



## Monitoring thiobarbituric acid-reactive substances (TBARs) as an assay for oxidative damage in neuronal cultures and central nervous system

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Received 1 October 2003; accepted 28 June 2004

### Abstract

Oxidative stress is a pivotal factor in neuronal degeneration. A simple method to quantify oxidative damage in culture and in situ is therefore important for studies of neurodegeneration. We present herein modifications of the standard assay for thiobarbituric acid-reactive substances (TBARs) for analyses of both cell cultures and brain tissue homogenates. Since the TBAR assay measures end-point oxidative damage, it is useful to assess the overall impact of oxidative stress-inducing and neuroprotective agents; interpretation is not potentially confounded by the presence or absence of transient products of oxidative damage.

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**Keywords:** Thiobarbituric acid-reactive substances; Oxidative stress; Antioxidants; Vitamin E; Nervous system; Neurodegeneration

### 1. Introduction

One factor contributing to the age-related decline in cognitive performance, including the hastened decline which accompanies Alzheimer's disease (AD), is increased oxidative stress (Berr, 2002; Butterfield and Lauderback, 2002; Floyd and Hensley, 2002; Perry et al., 2002a, 2002b; Miranda et al., 2000; Smith et al., 2000). Restoring or maintaining oxidative buffering capacity, and/or quenching reactive oxygen species (ROS), therefore represents one potential therapeutic approach to minimize neurodegeneration.

Whether or not supplementation with vitamins or antioxidants can be effective in chronic neurodegenerative conditions such as AD remains the subject of debate (Gilgun-Sherki et al., 2003; Martin, 2003; Solfrizzi et al., 2003). For

example, clinical studies demonstrated only limited efficacy of the antioxidant Vitamin E in delaying the severity of some clinical AD symptoms (Drachman and Leber, 1997; Sano et al., 1997). Additional studies have demonstrated that combined supplementation with Vitamin E and other agents is more effective than Vitamin E alone both in neuronal cultures and in central nervous system of normal and transgenic mice that are compromised in oxidative buffering capacity (Shea et al., 2002a; Veinbergs et al., 2000). Nutritional supplementation with fruits and vegetables rich in antioxidant potential has also been shown to provide neuroprotection (Joseph et al., 1998a, 1998b, 1999; Ortiz and Shea, 2003; Rogers et al., 2003; Smith et al., 1999).

Oxidative damage induces a cascade of downstream reactive oxygen species, some of which are relatively transient, such as hydroxynonenol, while others appear later and accumulate, such as malondialdehyde (Miyata and Smith, 1996; Pedersen et al., 2000; Keller et al., 2000; Subramaniam et al.,

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1998). Of use would be a single, simply assay that could be utilized to quantify the total burden of reactive oxygen species as well and the impact of any therapeutic approach. We describe the use of the thiobarbituric acid-reactive substances (TBARs) assay, which detects any thiobarbituric acid-reactive substance such as malondialdehyde (Meagher and Fitzgerald, 2000), for use in homogenates of cultured neuroblastoma and brain tissue.

## 2. Materials and methods

Cortical neurons were cultured from day 16 murine embryos as described previously (Ekinici et al., 1999). Neurons were cultured for 24 h in DMEM (high glucose formulation) supplemented B-27 containing antioxidants (Gibco-BRL), after which medium was replaced with medium containing B-27 without antioxidants. Cultures were treated with 10  $\mu$ M hydrogen peroxide for 30 min. Additional cultures received 0.0625 IU/mL Vitamin E alone, or along with 0.13 mg/mL Na pyruvate and 0.2 mg/mL separately or in combination for 30 min (Shea et al., 2002a). Cultures were scraped from the plate, homogenized, and analyzed by fluorescent spectrophotometry for TBARs according to Ohkawa et al. (1979) with modifications. A standard curve was prepared by diluting 1 mM tetraethoxypropane (TEP) in 0.01N HCl. Cultures were sonicated (3 s  $\times$  5 s bursts) in 1 ml cold KCl. Aliquots of homogenates or standards (0.5 ml) were combined with 0.22 ml of 8.1% SDS, followed by 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml 0.8% TBA, and samples were vortexed. Samples were then incubated at 95  $^{\circ}$ C for 60 min followed by cooling in tap water. Samples were combined with 4 ml butanol/pyridine (15:1), mixed and centrifuged (5 min at approx. 5000 rpm) in a tabletop centrifuge to facilitate separation of layers. The upper butanol/pyridine phase was then removed and fluorescence was quantified with an excitation wavelength of 520 nm and emission wavelength of 553 nm.

