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EXPERIMENTAL STUDY

PIXE analysis of iron in rabbit cerebellum after exposure to radiofrequency electromagnetic fields

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ABSTRACT

PURPOSE: We investigated iron accumulation and the possible mechanisms in the rabbit cerebellum after the exposure to the real GSM and generated radiofrequency electromagnetic fields (RF EMF) using inductively coupled plasma mass spectrometry (ICP MS) and particles induced X-ray emission (PIXE).

MATERIALS AND METHODS: Four groups of rabbits were exposed to the real EMF, generated EMF, combination of both the real and generated signals and the control group with no exposition. For determination of iron concentration in the four groups of cerebellum samples ICP MS was used. Iron accumulation in samples by PIXE analysis using the 3 MeV proton beam was carried out.

RESULTS: Iron concentration measured by ICP MS revealed no significant differences for all the groups. PIXE results showed a focal accumulation of iron with the size up to 3 mm. Highest concentration of iron after exposure to real signal was observed.

CONCLUSION: We suggest that the iron accumulation after the exposure to RF ELF is not the result of higher permeability of blood-brain barrier and leaking out of iron from the bloodstream into the brain cells and tissues. It could be the result of an iron actuation and its redistribution in the tissue *(Fig. 2, Ref. 86)*. Text in PDF *www.elis.sk*

KEY WORDS: brain, radiofrequency electromagnetic field, cell phone, ferritin.

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Introduction

Exposure of a human body to the radiofrequency electromagnetic field (RF EMF) has adverse health effect such as: altered production of enzymes and proteins, effect on physiological function of brain activity or structural changes of cerebellum (Kopani et al, 2018; Eker et al, 2018; Gevrek et al, 2017; Gumral et al, 2016). The blood-brain barrier (BBB) maintains a highly stable environment (Carpenter et al, 2014). It consists of the specialized tight junctions between the endothelial cells that line brain capillaries. Under some circumstances the BBB structure can be impaired what can lead to its higher permeability and leaking out of some elements from the bloodstream into the brain cells and the tissues. Several works deal with conflicting evidence about higher BBB permeability (Albert, 1979; Blasberg, 1979; Segal and Magin, 1982; Williams et al, 1984).

Iron as the most abundant metal in the brain and blood, can be regularly found in the brain tissue. It is responsible for a myelin production, growth and development. It also plays an important role in the development of neurodegenerative diseases and the aging processes (Rouault, 2013).

Ferritin as a primary iron storage is a protein situated in the cytoplasm of the cells and in small amounts in the blood circulation (Arosio and Levi, 2002). This protein shows spherical morphology

with a diameter of 12 nm. The core of ferritin consists of 6 nm Fe(III)-oxide particle stored in the form of ferrihydrite (5 Fe_2O_3 × 9 H_2O). Several studies showed that the core physiological ferritin is composed of ferrihydrite, magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3) and hematite (α-Fe_2O_3) (Cowley et al, 2000; Brem et al, 2006; Quintana, 2007; Gálvez et al, 2008). Ferritin iron core is enclosed by two types of polypeptides – heavy (Ft-H) form and light (Ft-L) polypeptide form.

Our previous work revealed iron accumulations in stratum granulosum of rabbit's cerebellum after the exposure to RF EMF (Kopani et al, 2018). This phenomenon can be the result of higher permeability of BBB and leaking out of iron from the bloodstream into the brain cells and tissues or the result of iron actuation and its redistribution – neuronal damage with subsequent release of iron sequestered in intracellular structures of neuronal cells. The aim of this study was to investigate the possible mechanisms of iron accumulation in rabbit cerebellum after the exposure to RF EMF analysed by Inductively coupled plasma mass spectrometry (ICP-MS) and Particles Induced X-ray Emission (PIXE) method.

Material and methods

Ethical declaration and compliance with ethical standards:

All the procedures were conducted in accordance with the Declaration of Helsinky and with the laws, rules, and regulations of the Slovak Republic, and Comenius University Bratislava, considering the Directive 2010/63/EU of the European Parliament. The research was approved by the Ethics Committee of Jessenius Faculty of Medicine, Comenius University in Martin (IRB00005636).

