Inhibition of Iron-Catalyzed Oxidations by Attainable Uric Acid and Ascorbic Acid Levels: Therapeutic Implications for Alzheimer's Disease and Late Cognitive Impairment

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Key Words

Brain iron • Longevity • Uric acid • Ascorbic acid • Alzheimer's disease • Fenton reaction

Abstract

Background: Alzheimer's disease (AD) has become one of the major health problems of the developed world. Previous studies have shown that oxidant-induced changes occur in cerebral tissue in AD and in late-onset amnesic mild cognitive impairment. The oxidative damage begins early and involves free radical-mediated effects that cause lipid peroxidations and oxidative protein and nucleic acid damages which begin before the cardinal neuropathologic manifestations. Impaired cerebral iron homeostasis and iron accumulation are postulated to be primary and seminal in the pathogenesis. **Objective:** To demonstrate that the Fenton reaction involving hydrogen peroxide and iron at very low concentrations as has been found in human plasma and cerebrospinal fluid may produce promptly oxidations which may be inhibited by preventive use of uric acid and ascorbic acid as hydrophilic antioxidants. **Methods:** A photometric study in vitro at physiologic pH using concentrations of uric acid and ascorbic acid readily attainable in human extracellular fluids. **Results:** Uric acid levels of 0.5 and 6.0 mg/dl (below the saturation level for urate precipitation) and ascorbic acid at a level readily attainable in blood plasma inhibited

significantly and completely, respectively, oxidations caused by reactions of 20 μM concentrations of hydrogen peroxide with free bivalent iron at 9.8 μM and at a low hemoglobin level of 12 mg/dl of saline. **Conclusion:** Results suggest that supplemental use orally of ascorbic acid combined with use of metabolic precursor to uric acid, like inosine or hypoxanthine, has the potential for preventing or attenuating the progression of AD and amnesic mild cognitive impairment.

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Introduction

Alzheimer's disease (AD) has become one of the major health problems in the United States and the entire developed world. Because the presence of AD clinically doubles with every 5 years of chronological age after 60, delaying onset by 5 years would reduce the prevalence by half [1, 2]. Prevention of mild cognitive decline preceding diagnosable AD and declines in AD dementia will probably be most effective when the intervention targets a process closely relevant to the disease pathogenesis [3].

Oxidative stress-mediated damage in cerebral tissue in AD, involving oxidation of nucleic acids, proteins, and lipids, are all prominent in the early stages of AD [4].

The oxidative stress changes have been shown to precede the cardinal neuropathologic manifestations of AD [4]. In addition, recent studies on patients with amnesic mild cognitive impairment (MCI) have shown increased lipid peroxidation and protein, DNA, and RNA oxidative changes in multiple brain regions [5]. These latter studies establish that free radical-mediated oxidative damage is involved as an early event in the neuron damage in AD [5].

Disruption of cerebral iron homeostasis with iron overloading is a likely primary seminal event in AD and in late MCI [6]. This theory for the pathogenesis of AD was first proposed by Goodman [7] in 1953 and is supported strongly by the studies of Connor et al. [8, 9] that the cerebral iron-regulatory system is dysfunctional in AD.

There is over-accumulation of iron in the hippocampus, cerebral cortex, and basal nucleus of Meynert [8, 9]. These affected brain areas are of special interest because they represent centers of memory and thought processes, all lost in the clinical picture of AD.

Brain iron is very toxic in its bivalent form when it reacts with hydrogen peroxide to produce highly reactive hydroxyl free radicals. This reaction between ferrous iron and hydrogen peroxide (which is diffusible) is termed the Fenton reaction. [10]. As soon as hydroxyl radicals are formed, they react with many different organic molecules in their immediate vicinity [10]. Hydroxyl radicals will react very quickly with almost every type of molecule found in living cells, e.g. sugars, amino acids, DNA bases, and organic acids [10]. Notably, iron is reported elevated in the cerebrospinal fluid (CSF) in AD compared to in control other elderly patients [11].

Uric acid is a strong scavenger of free radicals [12]. This end-product of purine metabolism in man is believed to be responsible in part for the longevity of humans because of its antioxidant properties [12]. Uric acid concentrations in serum and brain correlate with the highest maximum lifespan potential in man among mammals, perhaps concomitant with the antioxidant properties of ascorbic acid [13, 14].

