

Anxiety-like behavioural effects of extremely low-frequency electromagnetic field in rats

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Abstract In recent years, extremely low-frequency electromagnetic field (ELF-EMF) has received considerable attention for its potential biological effects. Numerous studies have shown the role of ELF-EMF in behaviour modulation. The aim of this study was to investigate the effect of short-term ELF-EMF (50 Hz) in the development of anxiety-like behaviour in rats through change hypothalamic oxidative stress and NO. Ten adult male rats (*Wistar albino*) were divided in two groups: control group—without exposure to ELF-EMF and experimental group—exposed to ELF-EMF during 7 days. After the exposure, time open field test and elevated plus maze were used to evaluate the anxiety-like behaviour of rats. Upon completion of the behavioural tests, concentrations of superoxide anion ($O_2^{\cdot-}$), nitrite (NO_2^- , as an indicator of NO) and peroxynitrite ($ONOO^-$) were determined in the hypothalamus of the animals. Obtained results show that ELF-EMF both induces anxiety-like behaviour and increases concentrations of $O_2^{\cdot-}$ and NO, whereas it did not effect on $ONOO^-$ concentration in hypothalamus of rats. In conclusion, the development of anxiety-like behaviour is mediated by oxidative stress and increased NO concentration in hypothalamus of rats exposed to ELF-EMF during 7 days.

Keywords Electromagnetic field · Anxiety · Nitric oxide · Oxidative stress · Hypothalamus

Introduction

The increasing use of modern technology on extremely low-frequency electromagnetic field (ELF-EMF, 50 Hz) has become widely present in the human environment due to power lines, power transporting cables and numerous household appliances. By the fact that different electromagnetic fields (EMFs) have beneficial and harmful biological effects (Consales et al. 2012; Kovacic and Somanathan 2010), and that the population is increasingly exposed, the concern about the effects of ELF-EMF on public and occupational health is raised. Literature data indicate that the EMF may cause a biological effect in target cells or tissues (Consales et al. 2012; Santini et al. 2009) but it is still not clarified whether it causes adverse effects on human health. Over the past decades, epidemiological and experimental studies indicate that EMF exposure disrupts the neuroendocrine activity of the organism. Possible association of EMF exposure with the development of certain neurodegenerative diseases (Alzheimer's disease, emotional depression and suicide) and increasing risk of cancer was suggested (Consales et al. 2012; The ELF Working Group 2005). In addition, electromagnetic stimulation has been widely used in clinical practice in treating of certain psychiatric and neurodegenerative disorders as well as in promoting the process of neural and bone regeneration (Consales et al. 2012; Kovacic and Somanathan 2010; Markov 2007). There are several reports indicating changes in behaviour, cognitive and brain activity caused by EMF (Rauš Balind et al. 2016; Foroozandeh et al. 2013; Consales et al. 2012; Barth et al. 2010). Behavioural and cognitive disorders are closely linked to most common psychiatric

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disorders such as anxiety (Herrero et al. 2006). The exact EMF's mechanism affecting the organism is still unknown.

Formation of free radical species and oxidative stress are one of the possible mechanisms of EMF influence. There are several reports indicating that the continuous exposure to EMF alters the metabolism of free radicals in different tissue: the heart, liver, kidney and brain (Consales et al. 2012; Martínez-Sámano et al. 2012; Martínez-Sámano et al. 2010; Kovacic and Somanathan 2010). Reactive oxygen (ROS) and reactive nitrogen (RNS) species are generated during oxidative metabolism of the cells and their production is under the control of the antioxidative defence system. Under physiological conditions, ROS act as cell signalling molecules (Sena and Chandel 2012). However, due to increased generation of ROS, redox status of cell is disturbed leading to oxidative stress, further to oxidation of nucleic acids, proteins and lipids and eventually to cell dysfunction and degradation. Reactive oxygen species include superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$). Superoxide anion radical is capable of reacting with nitric oxide (NO) forming highly toxic peroxynitrite ($ONOO^-$) (Halliwell and Gutteridge 2007).

