Original Article

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Can Salivary Acetylcholinesterase be a Diagnostic Biomarker for Alzheimer?

Dentistry Section

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ABSTRACT

Introduction: The loss of brain cholinergic activity is a key phenomenon in the biochemistry of Alzheimer's Disease (AD). Due to the specific biosynthesis of Acetylcholinesterase (AChE) of cholinergic neurons, the enzyme has been proposed as a potential biochemical marker of cholinergic activity. AChE is expressed not only in the Central Nervous System (CNS), Peripheral Nervous System (PNS) and muscles, but also on the surface of blood cells and saliva.

Aim: This study aimed to measure salivary AChE activity in AD and to determine the feasibility of creating a simple laboratory test for diagnosing such patients.

Materials and Methods: In this cross-sectional study, the recorded data were obtained from 15 Alzheimer's patients on memantine therapy and 15 healthy subjects. Unstimulated whole saliva samples were collected from the participants and salivary levels of AChE activity were determined by using the Ellman colorimetric method. The Mann Whitney U test was used to compare the average (median) of AChE activity between AD and controls. In order to adjust for possible confounding factors, partial correlation coefficient and multivariate linear regressions were used.

Results: Although the average of AChE activity in the saliva of people with AD was lower compared to the control group, we found no statistically significant differences using Mann Whitney U test (138 in control group vs. 175 in Alzheimer's patients, p value=0.25). Additionally, no significant differences were observed in the activity of this enzyme in both sexes or with increased age or duration of the disease. After adjusting for age and gender, there was no association between AChE activity and AD (regression coefficient β=0.08; p value= 0.67).

Conclusion: Saliva AChE activity was not significantly associated with AD. This study might help in introduce a new diagnostic aid for AD or monitor patients with AD.

Keywords: Cholinesterase, Dementia, Exploring, Fluid biomarker

INTRODUCTION

AD is a progressive neurodegenerative disorder that is characterized by the irreversible loss of memory and cognitive decline, and is associated with personality changes. [1] Aging is a major risk factor for the disease [2]. It is accounted that the prevalence is nearly 1.5% at age 65 years and doubles every four years to reach about 30% at age 80 years [3]. With the worldwide increase in life expectancy, AD has grown to be a socio economic and medical problem that is rapidly expanding [4], and with an aging global population has become a critical challenge in the field of public health [5]. AD severely affects the quality of life for millions of people and causes disastrous situations not only for patients but also for their families and the community [1].

Diagnosis takes time and requires a combination of clinical assessment, psychological tests, imaging and possibility of other neurological disorders to be eliminated [4]. AD can only be diagnosed with certainly through autopsy [6]. In fact the absence of tests for early diagnosis of AD has been considered as one of the main obstacle to the development of new treatments [7].

The chief neurochemical disorder in AD is the cholinergic defects in the CNS [2]. Cholinergic neurons are the most important group of neurons that are destroyed in the early stages of AD, leading to a significant reduction in the levels of Acetylcholine (Ach) [8]. A decrease in cholinergic activity is a key phenomenon in Alzheimer's neurochemistry. AChE is expressed not only in the CNS, PNS and muscles, but also on the surface of blood cells and saliva [1].

So far, most studies associated with biomarkers for AD have used Cerebro Spinal Fluid (CSF) samples obtained through lumbar puncture [9]. This is a specialized invasive procedure that has potential risks and is uncomfortable for patients. It is therefore, necessary to obtain their explicit consent before the procedure [8]. This method is not appropriate for screening the old people with risk factors for Alzheimer's or little cognitive defect [10]. Stress response and lumbar puncture lead to increased secretion of cortisol, which may be associated with any observed changes in biomarkers [11], As a result, the use of a body fluid with minimal discomfort for the patient can be beneficial [8].

Saliva is produced from salivary glands and a biological fluid that is easily obtained. Drug levels in saliva may also be indicative of changes in CSF [12-14]. Recent studies indicate a relation between AChE activity levels in saliva and AD [4]. These findings confirm practicality of markers of central cholinergic activity, which is a key process in Alzheimer's biochemistry.

In this context, the aim of the present study was to measure AChE activity in saliva and its relationship with AD in patients at Imam Hossein Hospital in Tehran, Iran and individuals who did not have Alzheimer's, to determine the feasibility of creating a simple laboratory test for diagnosing patients and monitoring of identified patients.

MATERIALS AND METHODS

Study Design: This cross-sectional, analytical study was performed on 15 patients diagnosed with AD who were referred to the neurology ward of Imam Hossein Hospital and were on memantine therapy. Memantine is a drug that is used to treat the cognitive problems of Alzheimer's patients, but it isn't an AChE inhibitor [15]. Thus, any possible effects of enzyme inhibitors on the activity of AChE enzyme in saliva were prevented. The control group was randomly selected from 15 elderly non demented subjects without neurological or cognitive disease.