Study group

Experiments were performed on 4 groups of New Zealand White Rabbits. Rabbits were initiated to the anaesthesia by combination of tiletaminum and zolazepamum (Zoletil 100, Virbac, France) in dose 30 mg/kg i.m. Recurrent supplemental anaesthesia was α-Chloralose (Sigma-Aldrich, USA) in dose 60 mg/kg i.p. as needed. The animals were allowed to breathe spontaneously, while end-tidal CO_2, respiration rate and body temperature were monitored continuously. The animals did not suffer from any disease. Each rabbit was individually placed into the Faraday exposure cage. During the exposure, the background extremely low frequency magnetic flux density did not exceed 80 nT for the frequency band of 5 – 100 kHz as measured by Narda EHP50-D (Narda Safety Test Solution, Pfullingen, Germany). The horizontal and vertical components of static magnetic field were 49 ± 4 μT and 80 ± 3 μT, respectively. Rabbit's head was placed 5 cm from the loop antenna. The occipital and parietal brain regions were primarily exposed during 150 min.

The rabbits were divided into four groups with 5 animals in each group:

1st group – exposed to real electromagnetic field with frequency band 1805 – 1870 MHz for 150 min. The signal was calibrated to intensity of electric field (E) 300 (V/m) measured by broadband meter Narda NBM550 (Narda Safety Test Solutions, Pfullingen, Germany).

2nd group – exposed to generated electromagnetic field with frequency 1788 MHz for 150 min. The signal was pulse modulated with duty cycle 50% under carrier frequency 1788 MHz generated by functional generator Agilent N9310A (Agilent Technologies, Santa Clara, USA) and amplified by laboratory 5 W amplifier AR 5S1G4 (Amplifier Research, Souderton, USA). Electric field (E) was calibrated to 160 (V/m).

3rd group – exposed to combination of both the real and generated signals for 150 min.

4th group – control without radio-frequency signal. Animals in sham group were held always at the same conditions as animals in groups 1, 2 or 3. Thus, they were affected with same anaesthetics and kept within the same exposure unit during the same time period.

Inductively coupled plasma mass spectrometry

For determination of the iron concentration in the volume of the cerebellum samples inductively coupled plasma mass spectrometry in Thermo iCAP Q v standard mode was carried out. Dried brain tissue was added to HNO_3 and H_2O_2 in Teflon digestion tubes, digested in a microwave digestion system Anton Paar Multiwave 3000. Calibration solutions from CRM Merck ICP multi-element standard solution XVI (100 mg/l) were prepared.

Particles induced X-ray emission

Four 5 μm thin slices of the cerebellum sections were placed on silicon wafer and then fixed on frame holders developed at the CENTA laboratory for PIXE analysis using the 3 MeV proton beam. The beam intensity was 0.5 nA, charge collection was set to 0.2 μC. The incident proton beam diameter was FWHM ~ 1 mm. Consequently, the measured spectra of samples were processed by the GUPIXWIN software to obtained dependency of Fe Kα peak area on the element concentration in each sample.

Results

Figure 1 reveals iron concentration in rabbit cerebellum after the exposure in four groups measured by ICP MS. From the results can be drawn non-significant differences in all the groups after the exposure.

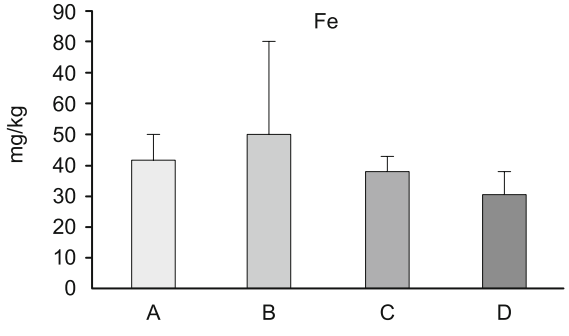

Fig. 1. Rabbit cerebellum, ICP MS. Iron concentration in rabbit cerebellum for four different groups: A) control group, B) generated signal, C) real signal and D) generated and real signal.

