Neuroprotective Effects of Low Intensity and Low Frequency Electromagnetic "*in vitro*" Stimulation on Glial Cells

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Degenerative neurological diseases (senile dementia, Alzheimer's disease, glaucoma), post-stroke sequelae are increasing as prevalence nowadays, in context of aging in general population, and there are strong evidences that astrocytes may play a significant role in neuroprotection. The study evaluates the effects of low intensity low frequency electromagnetic field (EMF) „in vitro" stimulation (195 mA, 7-8 Hz) on the glial cells, in different conditions, in order to identify the neuroprotective potential. Three cell lines were used for the study: the Clonetics (Lonza) line of normal human astrocytes, U87 glioblastoma tumor cells (ATCC) and L929 murine fibroblasts. The cell cultures were exposed to EMF stimulation for 2 hours/day, for 5 and 10 day, in absence and in the presence of oxidative stress stimuli. Cell viability, apoptotic potential, changes in cell phenotype, stimulation of cell proliferation for normal and malignant transformed cell lines, resilience to oxidative stress were investigated. The viability of the cells was investigated by MTS assay, and the Griess test was used to detect nitrite production as a marker of oxidation and inflammation. Inverted microscopy examination revealed that following exposure to the tested EMF, no morphological changes of the cells were recorded. In none of the cell lines tested, no cytotoxic or cytopathic effects were observed in the cultures exposed under low density conditions. In case of exposure of cell cultures for 10 days to the action of EMF, a viability of 98.2% of glial cells and 97% of exposed fibroblasts is observed, compared to unexposed cells. The viability of the cells exposed to both EMF and oxidative and inflammatory stimuli is increased by 0.96% in the case of H2O2 stimulation and in 3.76% in the case of LPS stimulation, suggesting a possible neuroprotective effect of EMF exposure. Low intensity low frequency EMF "in vitro" stimulation increased viability of the glial cells exposed to oxidative and inflammatory stimuli. No cytotoxic or cytopathic effects were observed in the exposed cultures under conditions of low density. Further tests are needed in order to elucidate the mechanisms of action of EMF on astrocytes, especially on astrocyte-neuron co-cultures to highlight the interactions between these cell types. Confirmation of the neuroprotective effect of low intensity and low frequency EMF opens the way for the development of an innovative, non-invasive therapy.

Keywords: astrocytes, electromagnetic field, neuroprotection

Degenerative neurological diseases (senile dementia, Alzheimer's disease, glaucoma), post-stroke sequelae are increasing as prevalence nowadays, in context of aging in general population and high incidence of cardio-vascular diseases. It is estimated that about 1 in 10 people over the age of 65 develop dementia or Alzheimer's disease [1]. The current medication fails to stop the disease or cure, and the pathophysiological processes involved seem to be increasingly connected, according to the latest findings [2], with the aging phenomenon of astrocytes, as a result of cumulative exposure over time to oxidative stress, inflammation and disturbances in cerebral microcirculation. Astrocytes have a well-known neuroprotective effect, through multiple mechanisms: angiogenetic, immunomodulatory, neurogenic, antioxidant and modulating synaptic transmission. This makes them excellent candidates as a source of neuroprotection in tissues affected by post-stroke ischemia/reperfusion syndrome [3, 4], but also in glaucomatous damage of the optic nerve.

Low intensity and low frequency electromagnetic stimulation (EMS) is currently used as an alternative therapy for the relief of a wide range of conditions (chronic pain associated with rheumatic, orthopedic, chronic sinusitis, periodontal disease, insomnia, stress control, anti-aging therapy, dermatology) based on its effects of stimulating microcirculation and improving cellular metabolism. Numerous experimental studies have shown the influence of the electromagnetic field (EMF) on important cellular processes, such as adhesion, proliferation, differentiation, directional migration, as well as division. There is also evidence of the beneficial effect of EMF stimulation in aging and Alzheimer's disease processes [5, 6].

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Also, clinical observations have shown that the therapy has favorable effects in regulating microcirculation and stabilizing the vascular wall, reducing tissue inflammation [7, 8].

The study evaluates the effects of low intensity low frequency electromagnetic field (EMF) „in vitro" stimulation (195 mA, 7-8 Hz) on the glial cells, in different conditions, in order to identify the neuroprotective potential.