Nitric oxide as an intracellular messenger with characteristics of free radicals is involved in a lot of physiological and pathological processes, especially in the brain. Some of its most important functions are neurotransmission, vasodilation, immune defence, learning and memory, development of anxiety and depression (Walton et al. 2013; Förstermann and Sessa 2012; Dhir and Kulkarni 2011). In the central nervous system (CNS), NO is formed by the enzymatic action of neural nitric oxide synthase (nNOS) (Förstermann and Sessa 2012). Under the oxidative stress conditions, excessive production of NO in the brain may induce change in the hypothalamic-pituitary-adrenal (HPA) axis function leading to depression and anxiety (Consales et al. 2012; Joung et al. 2012).

The aim of this study was to investigate the effect of short-termly ELF-EMF (50 Hz) in the development of anxiety-like behaviour in rats through change hypothalamic oxidative stress and NO.

Material and methods

Chemicals

All reagents used in this study were analytically graded and obtained from Sigma Chemical Company (St. Louis, USA) and Merck (Darmstadt, Germany).

Animals

All experimental procedures were approved by the Faculty Ethics Committee, University of Kragujevac. The subjects in

this study were ten male adult (3 months old) *Wistar albino* rats, weighting 250 ± 50 g each. The rats were housed one per cage under controlled temperature (21 ± 2 °C) and relative humidity (40–50%) condition with 12/12-h normal light-dark cycle (lights on at 08:00 to 20:00). Standard food and tap water were provided ad libitum. The rats were randomly divided into two groups, five animals in each: (I) no ELF-EMF exposure (control group) and (II) exposure to ELF-EMF (50 Hz), 24 h/day during 7 days.

The behaviour of control animals and animals exposed to ELF-EMF for 7 days was evaluated 24 h after the last treatment, using elevated plus maze (EPM) test and open field (OF) test. Both behaviour tests were conducted during the dark period, starting at 21 h.

Device for ELF-EMF formation

The ELF-EMF was generated from two coils with 11,300 copper windings each. The coils are appointed in vertical position, spaced apart at a distance equal to their radii (8 cm) and connected in parallel to the alternate current source. According to right hand screw rule, the achieved connection enabled superposition of magnetic field from both coils and maximum intensity in the centre between coils. The VC2002 function signal generator was used with output voltage of 9.1 V peak-to-peak value and frequency of 50 Hz and current amplifier up to 1A. Generated field was harmonic with frequency of 50 Hz with root mean square (RMS) value of 10 mT. Generated strength of ELF-EMF was measured by magnetic sensor MFS-3A (magnetic field 3 axis).

Elevated plus maze test

Elevated plus maze is a widely used test for the assessment of anxiety behaviour of experimental rats (Carola et al. 2002). The device is in the form of a cross consisting of four arms, two open (10 cm × 50 cm, length/width) and two closed arms (10 cm × 50 cm × 40 cm, length/width/height), which depart from the common central platform (10 cm × 10 cm). Closed arms simulate a safe environment whereas open arms simulate risky, unfamiliar environment. Animals were individually placed on the central platform of EPM facing one of the open arms and their behaviour was being recorded by video camera for 1 h. The level of anxiety was estimated based on the time that animals spent in the closed (presence of anxiety behaviour) and open arms. The elevated plus maze was cleaned with alcohol and washed with water after testing each animal.

Video recordings were translated into a table of coordinates using the *Tracker Open Source Physics* program. Obtained coordinates were transferred to *txt* and *ods* format and then data was transformed by calculation in percentages of the 60 min (recording time) that animals spent in movement/inaction and in closed/open arms of the EPM.

Open field test

Open field test is used to assess the level of locomotor activity and as an additional test for the assessment of anxiety behaviour (Carola et al. 2002). Open field is in the form of an open box, of dimensions 60 cm × 60 cm × 30 cm (length/width/height). The space of 20 cm from the border walls to the centre of box is marked as periphery, while the central part of dimensions 20 cm × 20 cm is marked as the centre area of open field. Animals were individually placed in the middle of the centre area and their behaviour was being continuously recorded by video camera for 1 h. Avoiding the central area of the open field and inaction on the periphery indicate reduced research ability and anxiety behaviour. The open field box was cleaned with alcohol and washed with water after testing each animal.

Video recordings were translated into a table of coordinates using the *Tracker Open Source Physics* program. Obtained coordinates were transferred to *txt* and *ods* format and then data was transformed by calculation in percentages of the 60 min (recording time) that animals spent in movement/inaction and in central area/periphery of the OF.

