACGAAGC TGGCGATGCAGAAACATAAG	1.99	17

[a] *Upper and lower sequences represent forward and reverse primers, respectively*

reference #1703940). Primary antibodies were a mouse monoclonal anti-MTCO1 antibody from Abcam (clone 1D6E1A8, reference ab14705) and a rabbit polyclonal anti-Hsp70 antibody from Abcam (reference ab66262), and were used at the indicated dilution. Secondary antibodies were peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies (Jackson ImmunoResearch Europe Ltd., Suffolk, UK). Western blots were revealed using the Enhanced Chemio Luminescence method (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) on a charge coupled device (CCD) camera (GeneGnome, Syngene Bio-Imaging, USA). Quantifications were done using the ImageJ software.

Statistical analysis

Significant differences in the number of RAPD probes' hybridising sites and frequency of PCR products when compared to control were determined with a Mann-Whitney U-test or a t-test depending on data normality. The same tests were applied to assess significant differences in relative gene expressions under different conditions. The statistical software used for all tests was Sigma stat 3.5. Both quantitative and qualitative qRAPD results are shown and statistical analysis was performed using the Mann-Whitney U-test. The following level of significance was reported: $P \leq 0.05$.

RESULTS

EMF triggers DNA modifications at the nucleotide level

In order to detect subtle DNA modifications, we used a RAPD-based methodology linked to quantitative PCR, in which creation or loss of hybridisation sites of probes can be quantified at the level of the genomic unit. Whenever exposure creates hybridisation sites, the number of PCR products increases; in case of a loss of hybridisation sites, the number of PCR products decreases, so that the increase or decrease in amplified DNA can be quantified.

The number of hybridisation sites in earthworms DNA increased two-times for both OPA9 and OPB10 decameric RAPD probes after two hour-exposure to 10 V m^{-1} or four hours to 23 V m^{-1} compared to control. This number decreased two-times for OPB10 probe after two hour-exposure to 41 V m^{-1} (Table 2). Even after 24 h of recovery following a two hour-exposure, the number of probe hybridisation sites displayed a significant two-fold decrease

Table 2 *Number of hybridisation sites per genome of RAPD probes on* Eisenia fetida *earthworms DNA exposed to EMF*[a]

Exposure	OPA9	OPB10
Control	0.11±0.01	0.032±0.005
10 V m^{-1}	0.20±0.03 *	0.079±0.017 *
23 V m^{-1} – 2 h	0.10±0.03	0.034±0.007
23 V m^{-1} modulated – 2 h	0.17±0.04	0.034±0.008
23 V m^{-1} – 4 h	0.19±0.02 *	0.035±0.010
41 V m^{-1}	0.06±0.02	0.014±0.002 *
12 V m^{-1}	0.11±0.02	0.044±0.005
Control – 24 h of rest	0.15±0.02	0.028±0.004
23 V m^{-1} – 2 h + 24 h recovery	0.07±0.01 *	0.012±0.002 *

[a] *Mean ± SEM (n=9). Asterisks indicate significantly different numbers of hybridisation sites compared to control as given by the Mann-Whitney U test (P<0.05)*

as compared to untreated control earthworms, implying a loss of hybridisation sites (Table 2).

The frequency of PCR product generation was also modified after exposure to EMF. PCR products of T*m* belonging to the temperature interval 74–76 °C showed a significant increase in the frequency of generation: from 0 for control up to 1 after four hour-exposure to 23 V m^{-1}, up to 0.9 after two hour-exposure to 41 V m^{-1} or modulated 23 V m^{-1}, and up to 0.4 after two hour-exposure to 120 V m^{-1} as assessed by the OPA9 probe (Table 3). PCR products of T*m* belonging to intervals 80–81 °C and 85–86 °C showed a significant decrease in the frequency of generation after two hour-exposure to 10 V m^{-1}, and 23 V m^{-1} with or without modulation as assessed by the OPB10 probe (Table 4). The same probe revealed significantly modified frequencies of PCR amplification after two hour-exposure to 120 V m^{-1} for T*m* belonging to intervals 74–76 °C, 80–81 °C, 83–84 °C, and 86–87 °C (Table 4).

EMF triggers the up-regulation of several genes involved in the general stress response

We addressed the expression of several genes involved in the response to general stress (such as HSP70 and MEKK1, the latter participating in signal transduction and involved in immune defence), oxidative stress (CAT, encoding catalase), chemical and immune defence (LYS, encoding lysenin, a lethal peptide for most insects, and MYD, encoding a myeloid differentiation factor).

