Effects of Neostigmine Methylsulfate on Enzyme Activity of Acetylcholinesterase in Rat Serum, Plasma, Muscle and Liver *in vivo*

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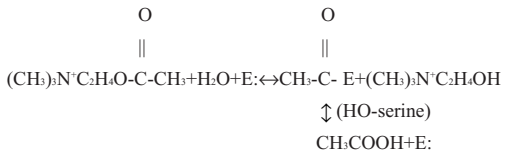
Summary
This study was designed to investigate the effects of neostigmine methylsulfate on enzyme activity of acetylcholinesterase (AChE) in rat serum, plasma, muscle and liver were investigated *in vivo*. Twelve Sprague-Dawley Albino male rats, weighing 150-200 g were housed in two groups, each group containing six rats. A single dose (0.05 mg/kg) of neostigmine methylsulfate was injected intramuscularly to the treatment rats. Control rats were given only the same amount of physiological saline. Blood samples for enzyme activity were obtained by cardiac puncture under ether anesthesia one, three, and six hours after the treatment. The tissues were dissected six hours after the treatment. The present study indicated that neostigmine methylsulfate possessed an inhibitory effect on AChE activity in serum, plasma, muscle and liver with the elapse of time. However, the greatest inhibition was found six hours after the injection.

Introduction
Acetylcholinesterase (AChE: E.C.3.1.1.7) catalyzes the hydrolysis of acetylcholine, a neurotransmitter substance which functions in certain parts of the nervous system. The reaction catalyzed by AChE occurs enzymically in two steps. In the first step, the enzyme serves as a powerful nucleophile. In the second step, the enzyme serves as an excellent leaving group aided by the nucleophilic hydroxyl group of a specific serine residue (*Aliriz & Turkoglu, 2003*).



AChE is an important component of cholinergic synapses in the peripheral and central nervous system, where it is partly responsible for terminating the actions of the neurotransmitter acetylcholine. Using histochemical techniques, it has been shown that AChE is localized in the region of nerve/muscle and nerve/nerve contacts in many electrophysiologically defined cholinergic systems (*Brimijoin & Koenigsberger, 1999*). Recent interest in AChE has been focused on the structures and relationships of different forms that have been observed in various tissues (*Massoulie & Bon, 1982; Rosenberry, 1982; Rosenberry, 1984*). Acetylcholinesterase is found in all conducting tissue in all species of animal, which have been investigated; it is mainly found in the brain, in nerve cells, in muscle and in erythrocytes (*Aliriz & Turkoglu, 2003; Riov & Jaffe, 1973; Earnst & Hartmann, 1980*).

In this study, effects of neostigmine methylsulfate on enzyme activity of AChE in rat serum, plasma, muscle and liver were investigated *in vivo*. Although neostigmine has been known as an AChE inhibitor for a long time, this study is the first to show its effect in plasma, serum and various organs in different time periods *in vivo*.

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Materials and Methods

The chemicals used in the present study were obtained from the indicated sources: DTNB, standard bovine serum albumin, Acetylthiocholiniodure, Coomassie brillant blue G-250 were purchased from Sigma Chem. Co. (St Louis, Missouri, USA). Sodium bicarbonate, Sodium citrate, NaOH, Sodium carbonate, NaCl, HCl, acetic acid, o-phosphoric acid, ethyl alcohol, saccarose, sodium phosphate, disodium phosphate were purchased from Merck A.G. (Darmstadt, Germany). Neostigmine methylsulfate was purchased from Adeka Co. (Istanbul, Turkey).

Treatment of Rats

Twelve outbred Sprague-Dawley Albino male rats, weighing 150-200 g were provided by the animal house of the Art and Science School of Yuzuncu Yil University and housed in two groups, each group containing six rats. The animals were fed a standard laboratory diet purchased from Van Animal feed factory (Van, Turkey), and the animals were housed at 20±2 °C in a daily 12h light/12h dark cycle. A single dose (0.05 mg/kg) of neostigmine methylsulfate was injected intramuscularly to the treatment rats. Control rats received only the same amount of physiological saline. Blood samples for enzyme activity were obtained by cardiac puncture under ether anesthesia one, three, and six hours after the treatment. Blood samples for obtaining serums were put immediately into ice-chilled siliconized disposable glass tubes. To obtain plasma, some of the blood was collected in heparinised tubes. Samples of serum and plasma were obtained by centrifuging blood samples at 3000 rpm for 15 min, at 4 °C.