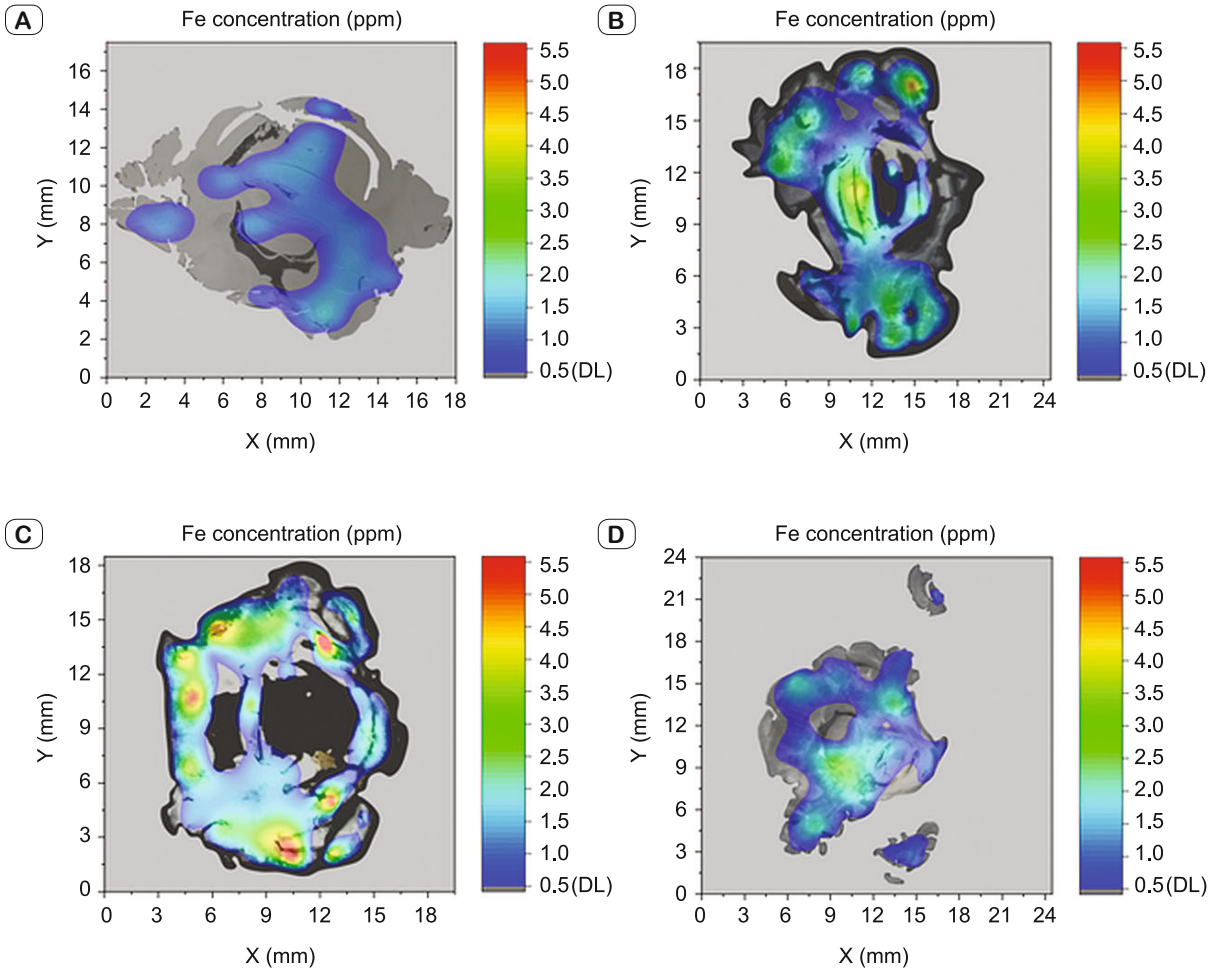

Fig. 2. Rabbit cerebellum, PIXE. Iron concentration in rabbit cerebellum for four different groups: A) control group, B) generated signal, C) real signal and D) generated and real signal. Detection limit is below 0.5 ppm.

Figure 2 shows spatial distribution of iron concentration in rabbit cerebellum in four groups measured by PIXE method: A) control group, B) generated signal, C) real signal and D) generated and real signal. The results show focal accumulation of iron with the size up to 3 mm. Fe concentration varied in the samples after the exposure in comparison with the control group. Highest concentration of iron in sample after the exposure to real signal (Fig. 2C) can be seen.

Discussion

Interaction of low energy RF EMF with cells and tissues is crucial from the view to understand the effect of this radiation. It is known that RF EMF has thermal and non-thermal effect (Repacholi, 1998; Habash et al, 2003). Both effects may cause structural and chemical changes, alter charge distribution of biological molecules or induction of eddy current in exposed cells and tissues (Céspedes and Ueno 2009). Some authors found a significant relationship between RF EMF exposure and structural changes of neurons what may assume serious neuronal damage (Salford et al, 2003; Eberhardt et al, 2008; Daniels et al, 2009). Our previous findings of structural changes of Purkinje cells in stratum granulosum of rabbit's cerebellum after the exposure to RF EMF can be explained from the view of mainly thermal effect. We consider that possible mechanism of iron accumulation may be the results of higher permeability of BBB and/or actuation and redistribution of endogenous iron in extra- and intracellular space via thermal and non-thermal effects.

