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RESEARCH ARTICLE

SODIUM SELENATE DECREASED ACETYLCHOLINESTERASE ACTIVITY UNDER 217HZ, 300µT ELECTROMAGNETIC FIELD

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ABSTRACT

Acetylcholinestrase is one of the most important enzymes in nervous system, and plays a role in the signal transduction in the somatic nervous system by termination of signal transduction in the synapse. It has been reported that the function of this enzyme plays a role in Alzimer's disease. Selenium is one of the most important micronutrient. Many investigations have been performed about the physiological, biochemical and behavioral effects of this element, such as postponing the Alzheimer's symptoms in the elderly and delaying the initiation signs of skin aging. Recent studies have shown that this element protects various enzymes against the toxicity caused by heavy metals such as; Pb, Al, Cu and Cd. Previous study demonstrated that Acetylcholinestrase activity was decreased under the influence of extremely low frequency electromagnetic field. In this study the effects of different concentrations of Sodium selenate (0, 390, 870, 1300 µM) on Acetylcholinestrase activity under 217 Hz, 300 µT investigated using UV-visible spectroscopy, fluorescence spectroscopy and circular dichroism spectroscopy. The main inactivation take place at a concentration of 1300 µM of Sodium selenate ($p<0.05$). The results have shown that under 217 Hz, 300 µT by increasing the cocentrations of sodium selenate the enzyme activity declined. Also, significant structural changes occurred in the secondary and tertiary structures of Acetylcholinestrase. In conclusion, according to changes observed in the secondary and tertiary structure of enzyme it is proposed that under 217 Hz, 300 µT, Sodium selenate are able to affect the activity of the Acetylcholinestrase Therefore, we suggest that the Sodium selenate could be useful in treatment of Alzimer's disease.

INTRODUCTION

Daily exposure to electromagnetic field is unavoidable as a consequence of living in a society that depends heavily on the use of the electricity. The exposure to extremely low frequency electromagnetic fields (ELF-EMFs) (frequencies less than 200-300 Hz) can alter biological systems like different enzymes, nucleic acids, cell proliferation and etc (Volpe, 2003; Zwirska-Korczala, 2005). The effects of these fields on human body depend on amperage, frequency and exposure duration (Seyyedi *et al.*, 2007). Small effects of ELF-EMFs on the activities of soluble enzymes such as antioxidative enzymes (Zwirska-Korczala, 2005) and membrane enzymes such as Na, K-ATPase or cytochrome oxidase and adenylate kinase (Ravera *et al.*, 2004; Morelli *et al.*, 2005), have been already reported. It has been recently reported that ELF-EMFs of 50, 100 and 217Hz and amplitude of 300 µT threshold, decreased Acetylcholinesterase (AChE) activity and the main inactivation take place in the 217 Hz ($p<0.05$)(Fathi and Farahzadi, 2012). Therefore, in the present study, the hypothesis is that under ELF-EMF of 217 Hz, 300µT Sodium selenate (Na_2SeO_4) affects the activity of AChE, which occurs at high specific activity in the brain and nervous tissues (Parveen *et al.*, 2004). AChE is usually found in several forms. Mammalian brain AChE consists almost exclusively of globular forms, principally the tetrameric one (Dvir *et al.*, 2007). AChE catalyzes the hydrolysis of acetylcholine (ACh) into acetate and choline at the cholinergic nervous terminals. It is, therefore, remarkable that the active site of this enzyme is found at the bottom of a deep and narrow gorge (Dvir *et al.*, 2007). We do not yet know whether this gorge provides a mechanism that somehow facilitates the rapid action of AChE. Selenium is an element that is essential for normal metabolic reactions in the body (Ani *et al.*, 2007), and seems to reduce the toxicity of several metals probably by forming the selenide complexes (Ani *et al.*, 2007; moshtaghie *et al.*, 2007). Selenium is also known to provide protection from reactive oxygen species (ROS) induced cell damages. The aim of this study was the most basic in this context: to determine the influence of Na_2SeO_4 on the activity and structure of AChE under ELF-EMF. The reason for this choice was both the availability of highly purified enzyme and substantial biochemical roll of Se in the body (Ortun˜o *et al.*, 1997; Aguilar *et al.*, 2009). Furthermore, AChE's enzymatic activity can easily be tested "in vitro" preparations, and thus, it provides a suitable model system to study the influence of trace mineral on cholinergic activity. Therefore, in this

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regards, the selenium has been shown to reverse some negative effects of EL-EMF on the AChE *in vitro*.

