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Evaluation of genotoxic potential of radiofrequency/microwave electromagnetic field (RF/MW EMF) using comet assay in earthworms (*Eisenia fetida*)

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Abstract- During the last twenty years exposure of living organisms to radiofrequency/microwave electromagnetic field (RF/MW EMF) has dramatically increased, mainly because of usage of different communication systems as well as modern electronic devices. Exposure to radiation of wireless mobile phones which operate at frequencies of 900 and 1800 MHz has risen. The evaluation of effects of this kind of radiation on all living organisms is thus of great importance. In this work genotoxic effects of RF/MW radiation on earthworms (*Eisenia fetida*) using Comet assay were investigated. Earthworms are soil invertebrates frequently used for assessing the toxicity of different stress conditions. They were exposed to RF/MW radiation at frequency of 900 MHz and electric field strengths of 10 and 23 V m^{-1} in GTEM cell during 2 hours. Obtained data showed an induction of DNA damage in earthworms exposed to RF/MW EMF at 23 V m^{-1} while no increase of DNA damage was observed at 10 V m^{-1} in comparison to control group. In conclusion, earthworms were proven to be sensitive model organisms for assessment of genotoxic effects of RF/MW radiation by the comet assay.

I. Introduction

With the widespread use of mobile phones operating in the 900 MHz, public attention has been drawn to possible adverse health effects of exposure to radiofrequency/microwave electromagnetic field (RF/MW EMF). Numerous studies have documented various biological effects of RF/MW radiation including changes in cell proliferation [1], enzyme activity [2], gene expression [3], cell membrane's permeability and ion homeostasis [4,] as well as oxidative stress [5, 6] and heat-shock response [7].
Although RF/MF EMFs are classified as nonionizing radiation that can not directly damage DNA there are some findings suggesting that they are genotoxic. The literature data available on the genotoxicity/mutagenicity of RF/MW radiation are mostly results from epidemiological, animal and in vitro studies using chromosomal aberrations, sister chromatid exchanges and micronucleus induction as genetic endpoints [8]. The majority of these investigations suggest that nonthermal exposure to microwaves is not genotoxic and that adverse effects are predominantly the result of hyperthermia. However, a number of reports gave some positive findings at moderate exposure levels [9, 10] demanding further investigations. The REFLEX final report [11] revealed that the RF-radiation caused a 10-fold increase in chromosomal gaps, and a 4-fold increase in chromosome breaks in fibroblasts. Moreover, Lai and Singh [12] reported that 2.45-GHz radiation caused an increase in DNA single- and double-strand breaks in rat brain cells. On the other hand, several authors reported no increase in DNA damage following exposure to RF/MW EMF [13, 14].
The single cell gel electrophoresis, also known as the comet assay has recently been used, mainly on animal cells, to detect possible adverse effect of RF/MF EMFs on the integrity of DNA molecule. Comet assay is a rapid and sensitive fluorescence microscopic method for the detection of DNA strand breakage in single cells and it has been suggested as a method for monitoring human exposure to genotoxic agents [15]. Comet assay is based on the principle of electophoretic migration of damaged DNA away from the nuclei immobilised in agarose gel. In its high alkaline version (pH > 13) it detects primarily strand breaks and abasic (alkaline labile) sites, both arising either as direct damage or intermediates of the DNA repair. The most commonly used parameters in comet assay are tail length, relative fluorescence intensity of head and tail (normally expressed as a percentage of DNA in tail), and tail moment [16].
The aim of this study has been to investigate the possibility of using the comet assay in coelomocytes of the earthworm *Eisenia fetida* (Annelida: Oligochaeta) as a method for detecting DNA damage caused by RF/MF EMF. It should be recognized that, whilst epidemiological and human laboratory studies directly address endpoints related to human health, cellular and animal studies are of value in

assessing causality and biological plausibility. Earthworms are considered to be one of the most important members of the soil biota. They play a crucial role in soil ecosystem. These invertebrates are frequently used for assessing the toxicity of different stress conditions because they have unique advantages such as easiness of handling and low cost [17, 18].

II. Exposure system

The earthworms *Eisenia fetida* (Figure 1) were obtained from an earthworm farm "Eršek" (Donja Bistra, Croatia) and maintained in jars with soil in the laboratory. They were kept in the dark at 21 ± 2 °C. The earthworms used in this assay were adults with well-developed clitella. As earthworms are hermaphrodite, no sexual differences were taken into account. Prior to experiment, earthworms were incubated for one day on wet filter paper at room temperature to empty their gut content. Before and during the exposure they were stored in plastic Petri dishes with small holes for air on a moist filter paper. For each exposure four Petri dishes with two animals in each were prepared.

Figure 1. Earthworms (*Eisenia fetida*). From Gregory [19].

