



ANTIOXIDANT CAPACITY IN THE LIPOPHILIC FRACTION OF ALZHEIMER'S BRAIN TISSUES

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ABSTRACT

The aim of this study was to investigate the antioxidant capacity (AC) in the lipophilic fraction of post-mortem motorcortex (MC), nucleus caudatus (NC) and gyrus temporalis (GT) from controls (C) and Alzheimer's disease (AD) patients. The initial samples consisted of 50 human brain tissues of AD and C. AC of the different region of human brain were measured by using the fluorescent method of the oxygen radical absorbance capacity (ORAC). Peroxyl and hydroxyl radical generators were used in the analysis. All ORAC analysis were carried out on the Perkin-Elmer spectrofluorometer LS 55 with fluorescent filters, Ex: 485 nm; Em: 520 nm. Final results were calculated using the differences between area under the quenching curve of fluorescein (FL), blank and analyzed biological samples. AC against peroxyl radicals (ORAC-ROO[•]) of lipophilic fraction in MC of AD was statistically significantly lower in comparison with MC of C ($p < 0,008$). No changes in the AC against hydroxyl radicals (ORAC-[•]OH) of lipophilic fraction of AD were found in comparison with C. Reduction of total protein in GT of AD ($p < 0,03$) was found. The results showed that in the MC of AD brain the balance between production of free radicals and the neutralization by a complex antioxidant system is disturbed. The manual fluorescent method for AC measurements proved to be sufficiently appropriate and sensitive for the AC measurements of lipophilic fraction of postmortem brain tissues from different pathological conditions.

KEY WORDS: antioxidant capacity, oxygen radical absorbance capacity (ORAC) assay, peroxyl radical, hydroxyl radical, oxidative stress, Alzheimer's disease.

INTRODUCTION

Damage and progressive cell death that occurs in neurodegenerative disorders such as Alzheimer's disease (AD) has been associated with activities of different free radicals (FR). However, there is growing evidence for a cascade of multiple deleterious factors, including oxidative stress, lipid peroxidation and altered iron metabolism leading to excess FR production, mitochondrial dysfunction and disturbed calcium homeostasis, which in turn result in cytoskeletal damage and cell death in AD. Oxidative stressors induce an imbalance between oxidants and antioxidants in favor of the former leading to the oxidative damage of the molecules like deoxyribonucleic acid, lipids and proteins (1, 2). Furthermore, brain aging in AD patients is accompanied by the alteration of several neurotransmitter systems with a pronounced deficit in the cholinergic system and abnormal accumulation of amyloid- β peptides and hyperphosphorylated tau. Nucleus basalis Maynert, hippocampus and cortex are mostly affected area in AD brain. The present study was aimed to investigate the AC in the lipophilic fraction of different brain regions, by means of ORAC method involving two different FR generators (2, 3, 4, 5, 6, 7, 8), modified by Sofic et al. (9). ORAC method has an advantage over other assays, because this method utilizes an area-under-curve technique and thus combines both inhibition time and inhibition degree of FR action by an antioxidant into a single quantity. Fluorescein (FL) was used as a target of FR attack, with 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH) as a peroxy radical (ROO°) generator, and hydrogen peroxide with cupric sulfate penta hydrate as an hydroxyl radical (OH°) generator. ROO° is a common FR found in the body and used in the antioxidant activity assays (3,4,5). It is slightly less reactive than OH° and thus possesses an "extended" half-life of seconds instead of nanoseconds. In original developed automated method beta - phycoerythrin (β -PE), a protein from *Porphyridium cruentum*, was used as a target of FR attack (2,3,4,5,6,7,8). An improved ORAC method has been developed and validated using FL as the photo sensor. Ou et al., (10) demonstrated that FL is superior to β -PE. In clinical studies where analysis of antioxidant status is important, ORAC method have been used to evaluate the hydrophilic and lipophilic antioxidants in postmortem brain tissue.

MATERIALS AND METHODS

Chemicals

Fluorescein, Standard Fluka for fluoresceine – free

acid was obtained from Fluka Chemie GmbH, Steinheim, Germany. 2,2'-azobis (2-amidino-propane) dihydrochloride, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma Aldrich Chemie GmbH Germany. N-hexane was obtained from Merck (Germany). Cupric sulphate penta hydrate and hydrogen peroxide were obtained from Kemika, Zagreb, Croatia.