Preparation of the Homogenates

At the end of the treatment, the rats were anesthetized with diethylether and sacrificed; six hours later, the tissues were dissected six hours after the treatment and put in petri dishes. After the tissues had been washed with physiological saline, samples were taken and kept at -78 °C until analysis. 10 g sample rat liver and muscle tissues were cut with a knife. Excess blood, foreign tissue and membranes were removed from the samples. Tissues were suspended in 100 ml of 5 mM phosphate buffer (pH 7.4) containing 458 mM saccharose and homogenized using a mixer at top speed for 3 min by an ultrasonic homogenizer for 40 min, at 12.100 rpm (21,200 x g) for 60 min, and the supernatant was removed. This process was repeated three times with the temperature maintained at 4 °C during the homogenization process.

Measurement of AChE Activity

AChE activity was determined by the method of Ellman and co-workers (*Ellman et al., 1961*), using thiocholine esters as substrates. The reaction mixture in a final volume of 3 ml contained 0.1 ml of 10 mM DTNB reagent [(prepared in 0.005 M Phosphate buffer, pH 7, containing 15 mg of $NaHCO_3$ (10 ml)], 0.1 ml of suitably dilute enzyme and 2.7 ml of buffer (0.05 M Sodium phosphate buffer, pH 8). The blank contained buffered substrate, DTNB and heated enzyme. The mixture was preincubated for 5 min at 37 °C and the reaction was initiated by the addition of 0.1 ml of 3 mM substrate (acetylthiocholiniodure). The mixture was incubated for 10 min and the increase in absorbance was measured at 412 nm.

Protein Determination

Quantitative protein determination was achieved by absorbance measurements at 595 nm according to Bradford method (*Bradford, 1976*), with bovine serum albumin as a standard (Figure 1).

Analysis of Data

All data were expressed as mean ± standard deviation (SD). For statistical analysis, the SPSS/PC+ package (SPSS/PC$^+$, Chicago, IL, USA) was applied. For all parameters, means and SD were calculated according to standard methods. The Mann-Whitney U-Test for differences between means of the treatment and the control rats was employed. The significance level was accepted at $p \leq 0.05$ for all tests.

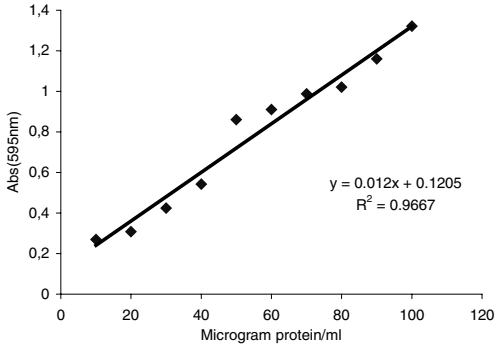

Figure 1. Standard Graph (Bradford Method, Bovine serum albumin, 595 nm).

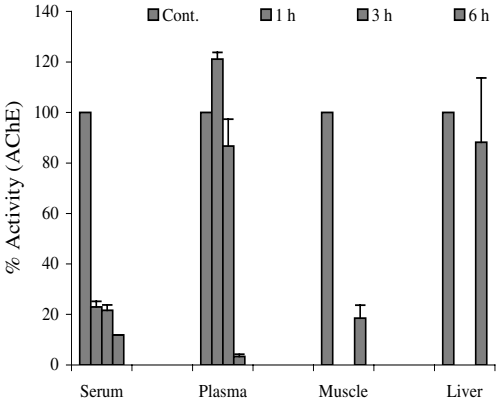

Figure 2. % Activity of AChE in serum, plasma, muscle and liver in first, third and sixth hours of experiment.

Results and Discussion

The aim of the present study was to investigate whether neostigmine methylsulfate could affect AChE activity in rats. Previous researches have shown that the toxicological or biological effects of AChE differ, and that the dose-effect relationship changes with different organisms. The present study indicated that neostigmine methylsulfate possessed an inhibitory effect on AChE activity. The AChE activity in the serum, plasma, muscle and liver of the control rats was determined as 7.80, 22.6, 3.99 and 1.42 Enzyme Units (EU), respectively. It was also found to 1.79±0.172 ($p<0.05$), 1.69±0.160 ($p<0.05$), and 0.93±0.0 ($p<0.05$) in serum, and 27.37±0.60 ($p<0.05$), 19.6±2.40 ($p<0.01$) and 0.76±0.20 ($p<0.05$) in plasma, one, three and six hours after the neostigmine treatment, respectively (Table 1 and Figure 2). The greatest inhibition was found six hours after the injection. AChE activity of muscle and liver tissues was found to be as 0.77±0.209 ($p<0.05$) and 1.25±0.358 ($p<0.01$), respectively six hours after the treatment (Table 1 and Figure 2).