Tissue preparation

After performing the tests of behaviour, animals were anaesthetised with ether and sacrificed by decapitation. Dissection of the brain and the isolation of the hypothalamus were carried out immediately on the ice. Hypothalamic tissue was washed with phosphate-buffered saline (PBS). We measured 100 mg of fresh tissue, homogenized it in 1 ml of PBS and then homogenate was centrifuged at 4000 rpm/min for 20 min. The supernatant was used for the quantification of superoxide anion ($O_2^{\cdot-}$), nitrites (NO_2^-) and peroxynitrite ($ONOO^-$).

Oxidative stress parameters

Concentration of $O_2^{\cdot-}$, NO_2^- and $ONOO^-$ in hypothalamus was determined after extraction using the following protocol: 2 vol 20 mM EDTA and ½ vol 3 M perchloric acid were added to 1 vol of supernatant. After extraction on ice (15 min) and centrifugation at 4000 rpm/min for 10 min, the extracts were neutralized using 2 M K_2CO_3 . The spectrophotometric determination of $O_2^{\cdot-}$ was based on the reduction of nitroblue tetrazolium (NBT) in the presence of $O_2^{\cdot-}$ (Auclair and Voisin 1985). Spectrophotometric determinations of NO_2^- , as an indicator of NO concentration, were performed using the Griess method (Green et al. 1982). Concentration of 3-nitrotyrosine (3-NT) as an indicator of the $ONOO^-$ (Herce-Pagliai et al. 1998) was determined using Riordan and Valle’s method (Riordan and Vallee 1972). The concentrations in all reactive species were expressed as nanomole per gram tissue.

Statistical analysis

All the data were analysed using software package SPSS for Windows (version 13.0). The results are presented as mean ± standard error of the mean (S.E.M). Statistical analysis was performed via independent-samples *t* test. Correlation between concentrations of NO_2^- and $O_2^{\cdot-}/ONOO^-$ in the hypothalamus of rats exposed to ELF-EMF was examined using Pearson’s correlation test and are expressed by the correlation coefficient *r*. The level of statistical significance was accepted at the $p \leq 0.05$ for all statistical analyses.

Results

Behavioural tests

Elevated plus maze test

Anxiety level was determined based on the time that animals spent in the closed arms of EPM. Effects of 7-day ELF-EMF exposure on behaviour of rats within an EPM are shown in Table 1. The obtained results show that the activity of rats after ELF-EMF exposure was significantly reduced in comparison to the control group rats. Exposed rats spent more time in closed arms and less time in open arms compared to the control group.

The open field test

The results of the open field test showed significant effects of ELF-EMF exposure on time spent in periphery and central area as well as on the activity of the rats (Table 2). Compared with the control group, activity of the exposed animals was significantly reduced. In addition, they spent more time at periphery and less time in central area of OF.

Concentrations of $O_2^{\cdot-}$, NO_2^- and $ONOO^-$ in hypothalamus

The effects of ELF-EMF on $O_2^{\cdot-}$, NO_2^- and $ONOO^-$ concentrations in hypothalamus of rats are shown in Figs. 1, 2 and 3.

Table 1 Effects of extremely low-frequency electromagnetic field (ELF-EMF) exposure on rats behaviour in the elevated plus maze

	Control	ELF-EMF exposed group
Movement (% of 60 min)	5.89 ± 0.82	3.28 ± 0.69*
Inaction (% of 60 min)	94.10 ± 0.82	96.71 ± 0.69*
Time spent in closed arms	87.05 ± 3.24	95.80 ± 1.26*
Time spent in open arms	12.94 ± 3.24	4.19 ± 1.26*

Values are means ± S.E.M, * $p \leq 0.05$ ELF-EMF exposed group versus control

Table 2 Effects of extremely low-frequency electromagnetic field (ELF-EMF) exposure on rats' behaviour in the open field

	Control	ELF-EMF exposed group
Movement (% of 60 min)	16.74 ± 1.64	2.68 ± 0.11*
Inaction (% of 60 min)	83.26 ± 1.64	97.31 ± 1.15*
Time spent in central area	99.53 ± 0.32	96.32 ± 0.39*
Time spent in periphery	0.47 ± 0.03	3.68 ± 0.03*

Values are means ± S.E.M, * $p \leq 0.05$ ELF-EMF exposed group versus control

The obtained results show that 7-day exposure to ELF-EMF induce an increase in concentrations of $O_2^{\cdot-}$ (Fig. 1) and NO_2^- (Fig. 2). The $ONOO^-$ concentration does not differ significantly between the control and EMF exposed rats (Fig. 3).

