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Effects of a radio frequency electromagnetic field on honey bee larvae (*Apis mellifera*) differ in relation to the experimental study design

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ABSTRACT

Exposure to radiofrequency electromagnetic fields (RF-EMF) at the operating frequencies of different communication devices can cause various biological effects. However, there is a lack of studies on the oxidative stress response and genotoxicity in the honey bee (*Apis mellifera*) after exposure to RF-EMF. In this study, we investigated the oxidative stress and DNA damage in honey bee larvae situated in waxcomb cells, exposed to modulated RF-EMF 23 Vm-1. The glutathione S-transferase activity decreased, whereas the catalase activity increased significantly in the honey bee larvae upon RF-EMF exposure. Superoxide dismutase activity, the level of lipid peroxidation, and DNA damage were not statistically altered in exposed honey bee larvae when compared to the control group. These results suggest that the biological effects of modulated RF-EMF in honey bee larvae depend on the exposure design.

Key words: antioxidative enzymes; genotoxicity honey bee larvae; oxidative stress; radiofrequency electromagnetic fields

[#]Both authors contributed equally to the manuscript

Introduction

Exposure to radiofrequency electromagnetic fields (RF-EMF) at the operating frequencies of wireless communication or mobile phones can induce various non-thermal biological effects in laboratory and in-field conditions (for reviews see RUEDIGER, 2009; CUCHURACHI et al., 2013). However, the same authors concluded that the results of many studies were deficient in design as well as in relation to the impact on the ecosystem.

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Some of the major RT-EMF effects include oxidative stress and DNA damage (genotoxicity). Even though RF-EMF has been classified as a "possible" human carcinogen (Group 2B) (IARC, 2011), there have been several studies that have failed to detect non-thermal biological effects after exposure to RF-EMF, or have some methodological shortcomings, especially when examining genotoxic effects (VERSCHAEVE, 2009; VIJAYALAXMI and PRIHODA, 2012).

Studies on the influence of RF-EMF on oxidative stress and/or genotoxicity in honey bees are very scarce, especially related to various developmental stages. Previous studies regarding the effects of RF-EMF in honey bee colonies mostly investigated its impacts on honey production and foraging activity, colony strength, the egg laying rate of queens, and the behavior of the honey bees (SHARMA and KUMAR, 2010; FAVRE, 2011; MALL and KUMAR, 2014; KUMAR et al., 2016; FAVRE, 2017; PATEL and MALL 2019; LOPATINA et al., 2019; TLAK GAJGER et al., 2019). However, there are still many uncertainties and controversies about the effect of RF-EM radiation on honey bee colonies.

The results of studies on mammals have shown that the effects of RF-EMF differ significantly in relation to various physical properties of radiofrequency radiation (frequency, strength, modulation), exposure time, as well as biological properties (species, developmental stage, age) (KWEE and RASKMARK, 1998; SARIMOV et al., 2004; LUUKKONEN et al., 2009). Moreover, the review by PANAGOPOULOS et al. (2015) emphasized that real-life exposure to RF-EMF is a very important aspect in any investigation of biological effects.

In our previous study (VILIĆ et al., 2017), we demonstrated that exposure of honey bee larvae to RF-EMF in Petri dishes only caused a change in the activity of antioxidant enzymes and DNA damage under certain conditions of RF-EMF field strength and modulation. Specifically, we found that a modulated (80% AM 1 kHz sinus) electric field, with strength at 23 Vm^{-1}, increases DNA damage in honey bee larvae.

However, as the results of the study could be influenced not only by the physical parameters of radiofrequency radiation, but also by manipulation of biological samples, we were interested to examine the effects of RF-EMF on honey bee larvae situated in their original honeybee waxcomb cells. According to our knowledge, no studies of the impact of RF-EMF on oxidative stress and genotoxicity in honey bee larvae in waxcomb cells have been performed so far.

