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Antioxidant Status, Lipid Peroxidation Products and Cystatin C as Potential Clinical Markers of Alzheimer's Disease in Systemic Circulation

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Abstract: Growing data suggest oxidative stress contributes to the pathogenesis of Alzheimer's disease (AD). The objective of this study was to assess the value of antioxidant potential (AOP), antioxidant status (AOS) and thiobarbituric acid reactive substances (TBARS) and the cysteine protease inhibitor Cystatin C that plays a role in the processing of amyloid _ as clinical markers of AD in peripheral circulation and to find out the agreement limits of AOP and AOS as clinical laboratory tests. To do this, 21 severely demented patients fitting the DSM-IV diagnostic criteria for Alzheimer's type dementia as well as through minimental state examination were compared to 20 age-matching healthy controls with respect to their serum AOP, AOS, Cystatin C and TBARS levels. The under-curve area detected from ROC curves for TBARS, AOS, AOP and Cystatin C were 0.801, 0.622, 0.475 and 0.65 respectively. Altman and Bland analysis showed that the values obtained by the AOP assay were 0.0149-0.04664 with a mean of 0.03081 units higher than those values obtained via AOS assay, indicating a low level of agreement. In addition, Pearson's correlation testing showed a significant negative correlation between AOP and AOS ($r=0.479$, $p<0.01$). As a conclusion, among the tests investigated in this study, TBARS may be used as a dependable peripheral marker of AD with a sensitivity of 0.85 and a specificity of 0.6 at the cut-off point of 0.77 nmol/ml. AOS should be preferred to AOP as a marker with a specificity and sensitivity of 0.7 at the cut-off point of 0.011nmol/ml. Further studies may be undertaken to reveal the pathogenetic relation of oxidant stress and the processing of amyloid β.

Key Words: Alzheimer's disease, serum oxidative stress marker, Cystatin C

Introduction

Oxidative stress refers to the cytopathologic consequences of a mismatch between the production of free radicals and the ability of a cell to defend against them (1). Growing data from experimental models and human brain studies suggest that oxidative stress may play an important role in neuronal degeneration in diseases such as Alzheimer's disease (AD)(2). Mitochondrial oxidative metabolism, nitric oxide, phospholipid metabolism and proteolytic pathways are potential sources of intracellular free radicals. Alterations in free radical defence systems may also contribute to oxidative stress. A net increase in reactive oxygen species can produce damage to lipids, proteins and DNA, and induce necrosis or apoptosis.

One of the neuropathologic hallmarks of AD is the deposition of extracellular aggregates of β-amyloid peptides (Aβ). The finding that Aβ can be toxic to neurons in vitro has led to the hypothesis that neuronal degeneration in AD is due to Aβ toxicity (3). In vitro evidence suggests that oxidative stress may contribute to Aβ toxicity. Increases in H_2O_2 were detected in cells following exposure to Aβ, and both vitamin E and catalase prevented cell death (4,5). Cystatin C deposition in AD has been reported as a secondary contributory event in the pathogenesis of the disease rather than an independent risk factor (6).

Oxidative stress may contribute to the aggregation of soluble Ab into insoluble plaques. Soluble Aβ has been found to aggregate in vitro by the addition of metal catalyzed oxidation systems (7).

Levels of oxidative cellular damage are higher in the brains of individuals with AD. Elevated products of lipid peroxidation occur in vulnerable cortical regions in brains with AD, and neurofibrillary tangle bearing neurons have enhanced immunoreactivity for malondialdehyde (MDA), the biochemical marker of lipid peroxidation (8). To date, it is not clear if this free radical generation is secondary to other initiating causes, though they are deleterious and

form a part of the cascade of events that can lead to neuron death suggesting that therapeutic efforts aimed at the removal of reactive oxygen species (ROS) or the prevention of their formation may be beneficial in AD.

The level of systematic oxidant stress has been questioned by several investigators in the literature (9-12) and different results have been reported. It has also been suggested that a decrease in blood brain barrier function is possible in dementia disorders (13). Cystatin C has been found to be enriched in the cerebrospinal fluid (CSF) of patients with AD and a decrease in blood brain barrier function in dementia disorders has been suggested as a possibility (14). In the light of these findings, we hypothesized that serum levels of Cystatin C may vary in relation to AD detected clinically.