Positive correlation was found between concentration of NO_2^- and concentration of $O_2^{\cdot-}$ in the hypothalamus of rats 7 days after ELF-EMF exposure ($r = 0.990$; $p < 0.01$). Positive correlation of NO_2^- concentration and $ONOO^-$ concentration in the hypothalamus during 7-day ELF-EMF exposure was not significant.

Discussion

In this study, we demonstrate that short-term exposure to ELF-EMF induces development of anxiety-like behaviour in rats through oxidative stress and increased NO concentration in hypothalamus of rats.

The effects of ELF-EMF on anxiety-like behaviour are still unclear. There are contradictory evidence in the literature suggesting that ELF-EMF exposure has anxiogenic effect in rats (Rauš Balind et al. 2016; Shehu et al. 2016) not affecting the anxiety-like behaviour development in rats (Lai et al. 2016). The conflict in the literature data may be result of the differences in experimental conditions such as age of rats, intensity and time exposure to ELF-EMF. Data obtained in this study

indicate that ELF-EMF exposure during 7 days decreased locomotors activity and time spent in OF central area and open arms of EPM. Decreased movements and decreased time spent in the OF central area and in the open arms of EPM represent indicators for anxiety-like behaviour (Carola et al. 2002).

Anxiety is an overemotional reaction of fear, uneasiness and worry in situation which objectively is not menacing. It is characterized by autonomic and neuroendocrine activation and specific behaviour patterns, such as a defensive behaviour. Anxiety is characterized by high activity of the hypothalamic-pituitary-adrenal axis followed by high production of corticotropin-releasing factor (CRF) and high concentration of plasma corticosterone. In adaptive response (acute stressful situations), corticosterone stimulates mobilization and divert of energy from reserves in various organs. In addition, corticosterone through negative feedback is binding to glucocorticoid receptor (GR) in the hypothalamus and lead to inhibition of the activity of the HPA axis and secretion of CRF. Reduced sensitivity of GR in the hypothalamus results in disruption of the negative feedback system leading to an excessive secretion of CRF and elevated of plasma corticosterone, binding to GR and inducing peripheral stress response (Herman et al. 2003). Sensitivity of GR in the hypothalamus and high production of CRF have an important role in the development of anxiety-like behaviour (Zhang et al. 2016).

Overproduction of ROS attenuates the corticosterone negative feedback system through damage of GR in hypothalamus and consequently leads to the increase of CRF secretion (Chu et al. 2016). Disruption of the redox homeostasis in an organism through overproduction of ROS and/or reduction of antioxidative capacity has been implicated as the key mediator in the ELF-EMF induced neurodegeneration (Consales et al. 2012). In this study, we demonstrated that ELF-EMF exposure during 7 days induces the increase of $O_2^{\cdot-}$ concentration in the hypothalamus of rats. The literature data show that ELF-EMF increases the production of $O_2^{\cdot-}$ by elevated NADPH oxidase activity (Golbach et al. 2015). Additionally, ELF-EMF may

Fig. 1 Effects of extremely low-frequency electromagnetic field (ELF-EMF) exposure on concentration of superoxide anion ($O_2^{\cdot-}$) in the hypothalamus of rats. Values are means ± S.E.M, * $p \leq 0.05$ ELF-EMF exposed rats versus control

