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Determination of Effect of Iron (Fe^{2+} and Fe^{3+}) on Human Erythrocyte AChE Activity

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Many metals for example iron, in small quantities is needed for physiological functions of the body, but in extreme quantities may cause serious damage and poisoning. Previous report shows that the activity of erythrocyte cholinesterase enzyme, trueoecholinesterase or acetylcholin esterase (AChE) among workers producing iron-zinc alloy has decreased in relation to the control group. With respect to all these cases and also with considering high consumption of iron in society, it is decided to test it's influence on the acetylcholinesterase activity in erythrocyte. Blood used in this study, were obtained (n = 24) from healthy male volunteers, treated with 5 various concentration of Fe^{2+} and Fe^{3+}. Activity of acetylcholinesterase in erythrocyte was assayed according to colorimetric Ellman procedure. In this method, thiocholin iodide was used as substrate and dithio nitro benzoic acid used as thiol indicator. The activity of acetylcholinesterase enzyme was calculated as micromole of hydrolyzed butyryl cholin iodide per minute in each gram of tissue. Student-Newman-Keuls test was used for statistical analysis and $p < 0.05$ was considered to be significant. The mean activity of acetylcholinesterase enzyme in the concentrations under 200 µg/dl iron (Fe^{2+} and Fe^{3+}) has decreased and there is a significant difference with control group ($p < 0.001$). In addition, the mean activity of acetylcholinesterase enzyme in the concentrations higher than 200 µg/dl iron (Fe^{2+} and Fe^{3+} ions) has increased.

Key Words: Ellman procedure, Ferrous, Ferric ion, Acetylcholinesterase, Erythrocyte cholinesterase activity.

INTRODUCTION

Various agents affect on the environment and human health. Other toxins, *e.g.*, pesticides, heavy metals, chemical sewage and wastewater work similary. These compounds act through the inhibition of acetyl cholinesterase (AChE), determination is the most reliable and widely used biological (indicators) of human and animal

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exposure to these agents. Determination of cholinesterase activity is the most reliable and widely used biological indicators of human exposure to OPs (3, 4). Studies on the toxic mechanisms of theses agents have historically focused on their interactions with serine hydrolases, particularly acetylcholinesterase (AChE, EC 3.1.1.7)[1-4].

There are two different types of cholinesterase in human body, which differ in their location in tissues, substrate affinity, physiological function and sensitivity toward various inhibitors: Acetylcholinesterase which mainly found in the brain, muscles, erythrocytes and cholinergic neurons. It plays a major role in the regulation of several physiological events by hydrolyzing the neurotransmitter acetylcholine in cholinergic synapses and butyrylcholinesterase (BChE; E.C. 3.1.1.8) originating mainly from the liver is capable of splitting several cholinesterases, including acetylcholine. The highest activity being found with butyrylcholine or pseudocholinesterase, non-specific cholinesterase[5-7]. which are a group of enzymes present not only in liver but also in intestine, kidney, heart, lung, serum and cholinergic synapses within CNS and plays a major role in the metabolism of ester containing compounds. Butyrylcholinesterase can also take the place of acetylcholinesterase in acetylcholine (ACh) degradation when acetylcholinesterase is inhibited or absent[3].

Knowledge of the cholinesterase status is thus crucial for the early diagnosis of OPs exposure or intoxication and for monitoring the therapeutic effects of reactivates[8]. Among the bivalent metal ions, the effects of Hg^{2+}, Cd^{2+}, Cu^{2+}, Mg^{2+} and Ca^{2+} on BChE from different sources have been also investigated. For brain BChE purified from sheep, it has been shown that Cd^{2+} and Zn^{2+} are hyperbolic mixed-type inhibitors of the enzyme. Ca^{2+} or Mg^{2+} reactivates the enzyme after the following Cd^{2+} or Zn^{2+} inhibition[9,10].

It was reported that human serum enzyme could be inhibited by Al^{3+} when propionylthiocholine was used as substrate, but Al^{3+} was a linear mixed-type (intersecting, linear noncompetitive) inhibitor of the sheep brain BChE. Since plasma cholinesterase can be depressed by inherited traits or by other causes, notably liver disease, estimation of AChE activity gives us more reliable information[11-13].