The relative genes' expressions are shown in Table 5 and differential expressions (ratio of the relative expression of exposed worms over that of control worms) are displayed in Table 6. CAT, HSP70, LYS, and MEKK1 genes were significantly up-regulated after a two hour-exposure to field levels of 10 V m^{-1} and a modulated field at 23 V m^{-1}, with levels of expression reaching 2.4 to 5.3-times those of control worms (Tables 5 and 6). HSP70 and LYS genes featured a significant three-fold up-regulation compared to control worms after 24 h of recovery following a two hour-exposure to a field level of 23 V m^{-1}, meaning that the effect of EMF exposure lasted for hours. The responsiveness to EMF of these selected genes was not directly related to the strength of the field, since at the field level of 41 V m^{-1} MT, MYD, and PGP genes showed a 8-, 15-, and 25-fold decreased expression compared to control worms and an expression similar to that of control worms at 120 V m^{-1}.

EMF triggers the induction of stress response and respiratory proteins

A Western blot was performed using antibodies directed against the proteins HSP70 and MTCO1 (MTCO1 is the subunit 1 of cytochrome *c* oxidase). The Coomassie blue staining indicates that an equal amount of protein material was loaded on each lane (Figure 1). Western blots showed an increased quantity of the HSP70 protein after exposure to a field level of 23 V m^{-1} (2.6±0.9 times more compared to controls), and of MTCO1 subunit after exposure to the field of 10 V m^{-1} (2.6±0.9 times more compared to controls), indicating the onset of general stress response and a mitochondrial impact.

DISCUSSION

Increased levels of reactive oxygen species (ROS), antioxidants, and cellular damage such as DNA fragmentation and oxidative damage to DNA were found after radiofrequency exposure in different studies including those on cell cultures, laboratory animals, and even in humans (reviewed in 27, 28). Not only common mobile phone EMF (900 MHz, 16-29 V m^{-1}, SAR equal to 0.17-0.58 W kg^{-1}), but also extremely low-frequency magnetic fields (100 mT, 50 Hz) were able to increase the level of protein carbonylation in the rat brain exposed two hours a day for ten months (29, 30). Here we show that CAT, HSP70, LYS, and MEKK1 genes are over-expressed in response to the two hour-exposure to EMF levels of 10 and modulated EMF at 23 V m^{-1}. This indicates the onset of oxidative and general stress, which is in line with the observed strong induction of three genes encoding metallothionein, lysenin, and coactosin-like protein in *E. fetida* worms exposed to a smelter polluted soil containing

Table 3 *Classification according to the temperature intervals of the PCR products obtained with OPA9 probe on individual genomic DNA from EMF-exposed earthworms*[a]

Temperature intervals	Control	10 V m^{-1}	23 V m^{-1} – 2 h	23 V m^{-1} – 4 h	23 V m^{-1} – modulation	41 V m^{-1}	120 V m^{-1}	Control – 24 h of rest	23 V m^{-1} – 2 h + 24 h recovery
[74-76]	0	0.3	0.3	1 *	0.9 *	0.9 *	0.4 *	0.4	0.3
[76-78]	0.2	0.7 *	0.6	0.8 *	0.5	0.2	0.6	0.6	0.2
[78-80]	0.1	0.4	0.4	0.2	0.3	0.4	0.3	0.2	0.4
[80-81]	0.2	0	0	0.3	0.4	0	0.1	0	0.2
[81-82]	0.1	0	0.2	0	0.1	0	0	0.1	0.1
[82-83]	0.1	0.1	0.2	0.5	0	0.4	0.3	0.2	0.4
[83-84]	0.4	0.1	0.2	0 *	0.1	0 *	0.2	0.1	0.1
[84-85]	0.5	0.9	0.5	1 *	0.8	0.9	0.8	0.9	0.9
[85-86]	0.2	0	0.1	0	0.1	0	0	0	0
[86-87]	0.4	0.8	0.7	0.6	0.9 *	0.6	0.7	1	0.8
[87-88]	0.2	0.2	0.3	0.1	0	0.4	0	0	0
[88-89]	0.4	0 *	0.3	0.3	0.1	0 *	0.3	0.2	0.2
[89-92]	0.4	0.8	0.6	0.5	0.8	0.5	0.6	0.6	0.5