C57Bl/6J mice between 10 and 14 months of age received a basal diet (“AIN-76”; Purina/Mother Hubbard Inc.) lacking lacking or supplemented with Vitamin E (as  $\alpha$ -tocopherol, 50 IU/kg), with and without Na pyruvate (30 g/500 g total diet), PC (5 g/500 g total diet) and/or a mixture of fish and corn oil (8% and 2% of the total diet wet weight, respectively) and iron (as ferric citrate; 4 g/500 g total wet weight; the combination of oil and iron promote oxidative stress; Shea et al., 2002a). After 1 month of these various dietary regimens, homogenates of total CNS tissue were examined for TBARs.

Mice were sacrificed by cervical dislocation, and total central nervous system (CNS) tissue was harvested, homogenized, normalized according to total protein and aliquots of unfractionated homogenates were analyzed for TBARs as follows. Brain tissue homogenates (50  $\mu$ g total protein) were mixed with 1  $\mu$ M copper sulfate in 5 mM HEPES (total volume 400  $\mu$ L). Samples then received 1 ml of a 0.375% TBA/15% trichloroacetic acid in 0.25N HCl, incubated for 30 min at 90  $^{\circ}$ C, and were clarified by centrifugation

(1500 rpm for 10 min). The resulting supernatants were aspirated and fluorescence quantified in a fluorescent spectrophotometer (excitation 520 nm, emission 553 nm) by comparison with a standard curve of tetramethoxypropane in HCl. Data were pooled from four separate experiments, each of which contained three to four normal mice of mixed gender per diet, for a combined total of 12–16 mice. Statistical analyses were conducted by individual comparisons among groups via Student’s *t*-test; values were considered statistically different if  $P \leq 0.05$ .

## 3. Results and discussion

Treatment of cultured cortical neurons with 10  $\mu$ M hydrogen peroxide increased ROS as evidenced by TBARs (Table 1). As shown previously using 2,7-dichlorofluorescein, co-treatment with a combination of Vitamin E, PC and pyruvate prevented the increase in ROS that accompanied hydrogen peroxide treatment, while Vitamin E alone was not effective (Shea et al., 2002a). The increased protective effect of Vitamin E, PC and pyruvate in combination versus that of Vitamin E alone was also revealed by analyses of TBARs (Table 1). Peroxide-treated cultures also receiving Vitamin E, PC and pyruvate displayed reduced TBARs versus those treated with peroxide alone ( $P < 0.02$ ; Student’s *t*-test). Cultures receiving peroxide along with Vitamin E did not display a reproducible reduction in TBARs versus those in cultures receiving peroxide alone ( $P < 0.60$ ).

The TBAR assay was also useful for quantifying oxidative species in CNS. TBARs were increased by approximately 50% following dietary challenge with iron in the absence of Vitamin E (Table 2). Vitamin E substantially diminished this increase. However, combined treatment with Vitamin E, PC and pyruvate was markedly more effective at quenching oxidative damage than was Vitamin E alone. The markedly differential TBARs observed among various diets demonstrate that TBARs are not derived simply by the presence of this level of dietary iron.

Table 1  
Quantification of oxidative damage using the TBARs assay in cultured neurons

	Mean	Standard deviation	% change
Control	34.5	0.7	–
Peroxide	49.1	6.4	42.3
Peroxide + Vitamin E	52.9	9.2	53.3
Peroxide + Vitamin E, PC, pyr	35.1	4.3	1.7%

Embryonic cortical neuronal cultures received 10  $\mu$ M hydrogen peroxide with and without 0.0625 IU/mL Vitamin E alone, or in combination with 0.13 mg/mL Na pyruvate and 0.20 mg/mL separately or in combination for 30 min, after which cultures were assayed for TBARs as described in Section 2. Values for peroxide-treated cultures, and those receiving Vitamin E, PC or pyruvate individually differed statistically from untreated controls ( $P < 0.05$ ); peroxide-treated cultures receiving all three were statistically identical to controls ( $P > 0.05$ ). Values are expressed as  $\mu$ M TBARs/mg total protein and % change as indicated.