Therefore, the objective of this study was to investigate the effects of modulated RF-EMF (80% AM 1 kHz sinus) of 23 V m^{-1} at a frequency of 900 MHz, on oxidative stress and DNA damage in honey bees larvae exposed whilst in its original waxcomb cells, as well as to determine differences with regard to sample manipulation.

Materials and methods

The study was reviewed and approved by the Ethics Committee of the Faculty of Veterinary Medicine University of Zagreb (Class: 640-01/13-17/76; Record Number: 251/61-01/139-13-9).

Animals and conditions of the experiment. Frames with honey bee waxcombs containing younger honey bee (*Apis meliffera*) larvae were taken from a single hive, type Langstroth - Root. After the frames were taken from the hive they were wrapped in moist flannel and transported in a cardboard box. Immediately upon delivery at the laboratory, four to six day old honeybee larvae, in their original waxcomb cells, were placed in a Gigahertz transversal electromagnetic (GTEM) cell and were exposed to a modulated (80% AM 1 kHz sinusoidal) radiofrequency electromagnetic field at a frequency of 900 MHz, and a field level of 23 V m^{-1} for 2 hours. For the exposure session, eight pieces of sealed waxcombs, each containing four honeybee larvae, were placed at the center of the GTEM cell. The surface where the larvae were placed had the most uniform field distribution ($\pm$ 0.1 dB), as measured with an electric probe (Holaday HI-4455), and verified by the finite element method (MALARIC et al., 2005). Unexposed waxcombs, with four honeybee larvae were kept in the GTEM cell without the activation of RF-EMF, and were used as a control. The analyses of the oxidative stress

parameters were performed on 6 composite samples of 4 larvae from each piece of waxcomb (n = 6, in total 24 larvae), while a comet assay was performed on 4 larvae from two pieces of waxcomb (n = 8). The exposure was performed at the microwave laboratory of the Faculty of Electrical Engineering and Computing in Zagreb, at a room temperature of 23 ± 1 °C. Additionally, the temperature in the waxcomb cells with the younger honey bee brood was measured prior to and after the exposure, with a K2 K/J Thermometer, Fluke. The difference in temperature varied less than 0.1 °C.

Assay of oxidative stress parameters. Tissue extracts were prepared using a composite sample from four honey bee larvae (n = 6). Tissue was homogenized in cold 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM EDTA, using TissueLyser II (QIAGEN), for 60 seconds at 15 Hz. The homogenate was centrifuged twice under the following conditions: 15 000 g and 4 °C for 15 mins. The protein concentration in the supernatant was determined according to BRADFORD (1976), using bovine serum albumin as standard. The resulting supernatants were used for biochemical assays.

The level of lipid peroxidation was measured as the formation of thiobarbituric acid reactive substance (TBARS), a by-product of lipid peroxidation that reacts with thiobarbituric acid (LEGEAY et al., 2005). Supernatants (300 µL) were mixed with 200 µL of cold 20% (w/v) trichloracetic acid to precipitate proteins. The precipitate was pelleted by centrifugation (10 000 g for 15 mins at 4 °C) and the supernatant obtained was reacted with 400 µL of 1% (w/v) thiobarbituric acid prepared in 20% TCA. After heating at 95 ºC for 30 min, the mixture was cooled in an ice bath. The absorbance of the supernatant was measured at 532 nm, and corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The content of TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} and expressed per mg of proteins.

Catalase (CAT) activity (EC 1.11.1.6) was assayed by measuring the decrease in absorbance at 240 nm (ε = 36 mM^{-1} cm^{-1}), according to AEBI (1984). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 25 µL of sample. CAT activity was expressed in units per mg of proteins. One unit was defined as the amount of enzyme that hydrolyzes 1 µmol of H_2O_2 per minute, at 25 °C and pH 7.0.