MATERIALS AND METHODS

AChE [(EC 3.1.1.7 from Electrophorus electricus (electric eel)] was purchased from Sigma TM Chemical and Biochemical Company. Sodium selenate (Na_2SeO_4) was synthesized as explained earlier. All other materials and reagents were of analytical grade. The reported results in this paper are the outcome of triplicate measurements.

Enzyme assay

AChE activity was determined at 25°C by the spectrophotometeric method of Ellman (Ellman *et al.*, 1961), based on the following colorimetric reactions:

Acetylthiocholine iodide $\xrightarrow{\text{AChE}}$ Thiocholine + Acetate
Thiocholine +Dithiobisnitrobenzoic acid (DTNB) $\longrightarrow$ 5-thio-2-nitro-benzoat (yellow)

The reaction mixture containing the 3µM AChE with the 12.5×10^{-6} M acetylthiocholine iodide (ACh iodide) with different concentrations of Na_2SeO_4 (0, 390, 870, 1300 µM) was kept in the electromagnetic fields 217 Hz, 300 µT for 30 min at 4°C and immediately after the DTNB was added to the reaction mixture the enzymatic activity was measured for 10 min in the absence of the field. Control sample without Na_2SeO_4 were run in the same experimental condition as above mentioned. The principle of the method is the measurement of the rate of production of thiocholine as acetylthiocholine iodide is hydrolyzed. This is accomplished by the continuous reaction of the thiol with DTNB (Ellman reagent) to produce the yellow anion of 5-thio-2-nitro-benzoat. The rate of color production was measured at 412 nm for 10 min with a UV-spectrophotometer Shimadzu, model UV-3100 with jacketed cell holders. All the experiments were run in phosphate buffer (0.1 M) at pH 7.4 in conventional thermostated quartz cell to maintain the temperature at 20± 0.1 °C. The initial velocity of enzyme was calculated using an absorption velocity of enzyme calculated using an absorption coefficient, $\varepsilon=1.36\times10^{4}$ M^{-1} cm^{-1} and one unit of enzymatic initial velocity is given as µmol/min (Hekmat *et al.*, 2008).

The rate calculated from the equations:

$$\text{Rate (µmol /min)} = \frac{\Delta \text{ absorbance/min}}{1.36\times10^{4}}$$

Circular dichroism (CD) spectroscopy

The far-UV CD region (190–260), which corresponds to peptide bond absorption, was analyzed by an Aviv model 215 Spectropolarimeter (Lakewood, NJ, USA) to give the content of the regular secondary structure in AChE. Protein solutions were prepared in the same buffer as used for the enzymatic assay. Protein solutions of 0.4 mg/ml were used to obtain the spectra incubation at different concentrations of Na_2SeO_4 (0, 390, 870, 1300 µM) in the presence ELF-EMF. All spectra were collected in a triplicate from 190 to 260 nm and a back ground-corrected against buffer blank. The results were expressed as ellipticity (cm^2 $dmol^{-1}$). The molar ellipticity was determined as $[\theta] = (100 \times (MRW) \times \theta_{obs}/cl)$, where θ_{obs} is the observed ellipticity in degrees at a given wavelength, c is the protein concentration in mg/ml and l is the length of the light path in cm. All experiments were carried out at 4^{o} C.

Intrinsic Fluorescence

Intrinsic fluorescence intensity measurements were carried out using a Hitachi spectrofluorimeter, MPF-4 model, equipped with a thermostatically controlled cuvette compartment. The intrinsic emission of 3 µM protein was seen at the excitation wavelength of 280 nm. The experiments were repeated in the presence of different concentrations of Na_2SeO_4 (0, 390, 870, 1300 µM) in the presence ELF-EMF. All experiments were carried out at 4^{o} C.