Patients and Controls

The initial sample of total 50 subjects consisted of 26 controls (C) brains and 24 Alzheimer's disease (AD) brains. Motorcortex (MC), gyrus temporalis (GT) and nucleus caudatus (NC) were collected and stored at -80°C until analyzed. Brains were matched for age (C $76,6 \pm 10,1$ years; AD $84,7 \pm 7,7$) and postmortem time (C 27 ± 18 hours; AD $36,3 \pm 24,6$). Control brains did not show any abnormal histopathological changes. The death of control subjects was mainly caused by cardiac and pulmonary arrest and by different tumours. Neuropathological diagnosis was based on histological examination of characteristic Alzheimer's degeneration, the number of senile plaques and neurofibrillary tangles were determined according to the graduation of Khachaturian (11). AD patients died from cardiac and pulmonary deficits. All patients, before death, were underwent psychopharmaceutic therapy and received antibiotics.

Sample preparation

The crude tissue extracts from postmortem brain were prepared by homogenizing the tissues in a 75 mM phosphate buffer pH = 7,3 (10 ml buffer per gram of tissue). 100 μl of brain tissue homogenate was transferred to a glass tube, 200 μl of ethanol and 100 μl of water was added and mixed, and then 400 μl of hexane was added, followed by mixing. The mixture was left to sit for 1-2 min or until two layers appeared, followed by centrifugation for 5 min at 14000 rpm. The hexane layer was removed and added to a separate amber tube. An additional 400 μl of hexane was added to the original tube, mixed, left to settle for 2min, and then centrifuged for 5 min at 14000 rpm. The hexane layer was removed and combined with first extract. 400 μl of 0,5 M perchloric acid was added in the combined hexane extract to precipitate the protein. The sample was then centrifuged for 5 min at 14000 rpm. From the supernatant, 200 μl was added to 800 μl of phosphate buffer, mixed and used for the further measurement. *The manual antioxidant radical absorbance capacity (ORAC) assay*

Manual ORAC analysis were performed on a Perkin Elmer spectrofluorometer LS 55 with a fluorescent filters (Ex: 485 nm; Em: 520 nm). In the final assay mix-

ture (2 ml total volume) FL (10,5 nM) was used as a target of FR attack, with AAPH (32 mM) as a peroxy radical generator (ORAC- ROO^\bullet assay), and H_2O_2 - Cu^{2+} (H_2O_2 0,3 %; $\text{CuSO}_4 \times 5\text{H}_2\text{O}$ 0,9 mM) as mainly a hydroxyl radical generator (ORAC- OH^\bullet assay). The spectrofluorometer was programmed to record the fluorescence of FL every 10 min after AAPH, or H_2O_2 - Cu^{2+} were added for as long as 180 min and the samples were thermostated at 37° C (KP 20-D Lauda, Lauda Koenigshofen). All fluorescence measurements were expressed relative to the initial reading. Final results were calculated using the differences of areas under the FL decay curves between the blank and a sample and expressed as a μmol per g of fresh brain tissue.

Biochemical analysis

Colorimetric determination of total protein was based on the principle of the Biuret reaction using bovine serum albumin as a standard. Cholesterol and triglycerides were measured using commercially available enzymatic kits, which are based on the spectrophotometric determination.

Statistical analysis

For statistical comparisons Student's test was performed.

RESULTS

The ORAC- ROO^\bullet and ORAC- OH^\bullet values from lipophilic NC, GT and MC fractions of C and AD are shown in Table 1 and Table 2.

Group	Brain region	ORAC- ROO^\bullet ($\mu\text{mol} / \text{g}$)
C (n = 6)	Nucleus caudatus	276,7 ± 139,6
AD (n = 9)		204,8 ± 140,7
C (n = 8)	Gyrus temporalis	273,4 ± 129,8
AD (n = 9)		185,4 ± 132,8
C (n = 8)	Motorcortex	257,5 ± 74,2
AD (n = 4)		101,1* ± 87,4

Results are expressed as means ±SD; Number of samples are given in parenthesis; * significantly different from C ($p < 0,008$ by Student's t-test)

TABLE 1. Antioxidant capacity against peroxy radical, ORAC- ROO^\bullet ($\mu\text{mol} / \text{g}$) in different brain regions of C and AD

In the MC of AD patients antioxidant capacity against peroxy radical was significantly lower than in the MC of C, $p < 0,008$ by Student's t-test (Table 1).

No changes in the antioxidant capacity were found in brain tissues of AD in comparison with C, when hydroxyl radical generator was used in the assay (Table 2).

Group	Brain region	ORAC- OH^\bullet ($\mu\text{mol} / \text{g}$)
C (n = 5)	Nucleus caudatus	126,2 ± 46,6
AD (n = 10)		83,0 ± 36,7
C (n = 9)	Gyrus temporalis	65,5 ± 22,7
AD (n = 9)		87,7 ± 40,0
C (n = 5)	Motorcortex	138,4 ± 60,3
AD (n = 3)		78,1 ± 31,0

Results are expressed as means ±SD; Number of samples are given in parenthesis;

TABLE 2. Antioxidant capacity against hydroxyl radical, ORAC- OH^\bullet ($\mu\text{mol} / \text{g}$) in different brain regions of C and AD

Total protein, cholesterol and triglycerides in different brain regions of C and AD are presented in Table 3.