The *in vitro* studies on the inhibition of AChE by different metals indicated that lead, cadmium and copper are the most predominant inhibitor and high concentration of these metals can be attributed to neurotoxice substance prevalant in those regions. The effect of iron (Fe^{2+} and Fe^{3+} ions) has many functions within the body including oxygen transport and enzyme activity. Iron's main function is to help make hemoglobin. Hemoglobin is the blood molecule that attracts oxygen from the breathing air. Through the lungs and circulatory system the oxygen rich blood is delivered to working muscles and other vital tissues in the body[14]. But effect of iron (Fe^{2+} and Fe^{3+} ions) on human erythrocyte AChE activity is not clear. Based on AChE activities despite BChE abundant presence in different tissues, the physiological function of this enzyme has not yet been established. While muscular or nervous tissue AChE is not accessible to direct measurement, erythrocyte AChE is regarded as a reliable surrogate[8].

A variety of methods (electrometry, tintometry, radiometry and colorimetry) have been developed, providing sensitive and specific assay for determination of AChE activity[8]. However routine use of these procedures is often hampered by many factors such as laborious sample preparation, long measuring time, insufficient specificity of substrate and disturbances by the sample matrix[5]. The calorimetric Ellman procedure is generally preferred method screening and therapeutic monitoring of pesticide-poisoned patients[9,15,16].

In continuation of our work on the determination of AChE activity in human and fishes and as a part of our continuing efforts in this area, present work was done. The purpose of this investigation is to evaluate the effect of Fe^{2+} and Fe^{3+} on activity of human erythrocyte AChE[17,18].

EXPERIMENTAL

Acetylthiocholine iodide (ATChI), dithio-*bis*(2-nitrobenzoic acid) (DTNB) were purchased from sigma (St. Louis, MO, USA). $FeSO_4$, $Fe_2(SO_4)_3$. All other compounds used in this study were prepared from Merck Chemical Co. (Germany). Water was distilled and deionized. All reagents needed for measurment of AChE activity (DTNB, substrate and stopping argents) in this study were prepared as described previously[9,17]. The estimation of AChE activity was performed according to the method of Ellman[9,15]. It was based on the inhibitory effect on acethylcholin-esterase (AChE) and the use of 5,5'-dithio*bis*(2-nitrobenzoic) acid (DTNB) as a chromogenic reagent for the thiocholine iodide (TChI) released from the acetyl-thiocholine iodide substrate[19]. The enzyme activity was calculated by measuring the increase in yellow colour produced by thiocholine when it reacts with dithio*bis*-nitrobenzoate ions. A Shimadzu UV-Mini 1240 colorimeter was used for estimating AChE activity. The analyses were performed at 25 ± 1 ºC at wavelength of 440 nm.

DTNB Reagent: Dithio-*bis*(2-nitrobenzoic acid) (DTNB) reagent was prepared in L of 33 mmol/L (5.74 g/L) K_2HPO_4 and 100 mL of 0.10 mol/L KH_2PO_4 (1.36 g/100 mL). Enough amounts of the latter were added to bring the pH of the former to 7.6. Then 0.26 mmol (107 mg) of DTNB and 20 mol (16 mg) of quinidine sulfate were prepared in 1 L of the buffer and stored in dark bottle, at 4 °C.

ATChI substrate (02 mol/L): 530 mg of acetylthiocholine iodide (ATChI) was dissolved in 10.0 mL of water and freezed for subsequent use. Before use,the substrate was thawed and diluted with 1.0 mL of water and mixed thoroughly.

Stopping reagent: This was 43 mmol/L (20 g/L) of hyamine 1622 in water, which was stored at 4 °C in dark bottle.

Ferric chloride stock solution: Ferric treatment was done by adding certain concentration of ferric stock solution (290.2290 mg/L of $FeCl_3 \cdot 6H_2O$) to prepare 50, 75, 100, 200, 250 µg/dl ferric per erythrocyte.

Ferrous sulfate stock solution: Ferrous treatment was done by adding certain concentration of ferrous stock solution (298.5188 mg/L of $FeSO_4 \cdot 7H_2O$) to prepare 50, 75, 100, 200, 250 µg/dl ferrous per erythrocyte.