Glutathione S-transferase (GST) activity (EC 1.8.1.7) was determined at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB) according to a method modified from BOCCHETTI and REGOLI (2006). The reaction mixture contained 2.5 mM glutathione (GSH), 100 mM potassium phosphate buffer (pH 6.5), 2 mM CDNB, and 50 µL of examined sample. GST activity was expressed in units *per* mg of proteins, where one unit was defined as the amount of enzyme producing 1 µmol of GS-DNB conjugate *per* min, under the experimental conditions described.

The activity of superoxide dismutase (SOD) (EC 1.15.1.1) was analyzed by the xanthine oxidase/cytochrome c method, modified according to McCORD and FRIDOVICH (1969). The reaction mixtures contained 0.01 mM cytochrome c and 0.5 mM xanthine in 50 mM potassium phosphate buffer (pH 7.8) with 0.1 mM EDTA. Reactions were started by adding xanthine oxidase in an amount sufficient to cause change in the absorbance of 0.025 per min. One unit of SOD inhibits the reduction rate of cytochrome c by 50% in a coupled system, using xanthine and xanthine oxidase at pH 7.8 at 25 °C.

Comet assay. The alkaline Comet assay (single cell gel electrophoresis assay) was performed according to the basic procedure of SINGH et al. (1988), with slight modifications. To obtain the cell suspension, single larvae were dilacerated with a Potter-Elvehjem tissue homogenizer (Braun Biotech, Sartorius, Goettingen, Germany) in 500 µL of phosphate buffer saline (PBS; 1.45 M NaCl, 60 mM Na_2HPO_4, 40 mM KH_2PO_4; pH 7.0). The homogenates obtained in this manner were filtered through a 70-µm sieve, centrifuged at 200 g, at 4 °C for 10 mins, resuspended in 400 µL of PBS, centrifuged again at 180 g for 10 mins at 4 °C, and finally resuspended in 60 µL of PBS. 50 µL of cell suspension was mixed with 50 µL of 0.5 % low melting point (LMP) agarose, and transferred to microscope slides pre-coated with 1 % normal melting point (NMP). After solidification for 2.5 min in a freezer, a third layer consisting of 80

μL of 0.5 % LMP agarose was added and left to solidify as described above. The cells were lysed in a freshly made lysis 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 in a horizontal gel box, covered with the cold alkaline buffer (0.3 M NaOH, 1 mM EDTA pH > 13), and left for 15 min. Electrophoresis was performed in the same buffer at 35 V (1.16 V cm-1) and 300 mA for 15 mins at 4 °C. After electrophoresis, the slides were neutralized in cold neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 2 × 5 min, then fixed in methanol: acetic acid (3:1) for 5 mins and stored in the dark, at room temperature. Prior to examination, the slides were rehydrated, stained with 10 μg/mL ethidium bromide, and examined using a Zeiss Axioplan epifluorescence microscope. For each slide 100 nuclei were analyzed. The extent of DNA migration was determined as the percentage of DNA in the tail (% tDNA) using the Comet 5 image analysis system (Kinetic Imaging Ltd., UK).

Statistical analysis. All results were expressed as means followed by the corresponding standard error (SE). Statistical analysis was performed using the Statistica 12 (StatSoft, Inc., USA) software package. After testing for normal distribution (Kolmogorov-Smirnov test of normality), the results were tested by Student's *t*-test, and the P value < 0.05 was selected to indicate significance.

Results

Oxidative stress parameters. The GST activity significantly decreased (P<0.05) whereas the CAT activity significantly increased in the honey bee larvae in their original waxcomb cells exposed to modulated RF-EMF (80% AM 1 kHz sinus) at a frequency of 900 MHz and electric field strength of 23 V m^{-1}, in comparison to the control (Fig. 1a and 1b). The SOD activity, however, did not differ significantly between the groups (Fig. 1c).

The TBARS concentration did not differ between the exposed honey bee larvae and the control larvae group (Fig. 2).