Earthworms were exposed in plastic Petri dishes to continuous waves EMF at field strengths of 10 and 23 V m^{-1} for 2 h in the Gigahertz Transversal Electromagnetic (GTEM) cell. A HP 8657A signal generator with a continuous wave and 5 W MiniCircuits amplifier was used to generate 900 MHz fields (Figure 2).

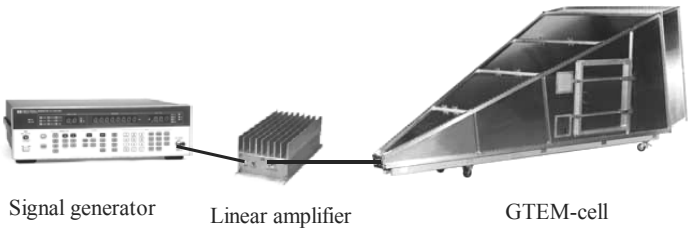

Figure. 2. Exposure system: GTEM cell, HP 8657A signal generator and 5 W MiniCircuits amplifier.

Four plastic Petri dishes with eight individuals in total were placed at the center of the GTEM cell below the septum, in the same plane, but perpendicular to the electric field (Figure 3A,B).

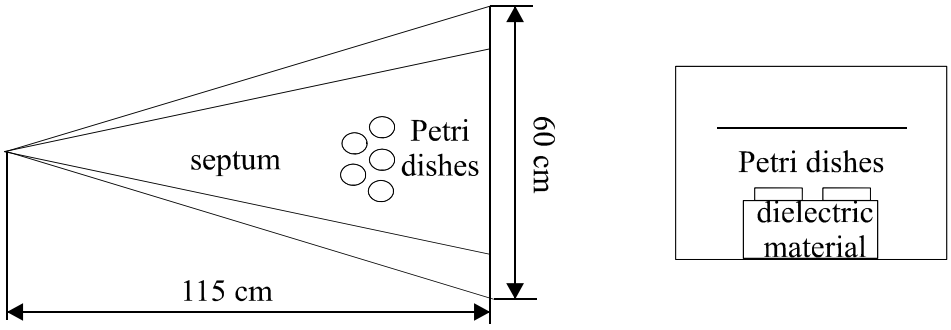

Figure 3. Position of Petri dishes in GTEM cell below the septum (A) and in respect to the direction of wave propagation, z (B).

The area where Petri dishes were placed had the most uniform field distribution (± 0.1 dB) as measured with electric probe (Holaday HI-4455) and verified with finite element method as described previously [20]. The power flux densities of the field were 0.3 and 1.4 W m^{-2} corresponding to specific absorption rates (SAR) of 5 and 26 mW kg^{-1}, respectively. Although applied power densities and calculated SARs exclude thermal stress [21], the temperature inside GTEM cell as well as in the animals was measured (K2 K/J Thermometer, Fluke) at the beginning and at the end of the exposure.
For each exposure treatment, control (non-exposed) animals were handled in the same way and kept in the same growth conditions (24 ± 2 °C, darkness) as the treated ones. In preliminary experiments, no significant differences between animals kept in the GTEM cell, but not connected with generator (sham control) and animals outside the GTEM cell were found.

III. Comet assay

Immediately after exposure earthworms were submersed in 2 mL of phosphate buffer saline and stimulated twice for 15 s with 4.5 V electric current, what resulted in extrusion of coelomocytes (Figure 4) through dorsal pores.

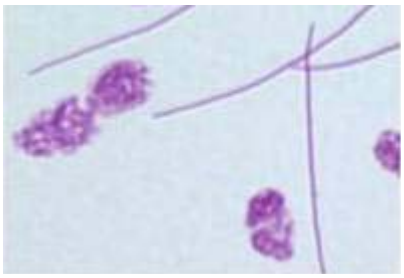

Figure 4. Feulgen-stained nuclei and spermatozoa from coelomocytes of *E. fetida*. From Gregory [19].

The comet assay was conducted following the procedure of Singh et al. [22] with slight modifications. 100 µl of aliquots of isolated coelomocytes in phosphate buffer mixed with 0.8% LMP agarose (low melting point) were placed on a 0.75% NMP agarose (normal melting point) precoated microscope slides. After solidifying for 2.5 min at 0 °C, third layer of 0.5% LMP agarose was added and left to solidify as described. The cells were lysed in freshly made lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 10% DMSO, 1% Triton X-100), pH 10, for 1 h at 4 °C. After rinsing with redistilled water, the slides were placed on the horizontal gel box, covered with cold alkaline buffer pH>13 (10 M NaOH, 200 mM EDTA), and left for 15 min. Electrophoresis was run in the same buffer at 35 V (1.16 V/cm) and 300 mA for 20 min at 4 °C. After electrophoresis slides were neutralised in the cold neutralisation buffer pH 7.5 (0.4 M Tris-HCl), 2×5 min, fixed in methanol:acetic acid (3:1) for 15 min and stored in the dark at room temperature. Prior to examination, the slides were rehydrated and stained with 10 µg/ml ethidium bromide and examined using Zeiss Axioplan epifluorescence microscope. Per every slide (per animal) 50 cells were examined, and the extent of DNA migration was determined as percentage of tail DNA using an image analysis system Komet 5, Kinetic Ltd. Cells with 50 and more percent of tail DNA were excluded from analysis. Such cells probably represent dead or dying cells, so the measurement of increase in DNA migration in their absence is more important for the evaluation of genotoxicity than an increase that depends on them [23].
Statistical analysis was performed by nonparametric Mann–Whitney U-test, using STATISTICA 7.1 (StatSoft, Inc., USA) software package, and differences between corresponding controls and exposure treatment were considered as statistically significant at $P < 0.05$.