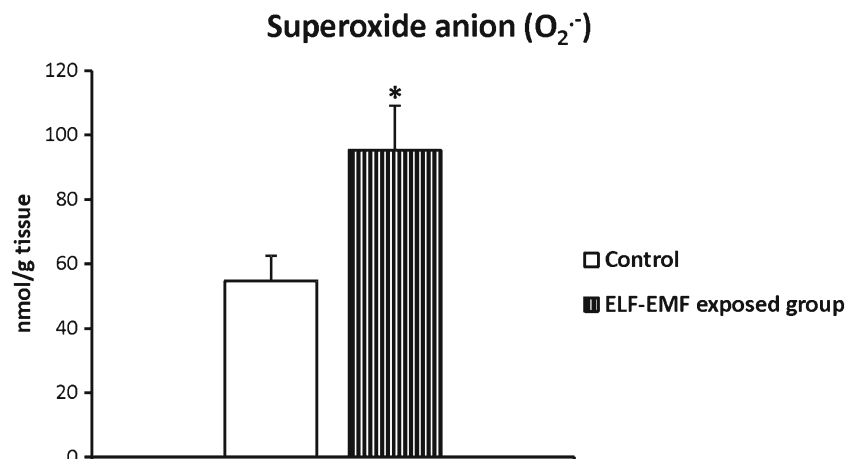
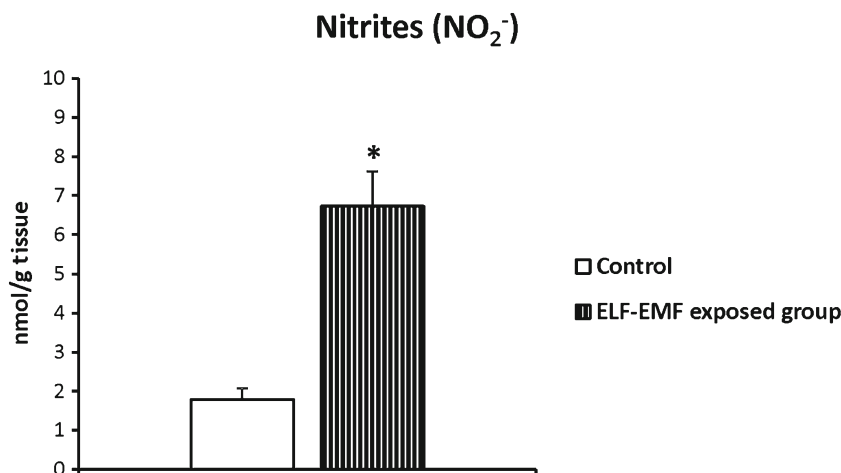


Fig. 2 Effects of extremely low-frequency electromagnetic field (ELF-EMF) exposure on concentration of nitrites (NO_2^-) in the hypothalamus of rats. Values are means \pm S.E.M, $*p \leq 0.05$ ELF-EMF exposed rats versus control



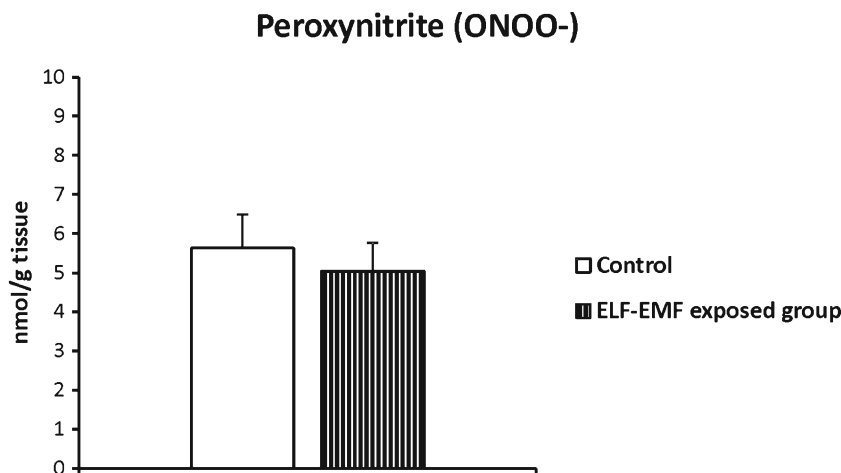
prolong the lifetime of $\text{O}_2^{\cdot-}$ by reducing the activity of superoxide dismutase and catalase, catalysing the neutralization of $\text{O}_2^{\cdot-}$ to H_2O (Martínez-Sámamo et al. 2012). In this way, ELF-EMF leads to accumulation of $\text{O}_2^{\cdot-}$, resulting in formation of oxidative stress in hypothalamus. High concentration of $\text{O}_2^{\cdot-}$ can damage of GR in hypothalamus and consequently contribute to maintenance of high concentration of CRF and corticosterone that cause anxiety-like behaviour. The importance of oxidative stress in the development of anxiety-like behaviour is indicated in the literature demonstrating a link between oxidative stress in the prefrontal cortex and hippocampus and anxiety-like behaviour (Filipović et al. 2016).