According to statistical sample size determination a minimum sample of 11 was required in each group, however 15 individuals were included to compensate for attrition.

Demographic information including age, sex and duration of disease was obtained by interviews.

The committee of medical ethics of Shahid Beheshti University of Medical Sciences approved the study procedure. The study was done

between 2014 and 2015 year at the Department of Oral Medicine and Neurology, Shahid Beheshti University of Medical Sciences and Imam Hossein Hospital, Tehran, Iran. Informed consent was given by their caregivers before the onset of the study.

Salivary Collection: Subjects were advised to avoid from eating, drinking or smoking one hour prior to sampling. They were then asked to rinse their mouth with water. After five minutes, 2 ml of whole unstimulated saliva samples were collected from the subjects in the sitting and relaxing position by spitting into 15 ml Falcon tubes. All samples were collected between the hours of 9 am and 12 am [16].Saliva samples were immediately transferred to the laboratory inside a chamber containing ice and were kept at the freezing temperature of -70°C.

On the day of testing, saliva samples were centrifuged for 10 minutes at 3000 rpm (Behdad, made in Iran) to separate any possible debris and squamous cells. The AChE activity levels in saliva were then determined using the Ellman colorimetric method [17]. About 50 µl from each sample was added to a buffer solution containing 0.1 mM dithionitrobenzene (Merck, made in Germany), 60 µg acetylthiocholine (Sigma, made in USA), and 10 mM phosphate buffer pH=8 at the total volume of 110 µl. The mixture was incubated for 15 minutes at 37°C [8].

The AChE in the reaction mixture broke into thiocholine and acetate due to the activity of the enzyme AChE. The obtained thiocholine reacted with the dithionitrobenzene producing 5-thio-2-nitro-benzoic acid anion, which produces a yellow color [14]. Catalytic activity levels were measured in the wavelength 412 nm by photometry using Cobas Mira auto analyzer (Roche, made in USA).

The protein content of saliva samples was measured by using the Bradford method, which is among the most accurate colorimetric methods [18]. A 25 µl from each sample was added to 250 ml Bradford containing 5% ethanol 97°, 0.01% Coomassie Brilliant Blue G-250 (Merck, made in Germany), 10% phosphoric acid 85%, glycerin and distilled water. As a result, the Coomassie Brilliant Blue G-250 dye binding to proteins caused maximum absorption to change from 465 nm (red color) to 595 nm (blue color) [19]. Finally, the degree of AChE enzyme activity in saliva samples was normalized with the protein content of the samples. Enzyme activity of saliva samples from AD and the non-Alzheimer's group was compared and concluded.

STATISTICAL ANALYSIS

Data analysis was performed using SPSS version 18.0. The p-value<0.05 was considered as level of significance. We used Mann Whitney U test to compare the average (median) of AChE activity between AD and controls. In order to adjust for possible confounding factors, partial correlation coefficient and multivariate linear regressions were used.

RESULTS

In this study, 15 patients with AD 9 (56.2%) males and 6 (42.9%) females aged 64 to 90 (average age 78.4 years) and 15 individuals who did not have AD 7 (43.8%) males and 8 (57.1%) females; aged 61 to 85 (average age 71 years) were tested. Mean duration of AD was 2.23±1.23 years and the average duration of disease was found to be longer in men than women.

According to the results of Mann Whitney test the overall salivary AchE in Alzheimer's patients was lower than that of the control group which clinically may be important (138 in control group vs. 175 in Alzheimer patients) but we found no significant differences in the enzyme activity between two groups; p value=0.25 [Table/Fig-1]. Enzyme activity was generally lower in males than in females. To evaluate the association between enzyme activity and age with respect to the variables of sex and disease, adjusted correlation coefficients were used and a positive, weak and no significant relationship was observed; partial correlation coefficient= -0.041; p-value=0.837 [Table/Fig-2].

There were no significant relationship between duration of AD and enzyme activity with respect to the age and sex; partial correlation coefficient = 0.192; p value=0.531 [Table/Fig-3].

In evaluation of enzyme activity with respect to the variables of disease, age and sex, no significant relationship was observed using multivariate linear regression models; regression coefficient β disease=0.256, β age=-0.044, β sex=0.033; p-value>0.05 [Table/Fig-4].

However, according to the average values (mean and median), the difference between the patient and healthy groups may be important in clinical terms.

DISCUSSION

Cholinergic dysfunction is a key biochemical event that is associated with the progression of AD. Currently, this is the target of most existing treatment plans [7]. The purpose of this study was to survey the activity of AChE in the saliva of patients with AD. A total of 30 eligible persons were selected, 15 patients with AD and 15 healthy subjects. After taking saliva samples from subjects, the AChE activity was evaluated by using Ellman colorimetric method and the enzyme activity of each sample was normalized with its protein content. This method had previously been used by other researchers such as Sayer R et al., and Boston PF et al., [8,20].