Experimental part

Materials and methods

Three cell lines were used for the study: the Clonetics (Lonza) line of normal human astrocytes, U87 glioblastoma tumor cells (ATCC) and L929 murine fibroblasts. The cell cultures were exposed to EMF stimulation for 2 hours / day. Cell viability, apoptotic potential, changes in cell phenotype, stimulation of cell proliferation for normal and malignant transformed cell lines, resilience to oxidative stress were investigated.

The viability assessment of the astrocyte cultures exposed to the electromagnetic field generator was performed by a colorimetric method using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (Promega, USA). The reaction is based on the ability to reduce [3- (4,5-dimethylthiazol-2-yl) -5- (3-carboxymethoxyphenyl) -2- (4-sulfophenyl) -2H-tetrazolium (MTS) to formazan, soluble in the culture environment. The absorbance of this compound is measured at 490 nm directly in the culture plate, without additional processing. The conversion of MTS to soluble, aqueous formazan is carried out by dehydrogenase enzymes from metabolically active cells. Thus, the amount of formazan measured is directly proportional to the number of viable cells in the culture.

The tests were performed in 2 sequences. The first stage aimed at highlighting the non-harmful character of EMF on cell cultures, and secondly, the protective role of EMF stimulation against oxidative stress was studied.

The experimental model for evaluating the safety of applying the low frequency low intensity EMF was designed by using normal glial cell cultures (astrocytes, Clonetics, Lonza) and malignant glial cells (glioblastoma line, ATCC U87 / U118MG).

The same steps were followed for the two cell lines: 2-plate 24-well seeding (Corning, USA) – one for the control, one for the test. The cells were cultured in the center of 24 well plates, 1.5 $x10^3$ cells / well in complete medium and incubated at 37°C with 5% CO_2. Daily, for 2 h the test plates were exposed to EMF; In parallel, two control plates were not exposed to EMF. On the 5th day, the MTS test was performed to determine the degree of proliferation and to evaluate the cellular apoptosis. Exposure of normal glial cells was repeated for 2 hours / day for 10 consecutive days, sown in 24-well plates. On the 11th day, the MTS test was repeated to determine the degree of proliferation.

To verify the experimental results, the viability was also tested on a line of murine fibroblasts (L929), following the same steps: sowing 2 plates with 24 wells and 96 wells (Corning, USA) -1 for control, 1 for test. The plates (test and control) were exposed to EMF for 2 hours / day. On day 11, the MTS test was performed to determine the degree of proliferation.

In the second sequence, an experimental model was created for evaluating the neuroprotective effect induced by the exposure of normal glial cells (astrocytes) subjected to oxidative stress in the same low frequency low intensity EMF. Normal human astrocytes and murine fibroblasts were seeded into 96-well culture plates (Corning, USA) -one for control, one for test. The cells were cultured in the center of the plates with 96 wells, 5$x10^3$ cells/well in the case of fibroblasts, respectively 1.5$x10^3$ cells / well for astrocytes in complete medium and incubated at 37°C with 5% CO_2.

At 48 hours after sowing, oxidative and inflammatory stimuli were applied to representative batches of astrocyte wells - 10mM H_2O_2 and 10 µg / mL LPS. The plates (test and control) were exposed to EMF 2 hours/day, for 3 days.

In the 4th day, the MTS test was performed to determine the degree of proliferation and Griess test to detect nitrite production as a marker of oxidation and inflammation.

The nitrate concentration in a sample is measured by a diazotation reaction using Griess reagent (1% sulfanamide and 0.1% N- (1-naphthyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4). In order to evaluate the total amount of nitrite, it is necessary in an initial phase the enzymatic conversion of nitrate to nitrite or reduction with metallic cadmium, after which nitrite from the sample is measured by the diazotation reaction with the Griess reagent. In this case, the quantity nitrite was evaluated in the absence of an enzyme substrate. The results were reported to a standard sodium nitrite ($NaNO_2$) curve.

Results and discussions

In the present study, the effects of a low intensity, low frequency EMF was tested on 3 cell lines: normal human astrocytes, U87 glioblastoma cells, as well as L929 murine fibroblasts.

Inverted microscopy examination revealed that following exposure to the tested EMF, no morphological changes of the cells are recorded. No cytotoxic or cytopathic effects were observed in the exposed cultures under conditions of low density. The viability of all exposed cells, regardless of the device type, exceeded 90% compared to the unexposed cells.

It has been observed a slight stimulation of cell proliferation in normal human astrocyte culture (0.78%), while in glioblastoma cell culture the difference from unexposed control is insignificant (0.1%) (Tables 1, 2).