Table 2

Oxidative damage in normal and ApoE-knockout mice following dietary challenge with iron in the presence and absence of Vitamin E

	Mean	Standard deviation	% change
Basal diet	10.475	4	–
Basal diet + iron	17.725	2.85	69.2
Vitamin E+ iron	15.925	2	52.0
Vitamin E, PC, Pyr + iron	11.3	2.1	7.9

Mice ( $n = 4$  of each strain per diet) received a basal diet (“AIN-76”; Purina) with and without Vitamin E (as tocopherol acetate, 50 IU/kg), Na pyruvate: (30 g/500 g total diet) PC (5 g/500 g total diet) and/or a mixture of fish and corn oil (8 and 2% of the total diet wet weight, respectively) and iron (as ferric citrate; 4 g/500 g total wet weight) for 1 month. Total brain tissue was harvested and analyzed for TBARs as described in Materials and Methods. Values represent the  $\mu\text{MTBARS}/\text{mg}$  total protein (mean  $\pm$  standard error of the mean) or the percent change as indicated.

These findings demonstrate the efficacy of the TBARs assay for monitoring conditions that increase ROS as well as therapeutic antioxidant treatments. We have also utilized the TBAR test to demonstrate that supplementation with dietary folate is neuroprotective in vivo (Shea and Rogers, 2002; Shea et al., 2002b); results obtained in these studies using the TBAR assay were corroborated by total antioxidant levels within CNS as determined by the “tocopherol equivalent antioxidant assay” (Shea et al., 2003) and by glutathione levels within CNS as determined by HPLC within CNS (Shea et al., 2002b). The TBAR assay has also been utilized to quantify differences in steady-state oxidative species in nervous tissue in transgenic mice compromised in oxidative buffering capacity (Shea and Rogers, 2002) and in AD (Lovell et al., 1995). These findings support the use of the TBAR assay for analyses of oxidative damage in nervous tissue and in cultured neurons.

An additional potential modification for the use of the TBARs assay for tissue homogenates would be to quench any additional formation of malondialdehydes from other “upstream” free radicals during the assay itself; this can be accomplished by inclusion of 0.1% butylated hydroxytoluene (BHT; Jo and Ahn, 1998). Since our intent is to quantify total products of oxidative damage, we did not incorporate BHT into our protocol. We note, however, that inclusion of BHT in half of duplicate samples would give an index of total malondialdehydes at the time of harvest, as well as an index of additional transient oxidative species within the sample.

## References

Berr C. Oxidative stress and cognitive impairment in the elderly. *J Nutr Health Aging* 2002;6:261–6.

Butterfield DA, Lauderback CM. Lipid peroxidation and protein oxidation in Alzheimer’s disease: potential causes and consequences involving amyloid-beta peptide-associated free radical oxidative stress. *Free Radic Biol Med* 2002;32:1050–60.

Drachman DA, Leber P. Treatment of Alzheimer’s disease — searching for a breakthrough, settling for less. *N Engl J Med* 1997;336:1245–7.

Ekinci FJ, Malik KM, Shea TB. beta-amyloid induces calcium influx and neurodegeneration by MAP kinase-mediated activation of the L voltage-sensitive calcium channel. *J Biol Chem* 1999;274:30322–7.

Floyd RA, Hensley K. Oxidative stress in brain aging. Implications for therapeutics of neurodegenerative diseases. *Neurobiol Aging* 2002;23:795–807.

Gilgun-Sherki Y, Melamed E, Offen D. Antioxidant treatment in Alzheimer’s disease: current state. *J Mol Neurosci* 2003;21(1):1–12.

Jo C, Ahn DU. Fluorometric analysis of 2-thiobarbituric acid-reactive substances in Turkey. *Poul Sci* 1998;77:475–80.

Joseph JA, Shukitt-Hale B, Denisova NA, Prior RL, Cao G, Martin A, et al. Long-term dietary strawberry, spinach, or Vitamin E supplementation retards the onset of age-related neuronal signal-transduction and cognitive behavioral deficits. *J Neurosci* 1998;18:8047–55.

Joseph JA, Shukitt-Hale B, Denisova NA, Prior RL, Cao G, Martin A, et al. Long-term dietary strawberry, spinach, or Vitamin E supplementation retards the onset of age-related neuronal signal-transduction and cognitive behavioral deficits. *J Neurosci* 1998;18:8047–55.

Joseph JA, Shukitt-Hale B, Denisova NA, Bielinski D, Martin A, McEwen JJ, et al. Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. *J Neurosci* 1999;19:8114–21.

Keller JN, Lauderback CM, Butterfield DA, Kindy MS, Markesbery WR. Amyloid-beta peptide effects on synaptosomes from Apolipoprotein E-deficient mice. *J Neurochem* 2000;74:1579–86.

Lovell MA, Ehmann WD, Butler SM, Markesbery WR. Elevated 4-hydroxynonenal in ventricular fluid in Alzheimer’s disease. *Neurobiol Aging* 1995;18:457–61.