IV. Results and discussion

DNA damage in coelomocytes of *Eisenia fetida* exposed in vivo for 2 hours to RF/MW EMF of 900 MHz measured by the comet assay was determined as percentage of DNA in tail. Although tail length and tail moment could also be used as parameter of DNA damage, relative tail intensity (expressed as a percentage of DNA in tail) is the most useful parameter, as it bears a linear increase in percentage of DNA in tail that corresponds to DNA damage up to about 2.5 breaks per 109 Dalton. It is relatively unaffected by threshold settings, and allows discrimination of damage over the widest possible range

(in theory, from 0 to 100% DNA in tail). It also gives a very clear indication of what the comets actually looked like [16].
Statistically significant increase in DNA damage was detected after exposure to field strength of 23 V m^{-1} compared to non-exposed animals. In earthworms exposed to RF/MW EMF at lower field strength (10 V m^{-1}) no increase of DNA damage was observed in comparison to control group (Figure 5).
For trained eye it is also possible to discriminate degrees of damage according to comet appearance and according to [16] 5 classes, from 0 (no tail) to 4 (almost all DNA in tail) give sufficient resolution. As seen in Figure 6 after exposure to RF/MW EMF at 23 V m^{-1} there was visible extent of DNA in tail (C) in comparison to non-exposed animals (A) and animals exposed to RF/MW EMF at 10 V m^{-1} (B).
Furthermore, temperature was monitored in earthworm tissue prior and after exposure to radiation. During the radiation at 23 V m^{-1} the temperature in earthworm did not raised more than 0.1 °C what indicates that different mechanisms apart from hyperthermia are involved in the generation of the DNA damage. There is some evidence for an excitation of resonant oscillations in chain molecules, e.g. for certain proteins and DNA molecules, within the RF signal range [24] which may lead to structural changes and chain breaks. Another possible mechanism of non-thermal RF-EMF biological effects could be achieved through increased level of reactive oxygen species such as superoxide radical, hydroxyl radical and hydrogen peroxide [5, 6].

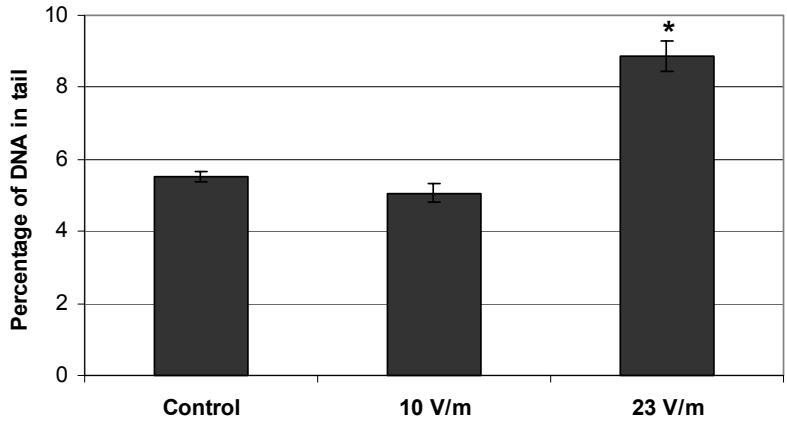

Figure 5. DNA damage in coelomocytes of *Eisenia fetida* exposed in vivo to EMF of 900 MHz at field strength of 10 and 23 V m^{-1}. * $P < 0.01$.

Figure 6. Images illustrating the induction of DNA damage in the comet assay. Nuclei of coelomocytes of non exposed earthworms (control group) (A). Nuclei of coelomocytes of *Eisenia fetida* in vivo exposed to EMF of 900 MHz at field strength of 10 V m^{-1} (B) and at field strength of 23 V m^{-1} (C).

V. Conclusions

Our preliminary results showed that nonthermal exposure to RF/MW EMF of 900 MHz at electric field strength of 23 V m^{-1} resulted in statistically significant increase in DNA damage. Our findings clearly indicate that earthworms may be useful indicator organisms to assess the genotoxic risks of RF/MW EMF and that the comet assay is a useful tool to use as biomarker of genotoxic effects of RF/MW EMF.

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