[a]*Numbers indicate the frequency of apparition of a PCR product in each temperature interval as recorded among 10 earthworms of a given exposure condition. Asterisks indicate significant differences between the contaminated earthworm DNAs and control ones (as assessed by the Mann-Whitney U test, P<0.05)*

Table 4 *Classification according to the temperature intervals of the PCR products obtained with OPB10 probe on individual genomic DNA from EMF-exposed earthworms*[a]

Temperature intervals	Control	10 V m^{-1}	23 V m^{-1} – 2 h	23 V m^{-1} – 4 h	23 V m^{-1} – modulation	41 V m^{-1}	120 V m^{-1}	Control – 24 h of rest	23 V m^{-1} – 2 h + 24 h recovery
[74-76[	0.5	0.8	0.6	1 *	0.6	1 *	1 *	0.7	0.8
[76-78[	0.6	0.7	0.4	1	0.5	0.8	0.7	0.7	0.7
[78-80[	0.1	0.3	0.2	0	0.4	0	0.3	0.2	0.3
[80-81[	0.9	0.4 *	0.3 *	0.7	0.4 *	0.7	0.4 *	0.8	0.5
[81-82[	0	0.2	0.2	0.1	0.1	0.2	0.3	0.2	0.2
[82-83[	0.2	0.2	0.1	0.1	0.4	0.3	0.3	0.1	0.2
[83-84[	0.4	0.6	0.5	0.2	0.3	0.2	0 *	0.1	0.3
[84-85[	0	0.1	0.3	0.2	0.3	0.2	0.3	0.2	0.1
[85-86[	1	0.6 *	0.6 *	0.6	0.5 *	0.8	0.7	0.7	0.5
[86-87[	0	0.3	0.5 *	0.4	0.4	0.2	0.5 *	0.4	0.6
[87-88[	0.1	0.2	0.1	0	0.2	0	0	0.1	0
[88-89[	0	0	0	0	0	0	0	0	0
[89-92[	0.9	0.9	1	0.7	1	0.9	0.8	0.4	0.8

[a]*Numbers indicate the frequency of apparition of a PCR product in each temperature interval as recorded among 10 earthworms of a given exposure condition. Asterisks indicate significant differences between the contaminated earthworm DNAs and control ones (as assessed by the Mann-Whitney U test, P<0.05)*

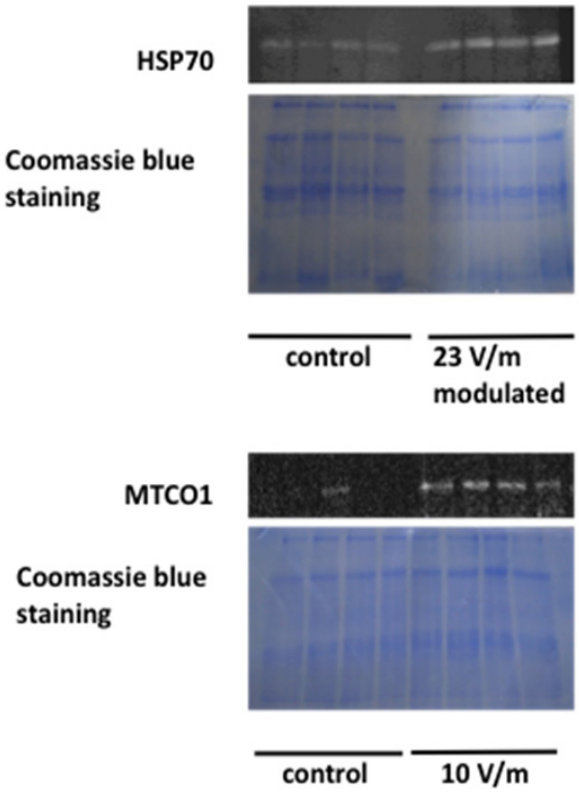

Figure 1 *Western blotting of HSP70 chaperon protein and of MTCO1 (subunit 1 of cytochrome c oxidase) in* E. fetida *body. Tissues were sampled from four animals. 10 µg of protein was loaded per lane and after transfer, antibodies directed against the indicated proteins were used. Coomassie blue staining was performed in parallel as a control of equivalent loading in lanes. Only the significant differential exposure instances are shown. All other exposure scenarios resulted in equivalent signals between the exposed and control earthworms (data not shown)*