Martin A. Antioxidant vitamins E and C and risk of Alzheimer’s disease. *Nutr Rev* 2003;61(2):69–73.

Meagher EA, Fitzgerald GA. Indices of lipid Peroxidation in vivo: strengths and limitations. *Free Radic Biol Med* 2000;28:1745–50.

Miranda S, Opazo C, Larrondo LF, Munoz FJ, Ruiz F, Leighton F, et al. The role of oxidative stress in the toxicity induced by amyloid beta peptide in Alzheimer’s disease. *Prog Neurobiol* 2000;62:633–48.

Miyata M, Smith JD. Apolipoprotein E allele-specific antioxidant activity and effects on cytotoxicity by oxidative insults and beta-amyloid peptides. *Nat Gen* 1996;14:55–61.

Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979;95:351–8.

Ortiz D, Shea TB. Apple juice prevents oxidative stress induced by amyloid-beta in culture. *J Alzheimers Dis* 2003 [in press].

Pedersen WA, Chan SL, Mattson MP. A mechanism for the neuroprotective effect of Apolipoprotein E: isoform-specific modification by the lipid peroxidation product 4-hydroxynonenal. *J Neurochem* 2000;74:1426–33.

Perry G, Cash AD, Smith MA. Alzheimer disease and oxidative stress. *J Biomed Biotechnol* 2002;2:120–3.

Perry G, Nunomura A, Hirai K, Zhu X, Prez M, Avila J, et al. Is oxidative damage the fundamental pathogenic mechanism of Alzheimer’s and other neurodegenerative diseases? *Free Radic Biol Med* 2002;33:1475–9.

Rogers EJ, Mihalick S, Ortiz D, Shea TB. Apple juice prevents oxidative stress and impaired cognitive performance caused by genetic and dietary deficiencies in mice. *J Nutr Health Aging* 2003 [in press].

Sano M, Ernesto C, Thomas RG, Klauber MR, Schafer K, Grundman M, et al. A controlled trial of selenine, alpha-tocopherol and both as a treatment for Alzheimer’s disease: The Alzheimer’s disease cooperative study. *N Engl J Med* 1997;336:1216–22.

Shea TB, Rogers E. Homocysteine as a risk factor for Alzheimer’s disease. *N Engl J Med* 2002;25:2007.

Shea TB, Ekinci FJ, Ortiz D, Dawn-Linsley M, Wilson TO, Nicolosi RJ. Efficacy of vitamin E, phosphatidyl choline and pyruvate on buffering neuronal degeneration and oxidative stress in cultured cortical neurons and central nervous tissue of apolipoprotein E-deficient mice. *Free Radic Biol Med* 2002;33:276–82.

- 227 Shea TB, Rogers E, Ortiz D, Sheu M-S. Apolipoprotein E deficiency pro- 237  
228 motes increased oxidative stress and compensatory increases in antioxi- 238  
229 dants in brain tissue. *Free Radic Biol Med* 2002;33:1115–20. 239
- 230 Shea TB, Ortiz D, Sheu M-S. Quantification of antioxidant activity in brain 240  
231 tissue homogenates using the “total equivalent antioxidant capacity”. *J* 241  
232 *Neurosci Meth* 2003;125:55–8. 242
- 233 Smith MA, Petot GJ, Perry G. Diet and oxidative stress: a novel synthe- 243  
234 sis of epidemiological data on Alzheimer’s disease. *J Alzheimers Dis* 244  
235 1999;1(4–5):203–6. 245
- 236 Smith MA, Rottkamp CA, Nunomura A, Raina AK, Perry G. Oxidative stress 246  
in Alzheimer’s disease. *Biochim Biophys Acta* 2000;1502:139–44. 247
- Solfrizzi V, Panza F, Capurso A. The role of diet in cognitive decline. *Neural* 237  
*Transm* 2003;110(1):95–110. 238
- Subramaniam R, Koppal T, Green M, Yatin S, Jordan B, Drake J, et al. 239  
The free radical antioxidant vitamin E protects cortical synaptosomal 240  
membranes from amyloid beta-peptide (25–35) toxicity but not from 241  
hydroxynonenol toxicity: Relevance to the free radical hypothesis of 242  
Alzheimer’s disease. *Neurochem Res* 1998;23:1403–10. 243
- Veinbergs I, Mallory M, Sagara Y, Masliah E. Vitamin E supple- 244  
mentation prevents spatial learning deficits and dendritic alterations 245  
in aged apolipoprotein E-deficient mice. *Eur J Neurosci* 2000;12: 246  
4541–6. 247

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