Preparation of Sodium selenate

7.9 grams of so-called standard Se (99.9%) were dissolved in 13ml of warm 65%-nitric acid. The selenious acid, H_2SeO_3, obtained, 1.2 grams, was then admixed with a stoichiometric amount of NaOH, 8 grams, as a 50%-solution of sodium hydroxide, there being obtained 17.3 grams of Sodium selenite and a further 3.6 grams of water. The pH was adjusted to 11, by adding a further minor amount of NaOH. Hydrogen peroxide (30%) was then stirred into the Sodium selenite solution in a stoichiometric excess, at a temperature of 70°C., the Sodium selenite being oxidized to Na_2SeO_4. The resultant solution, contained Sodium selenite, and then we put down on a flat place and the supernatant solution was separated, then its concentration determined by Inductively Coupled Plasma (ICP, Varian VISTA-MPX).

The chemical reactions involved in the method are:

$Se + HNO_3 \longrightarrow H_2SeO_3 + NOXH_2SeO_3 + 2NaOH \longrightarrow Na_2SeO_3 + 2H_2O$;
Or alternatively: $H_2SeO_3 + Na_2CO_3 \longrightarrow Na_2SeO_3 + CO_2Na\ Na_2SeO_3 + H_2O_2 \longrightarrow Na_2SeO_4 + H_2O$

ELF-EMF

ELF-EMF was generated by a parallel set of Helmholtz solenoids with 500 turns of 0.7 mm coated copper wire. Each solenoid diameter was 27 cm. The coils were driven by 217 Hz power through a variable transformer and generated a magnetic flux density of 300 µT. The samples were placed in the center half way between the plains of coils to receive a uniform field for 30 min.

Statistical analysis

Statistical analysis was performed by SPSS software using one way variance analysis ANOVA (Ver. 16, IBM Corporation, USA). Data were reported as mean ± standard deviation at significance level of $p < 0.05$.

RESULTS

Enzyme activity measurements

Absorption spectra of AChE in the presence of different concentrations of Na_2SeO_4 under ELF- EMF were recorded, as described by the Fig.1.According to this Figure AChE activity decreased by increasing the Na_2SeO_4 concentrations. under ELF-EMF with increasing the concentrations of Na_2SeO_4 the average rates of hydrolysis of ACh iodide was reduced, indicating decline in AChE activity.

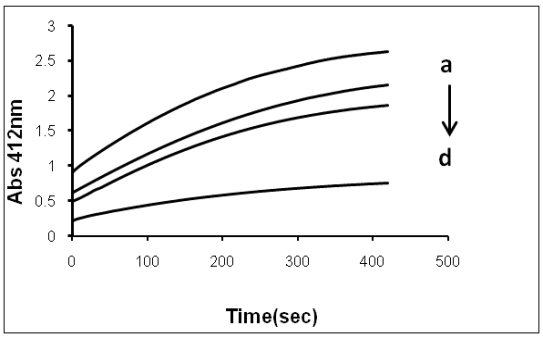

Figure 1. Absorption spectra of AChE in the presence of different concentrations of sodium selenate: a: 0, b: 390, c: 870, d: 1300 µM after incubation for 30 min under the frequency of 217 Hz and the intensity of 300 µT at 4 °C. The mean ± SD of three measurements for a, b, c and d were 0.34 ± 0.03, 0.17 ± 0.02, 0.11 ± 0.01 and 0.04 ± 0.01, respectively.