high amounts of cadmium, lead, and zinc (15). The over expression of the HSP70 gene is also in agreement with the observed induction of small-heat shock protein hsp12 family genes and hsp16 gene following exposure to 3 or 5 T static magnetic field in the nematode *Caenorhabditis elegans* (31). The increased HSP70 protein level after two hour-exposure to a field level of 23 V m^{-1} was consistent with such general stress response induction. The HSP70 promoter contains two different DNA regions that are specifically sensitive to different stressors, thermal and non-thermal (32). The non-thermal EMF responsive domain contains three electromagnetic response elements (EMRE) and differs from the consensus sequence in the temperature or thermal domain. These EMRE are not sensitive to increased temperature and the two domains, thermal and magnetic, function separately. Inserting EMRE into a promoter that does not have these sequences makes that gene responsive to the electromagnetic field (33). Both low frequency EMF and mobile phone exposure have been shown to induce HSP70 levels. For instance, in *Drosophila melanogaster* flies exposed to a mobile phone frequency (900 MHz; SAR around 1.4 W kg^{-1}) during a 10-day developmental period, from egg laying through pupation, HSP70 levels increased within minutes (34). Extremely low EMF (2 mT; frequency, 75 Hz) also proved to induce the HSP70 chaperone along with the cytoplasmic free radical scavenger superoxide dismutase enzyme (SOD1) on neuronal cells exposed for 72 h (35).

In the present work, the modulation of EMF presented a strong influence on the genes' response and this is most probably due to the fact that the modulated waves are more aggressive. Indeed, when astroglial cells were exposed to a field strength of 10 V m^{-1} for 20 min to either 900 MHz continuous or modulated waves, a significant increase in ROS levels and DNA fragmentation was found only after exposure to modulated EMF (36). Exposure to EMF also triggered a genetic stress response in plants. In tomato plants *Lycopersicon esculentum* exposed for 10 min to an EMF of 900 MHz, 5 V m^{-1}, stress-related mRNAs (calmodulin calm-n6, calcium-dependent protein kinase lecdpk1 and proteinase inhibitor pin2, chloroplast mRNA-binding protein cmbp, basic leucine zipper protein bZIP1) accumulated in a rapid manner typical of an environmental stress response (37, 38).

The strong down-regulation of MT, MYD, and PGP genes at 41 V m^{-1} is reminiscent of what had been observed in human lens epithelial cells exposed to 1800 MHz EMF for one hour. The ROS and malondialdehyde levels significantly increased whereas the mRNA expression of CAT, SOD1, SOD2, and GPX1 genes (encoding catalase, cytoplasmic and mitochondrial superoxide dismutases, and glutathione peroxidase) along with the expression of the related four proteins decreased compared to the sham group. The authors have inferred that the increased production of ROS may be due to the inappropriate and counter adaptive down-regulation of these four antioxidant enzyme genes induced by EMF exposure (39).

In the present work, gene response data show a lack of dose-effect relationship. The information collected from 113 studies from original peer-reviewed publications showed that in 65 % of the studies, ecological effects of EMF were found both at high as well as at low dosages. No clear dose-effect relationship could be discerned (40). The same holds true in the case of the gene expression response to magnetic fields in *C. elegans* which was higher at lower field strength since 1041 genes were found to be up-regulated after exposure to 3 T for four hours versus 513 at 5 T (31). In tomato plants too, no differences occurred in the kinetics or levels of pin2 mRNA accumulation between 5 V m^{-1} and 40 V m^{-1} exposure, indicating the lack of a direct link between the amplitude of the stimulation and the amplitude of the plant response (37).

Genotoxic effects of EMF have been described in many instances *in vivo* and *in vitro* on animal and plant species. In most cases, the comet assay and the micronucleus test have been used, and less frequently chromosomal aberrations have been scrutinised. Quite alarming is the discovery that Wi-Fi devices (2.4 GHz, SAR equal to 0.14-

Table 5 *Relative gene expression in* Eisenia fetida *earthworms exposed to EMF (in V m^{-1}).* [a]