Samples: Blood samples were obtained from 24 healthy male volunteers (ages 28 to 35) who are not exposed to ferrous of ferric ion. Heparinized blood samples were centrifuged at 3000 rpm to separate erythrocytes from plasma (washed 3 times). 100 µL of the bottom layer was taken and added to 6.0 mL of distilled water. This procedure causes release of erythrocyte AChE. The homogenate were frozen until assayed by method described below.

Estimation of activity

A series: To 6 glass test tubes containingin 50.0 µL of erythrocyte, several concentration levels of ferric chloride stock solution or ferrous sulfate stock solution was added (298.5188 mg/L of $FeSO_4 \cdot 7H_2O$ and 290.2290 mg/L of $FeCl_3 \cdot 6H_2O$) (25, 37.5, 50, 100, 125) to prepare a solutions of 50, 75, 100, 200, 250 µg/dl of ferrous (Fe^{2+}) or ferric (Fe^{3+}) per erythrocyte. These solutions were incubated for 0.5 h in a water bath set at 37 ºC and then were used for enzyme activity assay. The activity was comparable to that of 1 glass tube (named O tube) or blank tube containingin 50.0 µL of erythrocyte without further addition.

B series: To 7 glass test tubes containingin 3 mL of DTNB reagent and 100 µL of acetylthiocholine iodide substrate was equilibrated. After 25 min interval of **A series** was added to start the reaction. Tubes were placed in the water bath set at 37 ºC. To 6 glass test tubes of **B series** 100 µL of a series samples was added. To number 7 glass test tubes of **B series** as control and without further addition (metals ion and plasma) 100 µL of distilled water was added. Exactly 10 min after mixing; 1.0 mL of hyamine 1622 stopping reagent was added. The tubes were placed for 0.5 h at room temperature. Absorbance of test solution *vs.* the respective blanks were measured at 440 nm. For calculate of the resulting erythrocyte cholinesterase activity (R) in µM ACh/hydrolyzed/min unit (U) per gram of packed erythrocyte, absorbance difference (ΔA) was multiplied by 1/C (primary substrate concentration and reaction time was 10 min) or R = 574 ΔA/C. Triplicate measurements were done for each sample and the mean value was taken. A t-test was performed to distinguish between workers and healthy volunteers. Plasma cholinesterase activity was done similarly. Only butyrylthiocholine iodide reagent used instead of acetylthiocholine iodide. Containingin water bath set at 37 ºC.

Analyses of the data: Statistical analysis was performed using SPSS for Windows (Ver.10, SPSS, Inc., Chicago, USA). Data were analyzed by one-way analysis of variance (ANOVA) and presented as mean ± standard error in the mean (SEM). Student-Newman-Keuls test was used for statistical analysis and $p < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Fig. 1 shows correlation the mean concentrations (mean ± SD) of Fe^{2+} and Fe^{3+} per erythrocyte *vs.* AChE activity of samples from healthy male volunteers. There was significantly difference between mean AChE activity in Fe^{2+} (4.060 ± 0.2502) and Fe^{3+} (3.225 ± 0.1364) volunteers. This activity in Fe^{2+} was significantly higher

than activity in Fe^{3+} of volunteers ones ($p < 0.05$). The effect was concentrated dependent but the activity was low in lower concentrates. As shown in this figure, both Fe^{2+} and Fe^{3+} treatments significantly ($p < 0.05$) decreased the AChE activity in all concentration. The activity was comparable to that of blank tube containing in 50.0 µL of erythrocyte without further addition.