The parameters of antioxidant potential (AOP) and antioxidant status (AOS) are used to assess antioxidants, but they do not give any information about the enzymatic and non-enzymatic constituents of the total balance. Circulatory thiobarbituric acid reactive substances (TBARS) give information on the level of lipid peroxidation that is the product of oxidative stress which could not be counterbalanced by antioxidants originating from other tissue in the body.

In this study, we aimed to test if AOP, AOS and TBARS determination in the systemic circulation of patients with AD could be used as a clinical index for diagnosis as well as comparing the statistical agreement of AOP and AOS assays.

Materials and Methods

Twenty one severely demented patients with a mean age of 81 (min: 71, max: 96) comprising 15 females and 6 males with a mean Mini Mental State Examination (MMSE) score of 9/30 (min: 3, max: 12) were included in the study, most of whom had been referred to the geropsychiatry unit due to their behavioural disturbances. Dementia diagnoses were applied through interviews with caregivers and other informants such as social workers and psychologists. The diagnosis of dementia was strengthened by applying DSM IV diagnostic criteria for dementia of Alzheimer's type and giving the patients an MMSE (15). Types of dementia other than AD were excluded by the presence of cerebrovascular accident history and stepwise deterioration.

Cranial Computerized Tomographies (CT) were performed primarily to rule out silent infarcts and to detect cortical atrophy and ventricular enlargement. Other causes of dementia such as Pick's disease were excluded by the presence of frontotemporal atrophy and early cognitive impairment in the conservation of personality at an early stage. Dementia of Huntington's disease and Parkinson's disease types were excluded by the presence of motor abnormalities. Other types of dementia were excluded via medical history, such as the presence of alcohol use, other metabolic and infectious disorders or a history of head injury.

Consent was obtained, and the study was conducted in accordance with the Helsinki Declaration. Blood was obtained in accordance with NCCLS recommendations. Sera was seperated from whole blood and stored at –80ºC until the assays were performed. Blood from 20 healthy age matching controls was simultaneously collected.

Biochemical Assays

Antioxidant defence potential was assessed as described previously (16). In brief, the reaction medium enriched with fish oil samples were exposed to superoxide radical produced by the xanthine/xanthine oxidase system for one hour and then malondialdehyde levels were measured as previously described. By using this reaction system, it is suggested that it is possible to obtain more precise information on the antioxidant potentials of the samples. The antioxidant potential value was assessed from the differences between MDA levels in blanks and samples, which was proportional to the AOP values of the samples. Results were expressed as nmol $ml^{-1}h^{-1}$. The analysis scheme is given in Table 1.

Total antioxidant status was assessed by the kit from Randox (Randox Laboratories Ltd., Antrim, UK) on a Technicon RA-XT autoanalyzer (Bayer, Germany). The assay principle depends on the incubation of 2,2' –Azino-di-(3-ethylbenzthiazoline sulphonate) (ABTS) with peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation $ABTS^{*+}$. This has a relatively stable blue colour, which is measured at 600 nm. Antioxidants in the added sample cause the suppression of this colour production to a degree which is proportional to their concentration (17).

TBARS was measured as described previously (18).

Table 1. Analysis scheme of the antioxidant defense potential (AOP) measurement.

	Sample (ml)	Blank (ml)
Sample	0.2	-
Fish Oil*	0.01	0.01
Xanthine (10mM)	0.1	0.1
Xanthine Oxidase**	0.1	0.1
	Incubation for 1 hr at room temperature (25oC)	
Sample	-	0.2
	MDA measurement	

* Prepared by dissolving 1 ml fish oil (with total lipid content of 0.706 g/ml) in 2 ml acetone

** Prepared by diluting Xanthine Oxidase (XO) (Sigma X-4500, 2.9 mg protein/ml, 1.6 U/mg protein) 40-fold in $(NH_4)_2SO_4$ solution of 2M. Final amount of XO in the assay medium was 0.012U