Table 1
INDICES OF CELL PROLIFERATION OF NORMAL HUMAN ASTROCYTES AFTER 5 DAYS OF EMF EXPOSURE

	Optical density 492nm	St. dev.
Exposed cells	1.1983	0.0568
control	1.1981	0.0494

Table 2
GLIOBLASTOMA TUMOR CELL PROLIFERATION INDICES AFTER 5 DAYS OF EMF EXPOSURE

	Optical density 492nm	St. dev.
Exposed cells	0.6912	0.0254
control	0.6858	0.0470

Since no signs of apoptosis were detected, quantification of caspase 3 by ELISA method could not be performed.

In case of exposure of cell cultures for 10 days to the action of EMF, a viability of 98.2% of glial cells and 97% of exposed fibroblasts is observed, compared to unexposed cells (Tables 3, 4). A viability of 108.5% of the fibroblasts exposed to the EMF is observed, compared to the unexposed cells, meaning a proliferation stimulation of 8.5% compared to the unexposed cells.

Table 3
INDICES OF CELL PROLIFERATION OF NORMAL HUMAN ASTROCYTES AFTER EXPOSURE TO 10 DAYS EMF

	Optical density 492 nm	St. dev.
Exposed cells	1.7504	0.0145
control	1.7193	0.0287

Table 4
L 929 FIBROBLAST CELL PROLIFERATION INDICES

	Optical density 492 nm	St. dev.
Exposed cells	1.3928	0.0435
control	1.2831	0.0231

Regarding the results of the second phase of the study, it is found that the viability of the cells exposed to both EMF and oxidative and inflammatory stimuli is increased by 0.96% in the case of H_2O_2 stimulation and in 3.76% in the case of LPS stimulation, suggesting a possible neuroprotective effect of EMF exposure (Table 5).

Table 5
PERCENTAGE VALUES OF CELL VIABILITY UNDER STIMULATION CONDITIONS WITH H_2O_2 AND LPS

	Viability of cells stimulated with H2O2 10mM, compared to the untreated cells (%)	Viability of cells stimulated with LPS 10μg/ mL , compared to the untreated cells (%)
Exposed cells	24.27	85.95
Unexposed cells	23.31	82.19

The evaluation of nitric oxide synthase (NOS) activity reflected in the degree of nitric oxide production in astrocyte culture was performed using the Griess colorimetric test. NO product was determined indirectly by quantifying the total

nitrite level in the sample. Regarding nitrite production, it is found that the exposure of the cells to the EMC also induces the basic production of nitrite, independent of oxidizing and / or inflammatory stimuli (Figure 1).

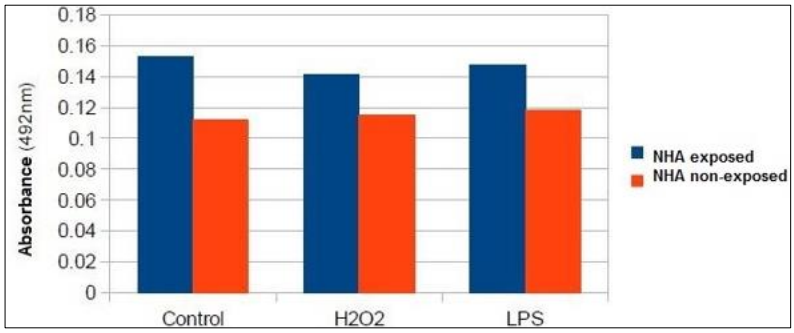

Fig. 1. Nitrit production in astrocytes (NHA) in exposed and non-exposed to EMF stimulation:

The measurements were made using a standard sodium nitrite curve as a control (Figure2).