To determine differences with regard to manipulation of samples we compared results measured in unexposed honey bee larvae situated in their original waxcomb cells (the control group in this study) with those placed in Petri dishes (the control group from a previous study, VILIĆ

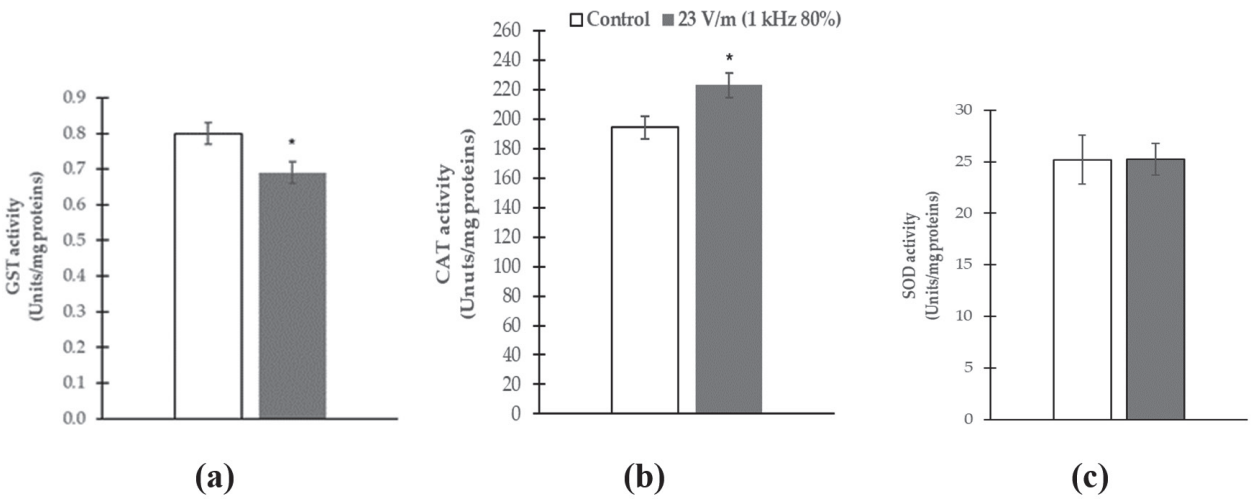

Fig. 1. (a) GST activity, (b) CAT activity and (c) SOD activity in the honey bee larvae exposed to modulated RF-EMF (80% AM 1 kHz sinus) at a frequency of 900 MHz and field level of 23 V m^{-1} in their waxcomb cells for 2 h. Results are presented as mean ± SE (n = 6). Columns with asterisk (*) are significantly different according to the Student's *t*-test at P< 0.05.

et al., 2017). The GST and CAT activities, as well as TBARS concentration in unexposed honey bee larvae from the original waxcomb cells were statistically lower ($P<0.05$) in comparison to the unexposed honey bee larvae in Petri dishes (Table 1).

Comet assay. The increase in DNA damage detected in honey bee larvae exposed to modulated RF-EMF (80% AM 1 kHz sinus), at a frequency of 900 MHz and electric field strength of 23 V m^{-1}, was not statistically different in comparison to the control group (Fig. 3).

Table 1. GST, CAT and SOD activity as well as TBARS concentrations in the honey bee larvae unexposed to RF-EMF in Petri dishes and the honey bee larvae unexposed to RF-EMF in its waxcomb cells for 2 h. Results are presented as mean ± SE.

	Control	
	[#]Petri dishes	Waxcomb
GST (U/mg proteins)	0.94 ± 0.06	0.80 ± 0.03*
CAT (U/mg proteins)	225.79 ± 5.87	194.30 ± 7.44*
SOD (U/mg proteins)	23.98 ± 1.42	25.20 ± 2.39
TBARS (nmol/mg proteins)	1.03 ± 0.10	0.67 ± 0.14*
DNA (%tDNA)	16.43 ± 1.67	22.10 ± 2.68

[#] VILIĆ et al., 2017; *denotes significance between groups (Student's *t*-test < 0.05).