Cystatin C is a serum protein that is known to be elevated in patients with deteriorated kidney functions (19). To rule out the presence of kidney function deterioration, and the dependent increase in serum Cystatin C levels, serum creatinine assays were done to each patient prior to inclusion in the study group. Two patients and 1 control were excluded from the study due to high serum creatinine levels. Serum creatinine assays were done by the Jaffe method (Beckman reagent, CA) on a CX7 autoanalyzer (Beckman, CA). The red colour complex formed on mixing samples with alkaline picrate reagent is monitored bichromatically by detectors at 520 and 560 nm. The observed rate of complex formation is directly proportional to the concentration of creatinine in the sample. Serum Cystatin C assay, were made by the latex particle enhanced turbidimetric (pet) immunoassay (DAKO, CA) on the CX7 analyzer (Beckman, CA). The DAKO Cystatin C pet kit contains polystyrene particles of uniform size, chemically coupled with rabbit antibody against human Cystatin C. A reaction between these immunoparticles and Cystatin C in a patient's specimen results in the formation of agglutinates and a concomittant change in the absorbance signal. The Cystatin C concentration of the patient's specimen is determined by interpolation on a calibration curve.

Statistics

Differences between the means was calculated by Student's t-test. ROC curves and Pearson's correlation analysis were assessed using SPSS 9.0 for Windows. Finally, the agreement between AOP and AOS was determined by the Blant and Altman method (20).

Student's t-test was applied to test the difference between the means. Furthermore, a ROC curve was generated to suggest a best cut-off point to discern patients with Alzheimer's disease from age matching controls considering the Cystatin C levels with best sensitivity and specificity.

Results

The comparison of the means and standard deviations of serum antioxidant potential, total antioxidant status and TBARS are shown in Figure 1 a-c. Accordingly, serum TBARS levels of AD patients were significantly higher than the controls ($p<0.01$). To test the value of TBARS as well as AOS and AOP as clinical laboratory markers of AD, we outlined the ROC curves in Figure 2. The demonstrated area under the curves, cut-off points, the sensitivity and the specificity of TBARS, AOP and AOS tests to detect AD are shown in Table 2. This study also demonstrated that there is a significant negative correlation between serum antioxidant potential and serum total antioxidant (Figure 3a). The agreement limits calculated by the Altman and Bland test is shown in Figure 3b. Accordingly, the agreement limits are between –0.04664 and –0.01497 with a mean of –0.03081 units. Thus, AOS gives better results than AOP at all times.

The means and standard deviations for both creatinine and Cystatin C are given in Table 3. The reference range suggested for Cystatin C for patients older than 50 years is 0.74-1.55 mg/L, whereas the reference range for creatinine was 0.6-1.3 mg/L. Accordingly, the difference between the means of the control group and the AD group was insignificant ($p=0.58$) for creatinine levels. This finding confirms that any change in the Cystatin C levels of the AD group would not be due to kidney dysfunction. However, there was a difference between the means of the AD group and the control group for serum Cystatin C levels ($p=0.05$). As shown in Figure 4, the area under the ROC curve for Cystatin C for AD patient determination was 65%. The sensitivity and 1-Specificity values for suggested cut-off points are shown in Table 4. Accordingly, with a cut off-point of 1.275 mg/L, AD was differentiated by Cystatin C levels with a sensitivity of 0.526 and a specificity of 0.895.

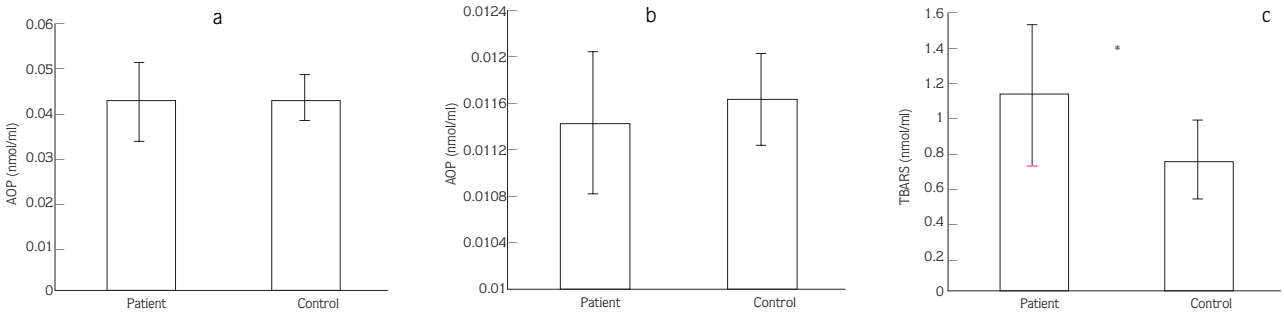

Figure 1. Circulatory levels of a. AOP, b. AOS and c. TBARS in Alzheimer's patients and control groups.