Table 1 Average rates of hydrolysis of ACh iodide

Samples	Rate (µmol /min)
AChE without Sodium selenate , under 217Hz, 300µT	1.09 ± 0.38
AChE and 390 µm Sodium selenate, under 217Hz, 300µT	0.86 ± 0.04
AChE and 870 µm Sodium selenate, under 217Hz, 300µT	0.58 ± 0.03
AChE and 13000 µm Sodium selenate, under 217Hz, 300µT	0.22 ± 0.01*

Each value represents the mean ± SD of three measurements
*Indicates $p<0.05$

Circular dichroism studies

Far-UV CD spectra (190-260 nm) are used for the determination of a protein secondary structure. The peptide bond is the main absorbing group. During the present analysis, in order to consider the effect of different concentrations of Na_2SeO_4, on the conformational changes of AChE under ELF-EMF, Far-UV CD technique was used and the spectra can be observed in Fig.2 shows the content of the secondary structure of AChE under ELF-EMF in the presence of different concentrations of Na_2SeO_4 (0, 390, 870, 1300 µM). Fig. 2 demonstrates the decrease of helicity in the presence of different concentrations of Na_2SeO_4. Within the wavelength region of 205–250 nm, the CD spectrum of a protein gives information about its conformation in relation to the secondary structure. From these results, it is apparent that increasing the concentration of Na_2SeO4 causes a conformational change of the protein with decreasing alpha helix and increasing beta structure. On the other hand, increasing the concentration of Na_2SeO4 in the presence of ELF-EMF leads to alpha-beta transition.

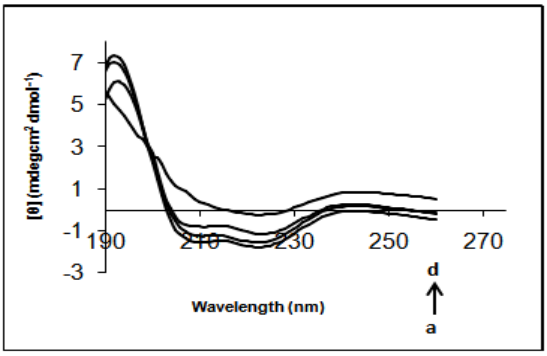

Figure 2. Far-UV-CD spectra of AChE in the presence of different concentrations of sodium selenate: a: 0, b: 390, c: 870, d: 1300 µM after incubation for 30 min under the frequency of 217 Hz and the intensity of 300 µT at 4 °C.

Table 2 Content of the secondary structure of the AChE in the presence of different concentrations of Sodium selenate using the Aviv program.

Samples	α-Helix (%)	β-Sheet (%)	Random coil (%)
AChE without Sodium selenate, under 217Hz, 300µT	59.3	16.8	20.9
AChE and 390 µm Sodium selenate, under 217Hz, 300µT	56.4	17.6	21.7
AChE and 870 µm Sodium selenate, under 217Hz, 300µT	50.8	19	23.7
AChE and 13000 µm Sodium selenate, under 217Hz, 300µT	35.9	28.3	29.3

Fluorescence studies

The fluorescence of proteins is caused by three intrinsic fluorophores present in the protein, tryptophan, tyrosine and phenylalanine residues. Because of very low quantum yield of phenylalanine and tyrosine, normally the fluorescence of tryptophan residue is investigated. Intrinsic Trp fluorescence was used to monitor changes in the protein conformation under ELF-EMF in the presence of different concentrations of Na_2SeO_4 after incubation for 30 min at 4 °C (Fig.3). Graphs a to d demonstrate a decrease in the intrinsic fluorescence by increasing the Na_2SeO_4 concentrations. This indicates that under ELF-EMF with increasing the concentration of Na_2SeO_4, the tryptophan residues are gradually exposed to more hydrophilic environment.

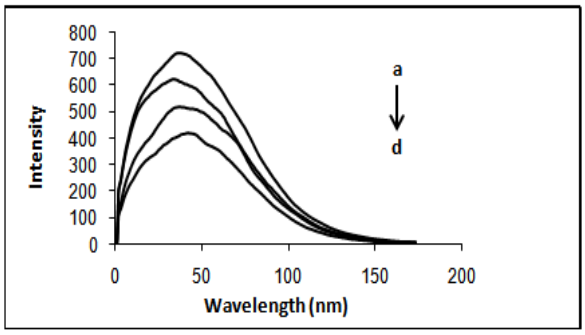

Figure 3. Intrinsic fluorescence spectra of AChE in the presence of different concentrations of sodium selenate: a: 0, b: 390, c: 870, d: 1300 µM after incubation for 30 min under the frequency of 217 Hz and the intensity of 300 µT at 4 °C.