Based on the current study results, the reduction in salivary AChE enzyme activity in patients was not significant compared to the control group.

Moreover, there was no significant difference in AChE activity levels in saliva by age or gender, and enzyme activity did not change significantly with the duration of the disease. The results of the present study are similar to the Boston PF et al., study [20]. Although in some studies, salivary tau species has been proposed as diagnostic biomarker specially in the early stage of AD [13,14].

Boston PF et al., studied saliva AChE activity in three groups of 15 subjects with AD, 13 subjects with vascular dementia and 13 healthy subjects matched for age as control group. No significant

Standardized Enzyme Activity Groups	N	Mean Rank	Sum of Ranks	p-value*
Alzheimer	15	13.70	205.50	0.25
Healthy	15	17.30	259.50	

[Table/Fig-1]: Comparison of enzyme activity in Alzheimer's disease and healthy groups.
*Mann Whitney U Test

Control Variable	Correlations	p*	r
Sex and groups (Alzheimer's disease and healthy)	Acetylcholinesterase and age	0.20	-0.24
	Standardized enzyme activity and age	0.837	-0.041

[Table/Fig-2]: Correlations of enzyme activity and age according to sex and disease.
*Pearson partial correlation coefficient

Control Variable	Correlations	p*	r
Age and Sex	Standardized enzyme activity and duration of Alzheimer's disease	0.531	0.192

[Table/Fig-3]: Correlations of duration of disease and enzyme activity according to the age and sex.
*Pearson partial correlation coefficient

Model		Beta	t	p-value*
1	Constant		0.233	0.817
	Age	-0.044	-0.207	0.837
	Sex	0.033	0.172	0.864
	Alzheimer's disease and Healthy	0.256	1.203	0.240

[Table/Fig-4]: Association of standardized enzyme activity and Alzheimer's disease adjusted for age and sex.
*Multivariate linear regressions

difference in enzyme activity was observed between the diagnostic groups. This means that the rate of changes in enzyme activity was not significant with respect to Mini Mental Examination Test (MMSE) scores. In addition, gender did not cause significant changes in enzyme activity, which is similar to the present study [20].

Sayer S et al., found the relationship between salivary AChE and AD and the response to cholinesterase inhibitors. In their study, the patients with AD were treated with donepezil at a rate of 5 mg per day and cognitive assessment tests were performed on them [8]. Saliva samples were taken after classification of the subjects based on their response to therapy and also forming a control group. The AChE enzyme activity was determined by using the Ellman colorimetric method [17]. The protein content of the samples was also measured and enzyme activity was normalized with the protein content of the samples. In this study, a significant reduction in the catalytic activity of enzymes associated with age was observed in the control group. Additionally, the enzyme activity in Alzheimer's patients who did not respond to treatment was significantly less than the control group matched for age. There was no difference between males and females in each group and changes in people with AD was not associated with age. There were also significant differences in enzyme activity between people who responded to treatment with AChE inhibitors and those who did not. The difference between the results of this study in comparison with our study could be due to differences in the study design. In this study, patients were treated with salivary AChE inhibitors and were classified based on whether they responded to treatment with inhibitors or not. Although, the inhibitor had been isolated from the saliva before sampling, it is possible that treatment with an inhibitor created the long term adaptive changes in the production of AchE. Alzheimer's patients who participated in our study were on drug memantine, which is not an AChE inhibitor.

Overall, enzyme activity was lower in men than women in the present study, although this difference was not statistically significant.

Thus, it appears that although the enzyme activity in patients with AD was lower than the control group, the opinion of experts in this field is required in order to determine whether these differences are clinically important and can be used for diagnosis of AD, even if they are not statistically significant. In order to achieve more reliable results, it seems beneficial to design a study in which subjects are categorized according to the severity of their disease, so that enzyme activity in each group can be investigated separately.

In the present study, cooperation of Alzheimer's patients was poor due to physical and mental disabilities. In future studies it is suggested to determine possible variables affecting the enzyme activity.

In addition, disease severity should be classified into mild, moderate and severe and the enzyme activity examined separately in each group.

CONCLUSION

We found that AChE activity levels in saliva vary in individuals with AD in comparison to healthy subjects. Considering confounding variables such as age and gender, the difference is not statistically significant, although it may be clinically important. Consequently, due to a lack of studies in this field, we cannot present a conclusion based on the evidence for the use of salivary AchE as a biomarker for diagnosing AD, moreover, AchE activity in the saliva of males and females does not change significantly with the duration of illness and age.

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