Table 2. The Cut-off Values, Sencitivity, Spesificity and Area Under the ROC Curve for TBARS, AOP and AOS in Alzheimer's Disease.

	Cut-off	Sensitivity	Spesificity	Area Under the Curve
TBARS	0.7705 nmol/ml	0.857	0.600	0.801
AOP	0.0443 nmol/ml	0.524	0.500	0.475
AOS	0.0115 nmol/ml	0.700	0.700	0.622

Table 3. Comparison of Creatinine and cystatin-C Levels in AD Patients versus Controls.

		Mean±Standard Deviation	p
CREATININE (mg/dl)	AD	0.810 ± 0.323	0.58
	Control	0.858 ± 0.186	
CYSTATIN-C (mg/L)	AD	1.227 ± 0.521	0.05
	Control	0.958 ± 0.279	

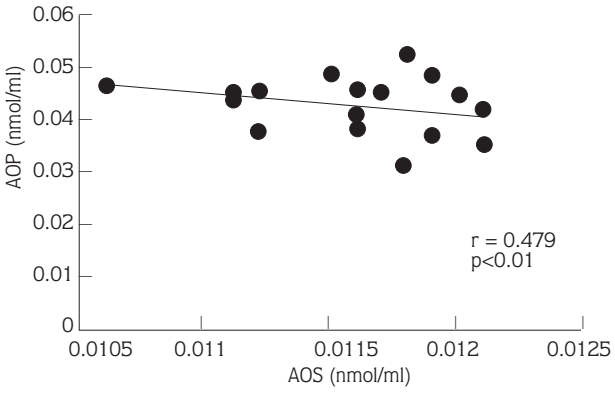

Figure 3a. Correlation analysis between AOP and AOS.

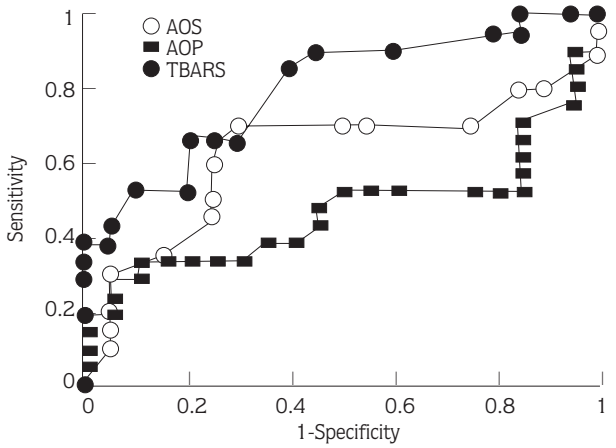

Figure 2. ROC curve for detection of sensitivity and specificity in tests of AOS, AOP and TBARS in Alzheimer's patients detected clinically as described in the text.

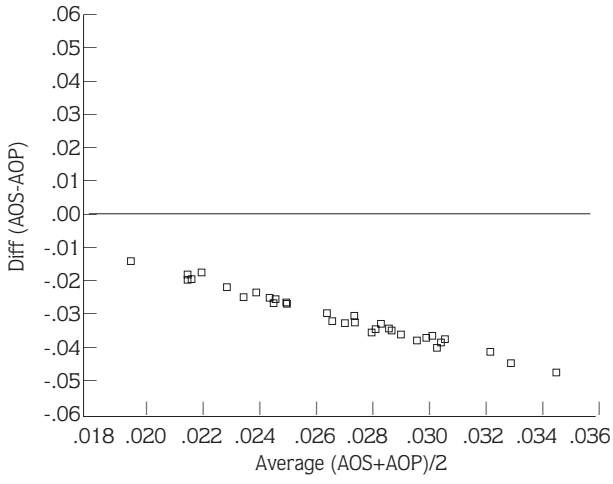

Figure 3b. Differences against mean for antioxidant status (potential).