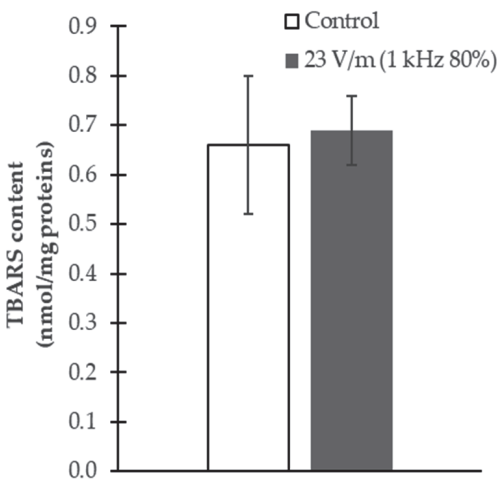

Fig. 2. TBARS concentration in the honey bee larvae exposed to modulated RF-EMF (80% AM 1 kHz sinus) at a frequency of 900 MHz and field level of 23 V m^{-1} in their waxcomb cells for 2 h. Results are presented as mean ± SE (n = 6).

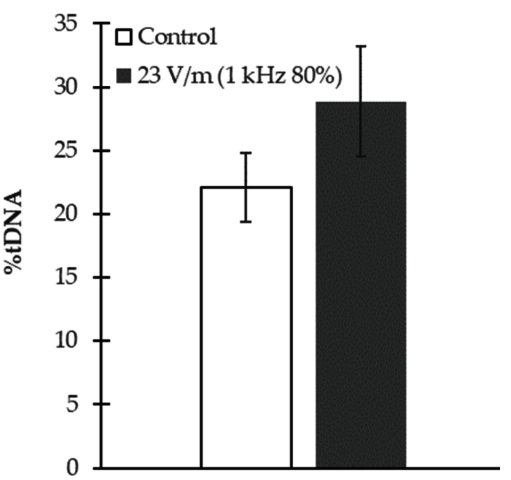

Fig. 3. DNA damage (% tDNA) in the honey bee larvae exposed to modulated RF-EMF (80% AM 1 kHz sinus) at 900 MHz and field levels of 23 V m^{-1} for 2 h. Results are presented as mean ± SE (n = 8).

Discussion

In this study we investigated the role of modulated (80% AM 1 kHz sinusoidal) RF-EMF at 900 MHz and field strength of 23 V m^{-1} on oxidative stress response and genotoxicity in honey bee larvae situated in their original waxcomb cells. Our results demonstrate that 2 h exposure affected the activity of some antioxidative enzymes (GST and CAT), but not SOD, and did not exert oxidative damage on DNA and lipids. Furthermore, oxidative stress parameters were statistically lower in the control group of honey bee larvae situated in

their own waxcomb cells, when compared to the control group of honey bee larvae kept in Petri dishes.

Changes to the antioxidant defense system parameters after RF-EMF exposure have been documented in different animal and plant species (AYDIN and AKAR, 2011; TKALEC et al., 2007; TKALEC et al., 2013). However, the results are often contradictory, and different outcomes regarding the activity of antioxidative enzymes and oxidative damage of macromolecules following RF-EMF exposure have been reported previously: (I) an increase in both the activity of antioxidant enzymes and lipid peroxidation level (YUREKLI et al., 2006; KHIRAZOVA et al., 2012; OZGUR et al., 2013); (II) a decrease in both parameters (JELODAR et al., 2013; AKBARI et al., 2014); (III) different outcomes of the activity of antioxidant enzymes and lipid peroxidation level (AYATA et al., 2004; TÜREDI et al., 2015), or (IV) no effects on the activity of antioxidant enzymes and lipid peroxidation levels (ZENI et al., 2005; STRONATI et al., 2006; SHEHU et al., 2016). Furthermore, concerning DNA damage, some authors reported significant effects, while others did not observe any increase in DNA damage (see review PANAGOPOULOS and MARGARITIS, 2008; RUEDIGER, 2009; MIYAKOSHI, 2013).