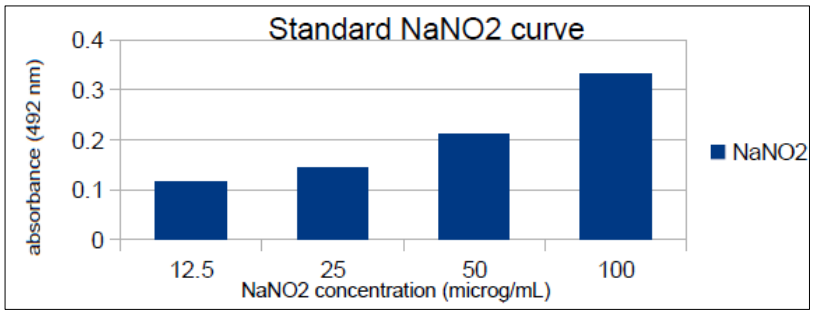

Fig. 2. Standard $NaNO_2$ curve

There is evidence that excessive nitric oxide (NO) and its metabolite or toxic peroxynitrite (ONOO) are involved in the pathogenesis of several neurodegenerative disorders, including multiple sclerosis (MS). Although astrocytes in culture show apparent resistance to these effects, neurons and oligodendrocytes seem particularly sensitive to NO / ONOO radicals - in vitro. It has been revealed that after exposure to cytokines, astrocytes show an increase in NO production and a corresponding increase to nitric oxide synthase (NOS) activity. Although the resulted NO / ONOO - radicals do not affect the survival of astrocytes, they can diffuse and cause mitochondrial injury and, possibly, cell death in neighboring NO / ONOO- sensitive cells, such as neurons and / or oligodendrocytes [9, 10].

Concomitant exposure to EMF and oxidizing stimuli of the H_2O_2 and LPS type of astrocytes leads to a slight increase in cell viability of up to 4% compared to cells not exposed to EMF, but stimulated oxidant-inflammatory, which could suggest a potential neuroprotective effect. Evaluation of nitrite production in astrocytes stimulated with H_2O_2 and LPS revealed an increase in its basal level in cells exposed to EMF. This fact has no implications on astrocyte culture, but at the nervous system level it could have a potentially effect on neurons. Nitric oxide (NO) is one of the most important signal molecules, involved in both physiological and pathological processes [11]. As a neurotransmitter in the central nervous system, NO regulates cerebral blood flow, neurogenesis, and synaptic plasticity. Cichon and col. showed that application of extremely low frequency EMF significantly increased 3-nitrotyrosine and nitrate/nitrite levels, while expression of NOS_2 was insignificantly decreased in both groups, with improving in functional and mental status and promoting recovery in poststroke patients [11]. There are also strong evidences about the role of oxidative stress in neurological degeneration and glaucoma [12- 15].

Studies of repetitive transcranial magnetic stimulation (rTMS) have led to novel attractive therapeutic approaches. Neural stem cells (NSCs) in adult human brain are able to self-renew and possess multidifferential ability to maintain homeostasis and repair damage after acute central nervous system [16]. Tsoy and col. showed that EMF is able to reduce Aβ42- and H_2O_2-induced cellular ROS, abrogate $A\beta_{42}$-induced production of mitochondrial ROS and the co-localization between the cytosolic (p47-phox) and membrane (gp91-phox) subunits of NADPH oxidase, while increasing MMP, and inhibiting H_2O_2–induced phosphorylation of p38MAPK and ERK1/2 in primary astrocytes, indicating the therapeutic potential of RF-EMF for the treatment of Alzheimer's disease [17]. Several studies also confirm that low-frequency EMF is a possible non-invasive therapeutic intervention for neurological rehabilitation secondary injury-induced events [18, 19].

Conclusions

Low intensity low frequency EMF ”in vitro” stimulation increased viability of the glial cells exposed to oxidative and inflammatory stimuli. No cytotoxic or cytopathic effects were observed in the exposed cultures under conditions of low density.

Further tests are needed in order to elucidate the mechanisms of action of EMF on astrocytes, especially on astrocyte-neuron co-cultures to highlight the interactions between these cell types. Confirmation of the neuroprotective effect of low intensity and low frequency EMF opens the way for the development of an innovative therapy in this field, completely non-invasive and without adverse effects.

The physiological process of aging associates a corresponding senescence of the glial and cerebral nerve tissue. Neurological disorders such as dementia and Alzheimer's disease are becoming more prevalent in the aging population, and these patients are associated with varying cognitive deficits, from mild to severe, needing help with daily care. Post stroke, post-traumatic trauma or surgery sequelae most often associate both motor deficiencies (paresis, paralysis), glaucoma, sensory and cognitive impairments, thus being invalid and interfering with daily activities, independence, mobility, quality of life and integration. of patients. Low intensity low frequency EMF stimulation may have a significant socio-economic impact, both by increasing the quality of life and maintaining the independence of these patients, and by reducing the costs of social assistance.

Declaration of interest

Funding: The EMF stimulation was performed by the Electronic Doctor device with application in dentistry and cosmetics, with the international patent application (PCT / IB2011 / 002804-WIPO) and national (A / 00201/2012) - "Equipment for the local application of a magnetic field of extremely low frequency in the oral cavity and its use", inventor Bogdan Vladila.

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