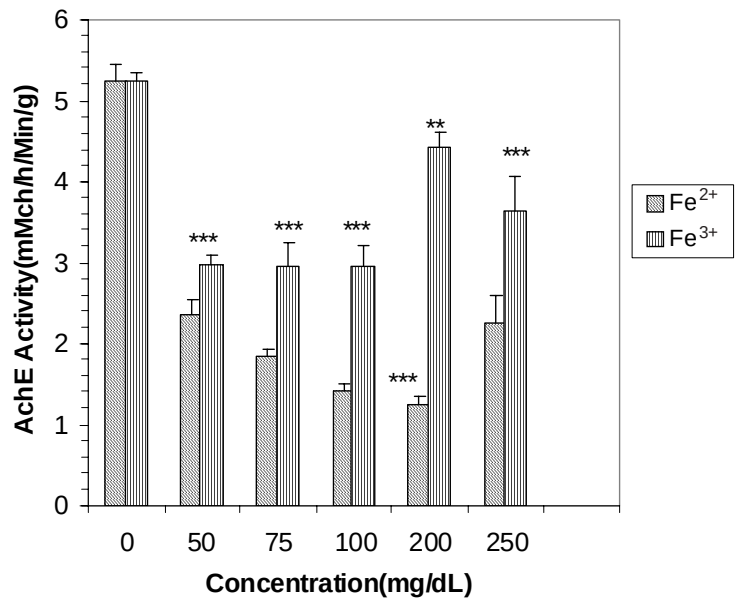

Fig. 1. Correlation the mean concentrations (mean ± SD) of ferric (Fe^{3+}), ferrous (Fe^{2+}) per erythrocyte *vs.* BChE activity of samples from healthy volunteers. Values are presented as mean ± SEM (N = 24), **$p < 0.05$ and ***$p < 0.001$ with respect to control, (ANOVA followed by Newman-Keuls multiple comparisons test)

The activity of AChE, an enzyme that modulates the amount of the neurotransmitter substance acetylcholine at the nerve cell junction (which also occurs even in non-nervous tissues in addition to nervous tissues), is reported to vary in different organs in response to enviromental stress, including heavy metal stress[20]. As determined in present study, the human erythrocyte exposed to 5 different concentrations of ferric (Fe^{3+}), ferrous (Fe^{2+}) ions relevant to human exposure exhibited remarkable variations with a decrease in AChE activity could be attributed to the structural damage to the cellular machinery concerned with enzyme production[21]. On other hand, the decrease in AChE activity could be due to the binding of Fe^{2+} and Fe^{3+} ions to lipid rich structural componets of mitochondria and subsequently affecting the activities of enzymes like AChE which are associated directly with lipid rich fraction, especially where intergrity of the structural componets is necessary for maximum catalytic activity.

Furthermore the decrease synthesis of the enzyme by the inhibitory nature of toxicants and also due to asphyxiation[20]. *In vivo* suppression in the activity of AChE is organ-dependent and is attributed to the organs. The magnitude of metal accumulation in the target tissues and biological half-life of the metal are cosidered of prime importance in the modulation or modification of the AChE[22].

It is revealed that the death occurs in fish when AChE activity falls below a critical level and inhibition of the brain AChE to the level of 70 to 80 % is critical to fishes. Next to the brain, the greater suppression in the activity of neuromuscular transmission and ionic fluxes is associated with its tension under imposed toxic metal stress[20].

Present results show no significant difference rate of AChE inhibition by ferric and ferrous ion. As shown in Fig. 1, both Fe^{2+} and Fe^{3+} ion show approximately similar trends of AChE inhibition. Ultimately *in vitro* depression of erythrocyte AChE activity can be caused by direct effect of metal ions, *i.e.* a decrease in quantity of the enzyme, or may be due to the interaction of metals and sulfhydryl groups of the enzyme. AChE binds its substrate by dipole/ionic interactions between charged and polar groups on the molecule. During the catalytic cycle, part of the substrate is also bound covalently.

Cofactors of metal ions as (Fe^{2+}) and (Fe^{3+}) ion, which are often bound by dipole interactions with histidine and other amino acids with lone-pairs in catalase[23,24]. And *in vitro* depression of erythrocyte AChE activity can be caused by indirect effect of metal ions, when the ratio of ferrous (Fe^{2+}) to ferric (Fe^{3+}) drops below 8:1. The ideal situation for the development of the hydroxyl radical exists. In the presence of H_2O_2, ferrous (Fe^{2+}) becomes oxidized to form ferric (Fe^{3+}), because it lost an electron. The H_2O_2 gains an electron and becomes reduced, by that forming the hydroxyl (OH) radical, a potential cause of lipid peroxidation[22-25].

The present paper represents the first record of the effect of Fe^{2+} and Fe^{3+} ions on AChE activity in human erythrocyte and reveals that both Fe^{2+} ion and Fe^{3+} ion an essential metal or ions, which belong to group VIIIB of the periodic table suppress AChE activity in human erythrocyte. The *in vitro* effects of Fe^{2+} and Fe^{3+} ion on human AChE activity remains to be cleared by further studies. The direct interaction of metal ions with AChE is proposed as a mechanism for depressed enzyme activity.

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