The results of the present study indicate decreased GST, but increased CAT activity, as well as the absence of DNA and lipid damage in honey bee larvae in waxcomb cells that were exposed to modulated RF-EMF at 900 MHz. To our knowledge, these are the first results considering the effect of modulated RF-EMF on honey bee larvae in their waxcomb cells. We suppose that the increased CAT activity observed in the five to six day old honey bee larvae, exposed in their waxcomb cells, could be a consequence of the specific profile and function of antioxidative enzymes during the larval developmental stage. Namely, it is known that CAT is the most important enzyme decomposing hydrogen peroxide in honey bee broods (FARJAN et al., 2012). However, in our previous study (VILIĆ et al., 2017) we found that the same exposure conditions (modulated RF-EMF at 23 V m^{-1}) increased DNA damage in honey bee larvae in comparison to the control group, while antioxidative enzyme activity remained at the control level. Differences in the observed effects could be attributed to variations in the manipulation of the larvae. Namely, in the previous study larvae were removed from their waxcombs and exposed to RF-EMF in Petri dishes. This is supported by the finding that the activity of CAT and GSH, as well as TBARS concentration, in unexposed larvae in waxcombs is lower compared to the larvae kept in Petri dishes (Tab. 1) (the larvae were randomly collected from comb cells using entomological tweezers), which could be related to manipulation-induced oxidative stress in the larvae. Namely, since honey bee larvae are considered to be at a vulnerable developmental stage and rather sensitive to stress, it is possible that the manipulation of larvae before and during exposure could cause oxidative stress and consequently DNA damage. These assertions agree with the study by PANAGOPOULOS et al. (2015), who demonstrated that a real-life exposure to RF-EMF is a very important aspect in studying its biological effects. ABOU-SHAARA (2018) also stated that the effects of many physical factors, such as electric, electromagnetic, or magnetic fields on honey bees, vary in relation to both the exposure and the experimental conditions. Furthermore, CUCURACHI et al. (2013) also note that the conditions applied in laboratory studies, in fact, do not always relate to realistic *in situ* exposure.

In conclusion, although there are no data on interactions between RF-EMF and some in-hive related materials, such as wooden frames and waxcombs, the results of our study show that the exposure of honey bee larvae to RF-EMF under the same conditions but with different experimental designs, may prove contrary. Therefore, results presented here should be considered in designing future studies on the effects of RF-EMF on younger honey bee broods.

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SAŽETAK

Izloženost radiofrekvencijskom elektromagnetskom polju (RF-EMF) na radnoj frekvenciji različitih komunikacijskih uređaja može imati razne biološke učinke. Nedostaju istraživanja odgovora na oksidacijski stres i genotoksičnost u medonosne pčele (*Apis mellifera*) poslije izloženosti RF-EMF-u. U ovom je radu istražen oksidacijski stres i DNA oštećenja u ličinkama medonosnih pčela smještenih u stanicama saća i izloženih modulirajućem RF-EMF 23 Vm-1. Aktivnost glutation S-transferaze smanjena je, dok je aktivnost katalaze značajno povećana u ličinkama medonosnih pčela izloženih RF-EMF-u. Nije bilo statistički značajne promjene u aktivnosti superoksid-dismutaze, razini lipidne peroksidacije i DNA oštećenju između ličinaka medonosne pčele i kontrolne skupine. Ovi rezultati pokazuju da biološki učinci modulirajućeg RF-EMF u ličinkama medonosne pčele ovise o oblikovanju izloženosti.

Ključne riječi: antiokdisativni enzimi; genotoksičnost; ličinke medonosnih pčela; oksidacijski stres; radiofrekvencijsko elektromagnetsko polje