	CAT	COA	HSP70	LYS	MEKK1 (x 10^{-3}) [b]	MT	MYD	PGP	PKC
Control	0.3±0.1	0.31±0.13	4.5±1.2	0.4±0.1	13±3	0.28±0.07	1.5±0.5	0.62±0.26	0.07±0.04
10	1.25±0.26*	0.07±0.01	13.1±1.2 *	1.4±0.35*	48±10*	1.7±1.0	4.8±1.1 *	0.5±0.1	0.08±0.04
23 – 2 h	0.92±0.21*	0.14±0.04	6.5±2.2	0.4±0.1	26±7	0.25±0.07	2.3±1.1	2.3±1.7	0.08±0.03
23 – 2 h modulated	1.6±0.4*	0.13±0.02	14.8±3.2 *	1.41±0.16*	31±2*	0.30±0.06	1.5±0.4	0.5±0.1	0.035±0.005
23 – 4 h	0.51±0.07	0.055±0.005	3.2±0.16	0.31±0.07	14±2	0.12±0.02	2.6±0.5	0.7±0.3	0.08±0.02
41	0.46±0.10	0.09±0.01	5.4±0.9	0.52±0.09	11±2	0.035±0.01*	0.10±0.05*	0.024±0.009*	0.014±0.008
120	0.57±0.17	0.08±0.03	5.8±1.3	0.5±0.25	23±7	0.20±0.05	3.1±1.1	0.6±0.2	0.13±0.06
Control – 24 h of rest	2.8±0.6	0.12±0.03	12.0±2.6	0.44±0.08	50±12	1.00±0.35	2.5±0.3	1.4±0.5	0.16±0.07
23 – 2 h + 24 h recovery	3.4±1.0	0.10±0.03	43±10 *	1.5±0.3 *	75±28	1.9±0.5	0.86±0.35 *	1.35±0.1	0.031±0.008

[a]The b-actin gene is the reference gene. The relative gene expressions are given as the mean±SEM (n=10 for control and n=5 for the exposed worms). Asterisks indicate significant differential expressions compared to controls as given by the Mann-Whitney U test (P<0.05). The abbreviated gene names stand for the following encoded proteins: CAT for catalase; COA for coactosin-like protein; HSP70 for a 70 kDa heat shock protein; LYS for lysenin; MEKK1 for MEK kinase 1 or MAP3K1; MT for metallothionein; MYD for myeloid differentiation factor 88; PGP for p-glycoprotein; PKC for protein kinase C1 (PKC1). [b]The values for the MEKK1 gene expression have been arbitrarily magnified 1000-times

7.1 mW kg^{-1}) are able to increase the level of DNA damage in the testes of rats exposed for 24 h a day for one year (41). Besides thermal effects, this genotoxic action of EMF has been linked to free radical generation and impairment of the DNA repair system (42-44). Here we show that the quantitative RAPD-PCR methodology can detect subtle effects of the exposure to EMF at the level of nucleotides since the gain or loss of probe hybridisation sites is due to the insertion, deletion, or substitution of single nucleotides, events that are different from single-, double-strand breaks or/and alkali-labile sites detected by the comet assay (6, 36, 45-47) or nuclear abnormalities detected by the micronucleus test.

EMF impinge on mitochondrial metabolism and physiology with the observed loss of cristae (48-49) or cristae degeneration (50), mitochondrial generation of ROS (51), oxidation of mitochondrial DNA and reduced mitochondrial DNA copy number (52), lowered mitochondrial respiration (35, 53), increased ADP/ATP ratio (54), and decreased mitochondrial membrane potential (53-55). In the present work, we show an increased quantity of the mitochondrial MTCO1/COX1 subunit 1 of cytochrome *c* oxidase, which points to a kind of a compensation mechanism. In another study, the reverse has been found with decreased levels of mitochondrial RNA transcripts (NADH dehydrogenase subunits 1 and 6, ND1 and ND6, and COX1) after EMF exposure (52). In this latter work, cortical neurons were exposed for 24 h to EMF at a modulated frequency of 1800 MHz at a SAR of 2 W kg^{-1}, a far greater SAR than that used in the present study (0.13 to 9.33 mW kg^{-1}), a difference likely to explain the observed discrepancy.

In the present work, EMF effects lasted after a 24 h recovery period following a two hour-exposure to 23 V m^{-1}. The same type of EMF effect has already been observed in tomato plants exposed for 10 min to 900 MHz, 5 V m^{-1}. The levels of three transcripts (calm-n6, cmbp, pin2) first increased four- to six-fold 15 min after the end of EMF stimulation, then dropped to initial levels after 30 min, and finally increased again at 60 min (37); the levels of the wound-inducible transcript, bzip, increased threefold by the end of the irradiation period, increased six-fold 5 min after the end of EMF stimulation, dropped to initial levels after 15 min, and then increased again at 30 min, and this level was maintained at 60 min (56).