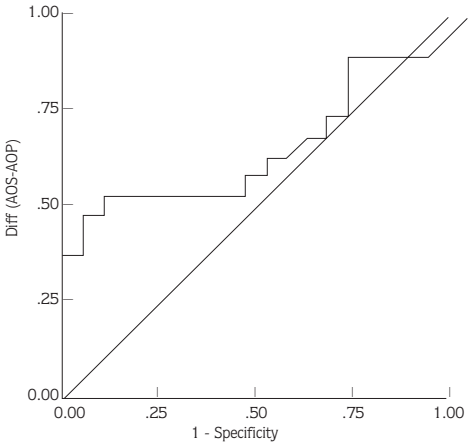

Figure 4. ROC curve for Cystatin C.

Table 4. Sensitivity and Specificities Attained with Suggested Cut-off Values for Cystatin-C.

Cut-off value (mg/L)	Sensitivity	Specificity
0.520	0.895	0.053
0.745	0.789	0.263
0.950	0.579	0.474
1.275	0.526	0.895
1.540	0.316	1
2.180	0.053	1
3.330	0	1

Discussion

Several investigators have determined the presence of increased lipid peroxidation products in the brain homogenates of patients with AD (21,22). However, to our knowledge, there are no studies investigating the value of serum AOS, AOP and TBARS as a laboratory test to diagnose AD. This study demonstrated that the peripheral levels of TBARS is a good indicator to differentiate AD. However, it should be noted that the patients included in this study were all severely demented. It has been suggested that at severe stages of dementia there may be a dysfunction of the blood-brain barrier (BBB), and the apparent increase in serum TBARS levels may be attributed to increased membrane peroxidation in the central nervous system detected peripherally due to a loss of BBB. With a cut off-point of 1.275 mg/L, AD could be differentiated by Cystatin C levels with a sensitivity of 0.526 and a specificity of 0.895. It is possible to plan larger studies to compare the Cystatin C test with other biochemical markers like Apo E, CSF tau (23), neuropeptides (24), diagnostic investigations like MRI, SPECT, PET, fMRI and MRS (25) and psychological tests like MMSE. With these results, we believe it is worthwhile to undertake further research that might include correlations of serum Cystatin C levels with CSF levels in early and late stages of dementia of an AD type supported by post-mortem immunopathology to decipher the mechanism of appearance of Cystatin C in peripheral circulation. Studies to be planned on larger groups will be informative either to support the cut-off points suggested in this study or to attain a better clinical utilization of Cystatin C to detect AD.

The other parameters tested as candidates for peripheral AD markers were AOS and AOP neither of which displayed a difference in means when compared to the control group. Previously, Sinclair et al. (26) have also investigated the difference in total antioxidant capacity (TAC) in the plasma of patients with AD and normal controls. Even though there was no difference in TAC, they observed lower vitamin E levels but similar vitamin C and β-carotene levels in AD patients when compared to controls. Riviera et al. (27), on the other hand, demonstrated decreased vitamin C and stable vitamin E levels in AD patients. The peripheral enzymatic antioxidant defence system is a manifestation of tissue degradation as these enzymes are intracellular. Thus, the AOP or AOS activity consists mostly of non-enzymatic anti-oxidant defense systems. Even though our study was also unable to prove any significant change in AOP or AOS, we believe it is worthwhile to further investigate the constituents of AOP or AOS to be able to suggest dietary interventions for the prevention of AD. The ROC curves showed that AOP and AOS are not as good indicators as TBARS for the differentiation of AD. In other words, these results imply that the systemic levels of antioxidants is not a dependable marker to reflect the buffering activity of neural antioxidant stress.

This study also provided evidence that, from a laboratory science point of view AOP and AOS tests have a low level of agreement. AOP is always higher than AOS, and they have significant negative correlation. This can be explained by the fact that these two assays are measuring chemically different substances, even though they were

both suggested to be measuring the total antioxidant capacity in the sample.

Studies to follow may target the utility of TBARS as a differentiator of AD from other etiologies of dementia as well as linking the severity of memory loss with TBARS levels in circulation. The response of AD patients at different stages to different therapeutic modalities may further illuminate the pathophysiologic cascades involving lipid peroxide generation.

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