Thermal effect

Electromagnetic radiation after impact on material can increase its temperature. Energy of radiation transforms into heat through the mechanism of Néel and Brown relaxation in magnetic materials occurring naturally in cells and tissues (Pankhurst et al, 2003; Deatsch and Evans, 2014). The former mechanism results in the magnetization rotating within the crystals. Brown relaxation results in mechanical rotation and alignment nanoparticle with the magnetic field.

Iron in ferritin nanoparticles present some extent of magnetism. Internal energy of these nanoparticles increases after exposure to RF EMF. Heat dissipated from them under RF EMF increases the temperature of surrounding microenvironment and locally influences the structure and biochemical reactions. It was observed that heat can increase the BBB permeability (Wijsman and Shivers, 1993; Kiyatkin and Sharma, 2009). BBB prevents damage to the brain through tight junction between endothelial cells. Altered tight junctions opening can change BBB permeability of macromolecules, nanoparticles or polymers and their distribution in the brain (Chen and Liu, 2012). For determination of BBB permeation after RF EMF various techniques were used such as horseradish peroxidase (Sutton and Carroll, 1979; Moriyama et al, 1991), Evans blue (Lin and Lin, 1982; Neilly and Lin, 1986; Ohmoto et al, 1996) and rubidium (Goldman et al, 1984). Some authors observed higher BBB permeation of rats even after low specific absorption rates (Persson et al, 1997; Salford et al, 2003; Eberhardt et al, 2008; Nittby et al, 2008; Nittby et al, 2009; Sirav and Seyhan, 2011). Poulletier de Gannes et al (2017) observed transient BBB leakage immediately after exposure at 0.26 W/kg. It is suggested that permeability depends on many factors – temperature rise, the specific absorption rate (SAR), energy of radiation, exposure duration and the rate of heat distribution and dissipation by the brain (Stam 2010). Riedinger et al (2013) observed temperature increasing to 45 °C at the surface of nanoparticles exposed to alternating magnetic field. The local hyperthermia around nanoparticles can rupture and influence the function of cell membrane, intracellular structures (lysosomes, endoplasmic reticulum, mitochondria, etc.) resulting in changes in structure and function. Rodrigues et al (2013) found that local hyperthermia has deleterious effect on integrity of cell membrane ant its viability.

However, the data on the occurrence of BBB permeation are contradictory. Many authors found no evidence of BBB leakage (Tsurita et al, 2000; Finnie et al, 2001; Kuribayashi et al, 2005; McQuade et al, 2009; Masuda et al, 2009).

Higher BBB permeation can explain the iron accumulation in stratum granulosum of rabbit's cerebellum after exposure to RF EMF. Iron from the blood can penetrate through the disrupted BBB and accumulate within the neurons and the glial cells. However, our ICP-MS obtained results show no significant change of iron concentration in cerebellum after exposure to RF EMF. This result may indicate that no BBB permeation occurs. Mass spectroscopy is a suitable method for determination of iron concentration in exposed part of the brain. ICP-MS was carried out on small groups - 5 individuals in each group. From the results we cannot consider any trend. Interindividual variability of iron concentration in this case play important role.

Non-thermal effect

Non-thermal effects of RF EMF in living organism are considered to be the biological mechanisms that are not related to increase the temperature higher than 0.01 °C. The discrimination of thermal and non-thermal effects is difficult and not precisely specified (la Hoz et al, 2006). The results of some reaction such as: reduction in reaction time, larger amount of product, higher rate of oxidation indicate that non-thermal effects can play an important role after RF EMF irradiation (Pagnotta et al, 1993; Zhang et al, 2001; Lamberto et al, 2003; Jachuck et al, 2006). Tanner et al (1967) defined non-thermal effects as change in cellular metabolism accompanied by a behavioural response. It is usually a combination of thermal and non-thermal effects. The crucial difference between them is in the matter of time scale. Similarly, like thermal effect on BBB permeability, the results of non-thermal effect are contradictory.

Reactive oxygen species generation as a non-thermal effects of RF EMF is well documented in Alzheimer disease, neuropsychiatric and behavioural disorders (Garcia et al, 2008; Davanipour and Sobel, 2009), changes in cardiac rhythm and blood pressure (Havas, 2013; Saili et al, 2015) or changes in function of immune system (Agarwal et al, 2009; Grigoriev et al, 2010; Sannino et al, 2011; Sannino et al, 2014).

Doubts and arguments against the existence of non-thermal effect on the cells and the tissues rise from the difficulties met in understanding the basic biological effects and molecular mechanisms (Porcelli et al, 1997; Belpomme et al, 2018). One possible mechanism is induction of eddy current in charged plasma membrane. Eddy currents induced after the exposure to RF EMF can excite it and bring about changes of the activity of Ca^{2+}-dependent K^{+}-channels, changes in membrane structure and in its permeability, displacement of ions, vibrations in bound changes, rotation and reorientation of dipolar molecules.