DISCUSSION

Investigation on the role of selenium in biological systems is very important. Many studies have been done over the years to demonstrate the effects of selenium: helps promote a healthy liver, acts as an antioxidant against free radicals (Abubakar *et al.*, 2004), may help prevent cancers (Fleet *et al.*, 1997), protects against toxic metals in the body including mercury (Ani *et al.*, 2007; moshtaghie *et al.*, 2007), protects against heart disease (Cardoso *et al.*, 2010). In the present study, UV-vis spectroscopy, CD and fluorescence techniques have been used to monitor the changes of structure of AChE under the frequency of 217 Hz and the intensity of 300 in the presence of different concentrations of Na_2SeO_4. Activity studies (Figure.1) showed that different concentrations of Na_2SeO_4

have an effect on the activity of AChE under ELF-EMF. Statistical evaluations elucidate significant differences in 1300µm Na_2SeO_4, in which changes in the structure occurs leading to the enzyme inactivation ($p<0.05$). CD has proved to be an ideal technique to monitor conformational changes in proteins, which can occur as a result of changes in experimental parameters such as pH, temperature, binding of ligands and so on (Kelly and Price, 2000). Far-UV CD studies of AChE under ELF-EMF at different concentrations of Na_2SeO_4 showed significant affected of Na_2SeO_4 on the secondary structure of the protein. The protein under 217Hz, 300µT has alpha/beta structure (Axelsen *et al.*, 1994; Fathi and Farahzadi, 2012) while in the presence of 390, 870 µM concentrations of Na_2SeO_4 the content of α-helix structure of AChE was decreased and at a concentration of 1300 µM of Na_2SeO_4 a transition from α-helix to β-structure was appeared (Figure 2) then, it can be concluded that with increasing the concentrations of Na_2SeO_4, the β-sheet structure can induce in the secondary structure of AChE under ELF-EMF and consequently prevent the access of the substrate (ACh iodide) to the enzyme active site. Taken together, the conclusion is reached that different concentrations of Na_2SeO_4 can bring about changes in enzyme structure, which in turn can affect AChE interaction with the substrate (ACh iodide), and its activity. Na_2SeO_4 induced changes were observed in intrinsic fluorescence intensity of the Trp residues in AChE under ELF-EMF: with increasing the concentrations of Na_2SeO_4, Trp fluorescence decreased. Combining the results from UV-vis, Far-UV CD and intrinsic fluorescence analysis, we conclude that the structure of AChE under ELF-EMF is altered by Na_2SeO_4. Peculiar characteristic of AChE is the presence of a large negative potential near the active site (gorge), due to the presence of negatively charged residues located at the entrance, midway down and near the gorge base. This charged group distribution leads to an electrostatic field, whose potential was calculated solving the Poisson-Boltzman equation by means of the finite difference method (Warwicker *et al.*, 1982) implemented in the Delphi algorithm (Gilson *et al.*, 1988) Cholinesterase have a rather strong first moment of 800-1800 Debye roughly aligned along the gorge axis, so that a positive charged substrate will be drawn to the active site by its electrostatic field, creating a selective and efficient substrate-binding site interaction. Neuropathological Studies have been reported that various antioxidants are decreased in different age-related degenerative diseases and thus, oxidative stress would have a central role in the pathogenesis of many disorders that involve neuronal degeneration, including Alzheimer's disease (AD). AD was associated with deficiency in the brain neurotransmitter, ACh (Tabet, 2006). The inhibition of AChE enzyme, which catalyzes the breakdown of ACh, may be one of the most realistic approaches to the symptomatic treatment of AD (Pangestuti and Kim, 2010). Therefore, we suggest that the Na_2SeO_4 in the presence of ELF-EMF could be useful in treatment of AD disease.

CONCLUSIONS

In conclusion, our results suggested that in the presence of 217Hz, 300µT and Na_2SeO_4 AChE activity decresed. The effects might be related to structural changes in the secondary and tertiary structure of the enzyme.

Acknowledgments

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