Many chemicals at relatively low dosages affect the metabolism by altering normal enzyme activity, particularly by inhibition of a specific enzyme (*Hochster et al., 1973*). The effect can be dramatic and systematic (*Chiristensen et al., 1982*). Many antibiotics are being used in therapies. However, there are few literature reports related to changes of enzyme activities (*Honjo & Wotanabe, 1984*). A few reports have indicated that some increases and decreases were found in human liver enzyme activity levels such as aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase (*Pickering et al., 1973; Singhvi et al., 1977; Turck et al 1973*).

The effects of sodium ampicillin, magnesium sulfate, and sodium dipyrone on human carbonic anhydrase isozymes have been investigated *in vitro* and from rat erythrocytes in vivo (*Beydemir et al., 2000*). Inhibitory effects of same antibiotics, such as sodium ceftizoxime, sodium ampicillin, sodium cefuroxime, sodium cefazolin, sodium cefoperazone, streptomycin sulphate, gentamicin sulphate, an netilmicin sulphate on glucose-6-phosphate dehydrogenase from human erythrocytes have been investigated (*Ciftci et al., 2000*). In addition, effects of metamizol and magnesium sulfate on enzyme activity of glucose-6-phosphate dehydrogenase from human erythrocytes *in vitro* and rat erythrocytes *in vivo* have been investigated (*Ciftci et al., 2001*).

The effects of handling and light on the levels of AChE in the preweaning rat brain have been

Table 1. The total protein, % activity and mean ± standard deviation shown results (In vivo effects of Neostigmine methylsulfate on rat serum, plasma, muscle and liver AChE enzyme activity).

	Total protein (mg/ml)	% Activity 1 h	3 h	6 h	X±SD 1 h	3 h	6 h	p
Control Serum	1.57	100	100	100	7.80	7.80	7.80	-
(Neostigmine Methylsulfate) Serum	0.94	22.95	21.67	11.92	1.79±0.172	1.69±0.160	0.93±0.0	P<0.05
Control Plasma	0.95	100	100	100	22.60	22.60	22.6	-
(Neostigmine Methylsulfate) Plasma	0.87	121.11	86.72	3.41	27.37±0.60	19.60±2.40	0.77±0.20	P<0.05
Control Muscle	1.15	100	100	100	-	-	3.99	-
(Neostigmine Methylsulfate) Muscle	1.06	-	-	18.54	-	-	0.76±0.21	P<0.05
Control Liver	0.95	100	100	100	-	-	1.42	-
(Neostigmine Methylsulfate) Liver	0.68	-	-	88.26	-	-	1.25±0.36	P<0.05

examined. In the normal rat, enzyme activity increased between tenth and twentieth day of life in the five brain areas studied. Handling during the first 10 days of life resulted in suppression and an augmentation of the developmental increase in enzyme activity in the neocortex and in the caudate nucleus, respectively (*Kling et al., 1965*). Chronic pyridostigmine administration at doses that reduces blood AChE by 50 % does not penetrate the blood brain barrier in chronically stressed rats or affect a test of working memory in rats (*Kant et al., 2001*). The influences of 2,5-Hexanedione have been investigated on rat brain *in vivo* (*Pareira et al., 2004*). It has been shown that the globular form of AChE, predominant in the mammalian brain and muscle, is a glycoprotein (*Rakonczay et al., 1981*). The purification of a soluble AChE from Japanese quail brain using affinity chromatography on concanavalin A-sepharose and edrophonium-sepharose has been described (*Son et al., 2002*). In addition effects of three pesticides from different chemical families have been demonstrated (carbamate, phenylurea, sulfonylurea) on AChE activity in goldfish *in vivo* (*Bretaud et al., 2000*). The results suggest that phenylmethylsulfonyl fluoride inhibition of AChE is a consequence of a selective inhibition of membrane-associated forms and that the apparent brain selectivity is related to the greater fraction of membrane-associated AChE in brain (*Skau & Shifley, 1999*).

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