Neubauer et al (1990) observed at SAR up to 2 W/kg increased uptake of rhodamine-ferritin in endothelial cells after exposure to 2.45 GHz 10-microseconds pulses for 30–120 min. They suggested a non-thermal pinocytotic-like mechanism responsible for observed effect. Many authors state that a higher permeability of BBB can lead to excess accumulation of iron and heavy metals in the brain (Castelnau et al, 1998; Thompson et al, 2001).

Other possible "source" of iron accumulations in *stratum granulosum* of rabbit's cerebellum after exposure to RF EMF can be iron release sequestered in intracellular structures (lysosomes) of neuronal cells and its redistribution. It is well known the effect of alternating magnetic field on magnetic (nano)particles (Ivkov et al, 2005; Mannix et al, 2008; Kumar and Mohammad, 2011; Creixell et al, 2011; Tseng et al, 2012; Cho et al, 2012; Domenech et al, 2013). Nanoparticles of ferritin core and iron sequestered in intracellular structures of neuronal cells increase their internal energy under exposure to RF EMF. This energy in the form of heat damages the protein polypeptide coat of ferritin and changes its molecular structure and function. Several studies confirm harmful bio-effect of RF EMF on molecular level (Cranfield et al, 2003; de Pomerai et al, 2003; Guney et al, 2007). The structural alteration in the ferritin light (Ft-L) polypeptide coat leads to the cellular iron mistreating and ferritin aggregation (Curtis et al, 2001; Muhoberac and Vidal, 2013). Two hours exposure of ferritin to RF EMF reduces its ability to bind an iron by 20 % (Céspedes and Ueno, 2009). They suggest intermolecular interaction in ferritin changes, degradation of protein core or charge distribution changes around the entry pores of ferritin coat. Authors suggest this non-thermal effect is mediated by a superpara-

magnetic nanoparticle of iron and can be important in human exposed to strong magnetic field. The result of this process can be free iron in the cells and extracellular space. Unbound iron is an inductor of oxygen radicals such as –OH and H_2O_2, which can impair cells.

Iron (nano)particles also damage cell membranes resulting in its higher permeability, disruption of lipid bilayer and afterwards lower activity of ion channels and receptors in plasma membrane (Kirschvink, 1992; Dobson and Pierre, 1996; Cartmell et al, 2002; Dobson et al, 2006; Dobson, 2008; Ghosh et al, 2012). The result of down-regulation of their activity is weakened cellular signal and responses. Impairment of lysosomal membrane causes extravasation of lysosomal content into the cytoplasm with a decrease the intracellular pH (Zhang et al, 2014). Biological effect of iron particles depends on the interaction between magnetic field and their number, the size, distribution and magnetic properties (Dobson and St. Pierre 1998). In addition, external magnetic field around nanoparticles may induce their aggregation due to their magnetism.

On the other hand, Glover et al (2007) concluded that MRI investigation in 7 T device has no effect in vestibular hair cells, what is potential vertigo-like sensation in the patient undergoing MRI. Dobson et al (2009) found that high-field MRI (9.4 T) has probably no biological effect and cannot bring about iron compounds mediated actuation.

The time period between exposure to RF EMF and a sacrifice of animal seems to be important both for penetration through BBB with subsequent accumulation and damage of neuronal cells by iron release. Rabbits in this study were sacrificed 15 minutes after the exposure. Although we conducted one-shot exposure experiment, we found moderate iron accumulation in neuronal tissue and cells. We suggest that our findings are the results of impairment of membrane and actuation and aggregation of endogenous iron in extra- and intracellular space through thermal and non-thermal effects. More severe iron accumulation will be probably observed after long-lasting and repeated exposures, longer time period between exposure and sacrifice of animals and higher intensity of electric and magnetic field.

Conclusion

It is well known that RF EMF application has adverse health effect on animals and humans. It may influence structure, chemical composition and thus cause functional changes. Our previous work revealed structural changes of Purkinje cells in *stratum granulosum* of rabbit's cerebellum after exposure to RF EMF. We suggest that changes probably reflect thermal effects of RF EMF on the cells and the tissues. We investigated higher iron accumulation as the result of either higher BBB permeability or actuation and redistribution of endogenous iron. ICP MS obtained results may indicate that no higher BBB permeation occurs. We suggest that iron accumulation may be result of impairment of membrane and actuation or aggregation of endogenous iron in extra- and intracellular space.

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