Under conditions of oxidative stress, high concentration of $\text{O}_2^{\cdot-}$ reduces bioavailability of NO with which it forms a highly toxic ONOO^- (Halliwell and Gutteridge 2007). Nitric oxide produced in paraventricular nucleus of hypothalamus mediates neuroendocrine function of hypothalamic-pituitary-adrenal axis and modulates different forms of behaviour (Joung et al. 2012). Maintaining the physiological concentrations of NO in the brain is very important for regulation of anxiety-like behaviour. A significant role of NO in the modulation of motor activity and anxiety-related behaviour were

shown. Numerous studies have investigated anxiolytic effects of NO synthase inhibitors (Joung et al. 2012; Sestakova et al. 2013). The results presented in this study indicate a significant increase of NO concentration, with no differences in ONOO^- concentration in the hypothalamus of rats exposed to ELF-EMF during the 7 days. ELF-EMF stimulates nNOS expression and activity resulting in the increased production of NO in the brain (Cho et al. 2012).

High concentration of NO stimulates the activity of hypothalamus paraventricular nucleus to secretion of CRF, stimulating pituitary to release adrenocorticotrophic hormone (ACTH), inducing corticosterone secretion from adrenal cortex of rats (Joung et al. 2012). Increased production of CRF induced by high concentrations of NO contributes to the development of anxiety-like behaviour in rats. Increased concentration of NO stimulates the activity of hypothalamus paraventricular nucleus to secretion of CRF and decreases sensitivity of GR in the hypothalamus (Joung et al. 2012; Zhou et al. 2011). This leads to elevated concentration of plasma corticosterone, through which the induced peripheral stress-response activity contributes to anxiety-like behaviour (Zhang et al. 2016; Joung et al. 2012; Herman et al. 2003).

Fig. 3 Effects of extremely low-frequency electromagnetic field (ELF-EMF) exposure on concentration of peroxynitrite (ONOO^-) in the hypothalamus of rats. Values are means \pm S.E.M, $*p \leq 0.05$ ELF-EMF exposed rats versus control



Peroxynitrite is a highly reactive molecule that can be reduced to peroxynitrite acid (HONOO) or can oxidize biomolecules, depending on the conditions in the cell. Within physiological pH values, HONOO is decomposed to $\cdot\text{OH}$ and NO_2^- (Beckman et al. 1990). Based on our results, ELF-EMF induces the increase of $\text{O}_2^{\cdot-}$ and NO concentration, while not changing concentration of ONOO^- , indicating the reduction of ONOO^- to HONOO, which after the decomposition leads to the increase of $\cdot\text{OH}$ and NO_2^- concentrations. In addition, we found a significantly positive correlation between concentration of NO and concentration of $\text{O}_2^{\cdot-}$, whereas the correlation between NO and $\text{O}_2^{\cdot-}$ concentrations and concentration of ONOO^- was not found. This indicates the decomposition of ONOO^- in hypothalamus of rats exposed ELF-EMF. Formed $\cdot\text{OH}$ oxidizes unsaturated fatty acids and starts the chain reaction of lipid peroxidation (Halliwell and Gutteridge 2007). This is supported by literature data suggesting that ELF-EMF induces lipid peroxidation (Martínez-Sámano 2012), which were found to be increased in patients with disorders associated to anxiety (Filipović et al. 2016). Ceylan et al. (2014) suggested that the lipid peroxide might be a potential biological marker for anxiety disorders. Nitrites formed by decomposition of ONOO^- in neural tissues could be reduced to NO by the neuroglobin (Shiva 2013), which leads to NO accumulation in hypothalamus.

In conclusion, the present study demonstrates that a 7-day exposure to ELF-EMF might have anxiogenic effects on rats. ELF-EMF induces anxiogenic effects on rats by increased concentration of $\text{O}_2^{\cdot-}$ and NO in hypothalamus. High concentrations of $\text{O}_2^{\cdot-}$ and NO decrease sensitivity of GR and CRF secretion in the hypothalamus leading to elevated concentration of plasma corticosterone, through which the induced peripheral stress-response activity contributes to anxiety-like behaviour (Zhang et al. 2016; Joung et al. 2012; Zhou et al. 2011; Herman et al. 2003). This conclusion suggests the need for future studies to examine the role of long-term exposure to ELF-EMF in pathophysiology of anxiety-like behaviour through the influence on the redox system of the hypothalamus.

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Compliance with ethical standards All experimental procedures were approved by the Faculty Ethics Committee, University of Kragujevac.

Conflict of interest The authors declare that they have no conflicts of interest.

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