Group	Brain region	Total protein (g / L)	Cholesterol (mmol / L)	Triglycerides (mmol / L)
C (n = 9)	Nucleus caudatus	9,3 ± 2,8	3,96 ± 1,82	0,62 ± 0,09
AD (n = 11)		10,1 ± 4,4	4,20 ± 1,43	0,65 ± 0,20
C (n = 11)	Gyrus temporalis	13,1 ± 6,8	2,89 ± 1,01	0,59 ± 0,12
AD (n = 11)		8,5* ± 2,7	3,45 ± 0,93	0,53 ± 0,14
C (n = 10)	Motorcortex	14,0 ± 7,1	3,73 ± 1,11	0,56 ± 0,12
AD (n = 10)		10,2 ± 6,3	3,94 ± 0,44	0,48 ± 0,07

Results are expressed as means ±SD; Number of samples are given in parenthesis; * significantly different from C ($p < 0,03$ by Student's t-test)

TABLE 3. Total protein, cholesterol and triglycerides in different brain regions of C and AD

Reduction of total protein in GT of AD, $p < 0,03$ was found. No changes of cholesterol and triglycerides were found in brain tissues of AD in comparison with C (Table 3).

DISCUSSION

FR and oxidative stress involvement in the pathophysiology of AD has been in the focus of AD research (12, 13). Multiple biochemical deficits have been observed in the postmortem brain tissue of AD patients (14, 15, 16) with altered neurotransmission (17, 18) and pronounced deficit in the presynaptic cholinergic system (19), reduced protein content (14, 20) and altered lipid composition (21) which may all be inducing factors of sequelae for cell death. Discovery of isoprostanes (22, 23) and data from the recent studies performed in living patients (24, 25), make important contribution to better understand-

ing and defining the role which oxygen radicals might play role in AD-pathogenesis. From the point of view AC is dependent of chemical composition of complex biological sample and upon FR generator used in assay. AC measured with different FR generators have different numerical values. Because of this fact it is necessary for estimation of antioxidant status of some sample to determine antioxidant score, which is a sum of ORAC- ROO° and ORAC- OH° in hydrophilic fraction and in lipophilic fraction. In this study, AC of lipophilic brain antioxidants was measured. Extracting the lipophilic components with hexane before analysis of the hydrophilic antioxidant fraction did not significantly alter the AC of the hydrophilic fraction (7). Thus, it appeared that

there was no carry over of the lipophilic components into the aqueous compartment, and one could obtain the same hydrophilic ORAC value, whether the samples were first extracted with hexane or not. Regarding our previously published data of decreased AC in hydrophilic fraction of different AD brain regions (9), AC in lipophilic fraction was about 20 – 30 % of AC measured in hydrophilic fraction. Protein content, which may be regarded an index of cell loss or atrophy (26) was reduced in GT of AD brains in comparison with C. However, no differences between AD and C were found with respect to the cholesterol and triglycerides investigated. Our results suggested that lipophilic antioxidant defense system in brain is region-dependently disturbed.

CONCLUSION

- Lipophilic fractions ORAC- ROO° in MC of AD was statistically significantly lower in comparison with MC of C ($p < 0,008$).
- No changes in the AD lipophilic fraction ORAC- OH° were found in comparison with C.
- Reduction of total protein in GT of AD ($p < 0,03$) in comparison with C was found.
- No changes of cholesterol and triglycerides were found in brain tissues of AD in comparison with C.
- The results showed that in the MC of AD brain the balance between production of FR and the neutralization by a complex lipophilic antioxidant system is disturbed.
- The manual fluorescent method for AC measurements proved to be sufficiently appropriate and sensitive for the AC measurements of lipophilic fraction of postmortem brain tissues.

List of Abbreviations

AC	-	antioxidant capacity
MC	-	motorcortex
NC	-	nucleus caudatus
GT	-	gyrus temporalis
C	-	controls
AD	-	Alzheimer's disease
ORAC	-	oxygen radical absorbance capacity
ORAC- ROO°	-	antioxidant capacity against peroxy radicals
ORAC- OH°	-	antioxidant capacity against hydroxyl radicals
FR	-	free radicals
AAPH	-	2, 2' – azobis (2 – amidino – propane) dihydrochloride
ROO $^\circ$	-	peroxy radical
OH°	-	hydroxyl radical
FL	-	fluorescein
β -PE	-	β - phycoerythrin

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