CONCLUSIONS

1/ In *E. fetida* not only did EMF trigger DNA strand breaks, as previously reported (7), but it also caused DNA modifications such as single base pairs substitutions, deletions, and insertions resulting in the modification of the hybridisation sites of RAPD probes.

2/ EMF exposure resulted in the up-regulation of the genes involved in the response to general and oxidative

Table 6 *Differential gene expression in* Eisenia fetida *earthworms exposed to EMF*[a]

	CAT	COA	HSP70	LYS	MEKK1	MT	MYD	PGP	PKC
10 V m^{-1}	4	=	2.9	3.5	3.7	=	3.2	=	=
23 V m^{-1} – 2 h	3	=	=	=	=	=	=	=	=
23 V m^{-1} modulated – 2 h	5.3	=	3.3	3.2	2.4	=	=	=	=
23 V m^{-1} – 4 h	=	=	=	=	=	=	=	=	=
41 V m^{-1}	=	=	=	=	=	1/8	1/15	1/25	=
120 V m^{-1}	=	=	=	=	=	=	=	=	=
23 V m^{-1} – 2 h + 24 h recovery [b]	=	=	3.6	3.4	=	=	1/3	=	=

[a]Only the significant differential expressions as compared to the control group are given (the statistical significance has been assessed by the Mann-Whitney U test; $P<0.05$, $n=10$ for control and $n=5$ for the exposed worms). The symbol = indicates a non-significant differential expression. [b]Control earthworms were unexposed animals (sham exposure in the apparatus) let to recover 24 h before sampling. The abbreviated gene names stand for the following encoded proteins: CAT for catalase; COA for coactosin-like protein; HSP70 for a 70 kDa heat shock protein; LYS for lysenin; MEKK1 for MEK kinase 1 or MAP3K1; MT for metallothionein; MYD for myeloid differentiation factor 88; PGP for p-glycoprotein; PKC for protein kinase C1 (PKC1)

stress, as well as in chemical and immune defence, which is in line with the previously observed onset of lipid peroxidation, protein carbonylation, and the increase of catalase activity (7).

3/ EMF effects lingered on after a 24 h recovery period following a two hour-exposure to 23 V m^{-1}.

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Elektromagnetsko polje na frekvenciji mobilnih telefona (900 MHz) izaziva stres i modifikacije DNA u gujavici *Eisenia fetida*

U ovom istraživanju gujavice vrste *Eisenia fetida* bile su izložene elektromagnetskom polju (EMP) na frekvenciji mobilnih telefona (900 MHz) te poljima jačine 10 do 120 V m^{-1} u dvosatnom razdoblju (što odgovara specifičnim ratama apsorpcije od 0,13 do 9,33 mW kg^{-1}). Utjecaj dužeg izlaganja (4 sata), modulacije polja te vrijeme oporavka od 24 sata nakon dva sata izlaganja proučavan je pri jačini polja od 23 V m^{-1}. Metoda kvantitativne nasumično umnožene polimorfne DNA (engl. *quantitative random amplified polymorphic DNA – qRAPD*) otkrila je značajne modifikacije DNA na svim proučavanim tretmanima. Čak i nakon 24-satnog oporavka broj hibridizacijskih mjesta bio je dvostruko manji u odnosu na broj zabilježen u kontrolnim gujavicama, što upozorava na gubitak hibridizacijskih mjesta i na dugoročan utjecaj EMP-a. Ekspresija gena uključenih u odgovor na stres (HSP70: kodira za 70kDa *heat shock* protein i MEKK1: uključen u provođenje signala), oksidacijski stres (CAT: kodira za katalazu) te kemijsku i imunosnu obranu (LYS: kodira za lysenin i MYD: kodira za faktor mijeloidne diferencijacije) bila je povišena nakon izlaganja polju jačine 10 V m^{-1} te moduliranome polju jačine 23 V m^{-1}. Western blot analiza potvrdila je odgovor na stres detekcijom povišene količine HSP70 i MTCO1 proteina. HSP70 i LYS geni imali su povišenu ekspresiju i nakon razdoblja oporavka, što upućuje na dugotrajan utjecaj EMP-a.

KLJUČNE RIJEČI: *modulacija polja; genotoksičnost; RAPD; HSP70; oksidacijski stres; imunosni odgovor*