Uric acid and ascorbic acid are two of the major hydrophilic antioxidants present in human plasma [15] and they may be likely relevant to the initial pathogenesis in AD and in late-onset MCI. Therefore, the following antioxidant experiments with uric acid and ascorbic acid, iron salt, and free human hemoglobin were performed in saline solutions mimetic of CSF except devoid of possible confounding other organic molecules.

Methods and Materials

Control and test solutions contained 9.0 mg/dl of *o*-dianisidine as a potential chromogenic hydrogen donor to be oxidized in saline solution of 140 mM NaCl and 3.0 mM phosphate buffer of $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$, pH 7.4, and either 9.8 μM of ferrous sulfate or 12 mg/dl of human hemoglobin. The ferrous sulfate or hemoglobin was added to the solutions shortly before photometric measurements. Hydrogen peroxide at a final level of 20 μM was added with vortex mixing for 2–3 s promptly before timed absorbance measurements in 12-mm diameter tube cuvettes in a Bausch & Lomb Spectronic 20 colorimeter at 460 nm. This was to detect oxidations of the included *o*-dianisidine as chromogen. The test solutions included uric acid at concentrations of 6.0 mg/dl (357 μM) or of 0.5 mg/dl (30 μM) or L-ascorbic acid at level of 1.6 mg/dl. In a few determinations, allantoin at 5.6 mg/dl (357 μM), or L-lysine free base at 0.44 mg/dl (30 μM), or L-arginine free base at 0.52 mg/dl (30 μM) was included instead as a test solution. Solution incubations and measurements were made at room temperatures of 25–28°C.

Chemicals

Employed were *o*-dianisidine dihydrochloride, L-ascorbic acid (cell culture tested), human hemoglobin (lyophilized), allantoin, L-arginine free base (cell culture tested), and L-lysine free base, all obtained from Sigma Chemical Co. (St. Louis, Mo., USA). Uric acid was from Fisher Scientific Co. (Fair Lawn, N.J., USA). Hydrogen peroxide, 3%, was used. Ferrous sulfate $7\text{H}_2\text{O}$ from Mallinckrodt Chemical Works (St. Louis, Mo., USA) and all other chemicals used were of analytical grade. Distilled water was employed.

Statistical Analysis

Paired Student's two-tailed *t* tests for mean differences between control data and the data of test solutions were used. Control solutions and test solutions were paired individually and measured concurrently at the same time intervals. Standard errors of means (SEM) are listed. *p* values ≤ 0.5 were considered to indicate statistical significant differences.

Results

Table 1 show the results when uric acid was used at the two listed concentrations to try to reduce the oxidations of dianisidine at the micromolar concentrations of 9.8 μM of ferrous sulfate and 20 μM of hydrogen peroxide. The control absorbance increases or measured oxidations of dianisidine became quickly maximal within 5 min at physiological pH of 7.4 (see table 1). At a urate level of 6.0 mg/dl (357 μM), the inhibition of oxidation averaged $75.3 \pm 4.8\%$. At a concentration 12-fold less or 8.3% of the higher urate level, i.e. at 0.5 mg/dl (30 μM), the mean inhibition was considerably less at $36.0 \pm 5.3\%$. At both concentrations of uric acid, the inhibition became mildly less during the next 5 min of incubation time.

Table 1. Uric acid inhibition of substrate oxidation induced by micromolar reaction of ferrous sulfate (9.8 μM) with hydrogen peroxide at 20 μM

	Time after H_2O_2	Absorbance increase after H_2O_2	% inhibition
Control	5 min	0.049 ± 0.004	
	10 min	0.049 ± 0.004	
Urate, 6.0 mg/dl (357 μM)	5 min	0.012 ± 0.003	$75.3 \pm 4.8^{**}$
	10 min	0.014 ± 0.003	$70.9 \pm 4.5^{**}$
Control	5 min	0.047 ± 0.008	
	10 min	0.047 ± 0.004	
Urate, 0.5 mg/dl (30 μM)	5 min	0.030 ± 0.003	$36.0 \pm 5.3^{**}$
	10 min	0.034 ± 0.017	$26.5 \pm 5.5^*$

Mixtures of 5.085 ml were incubated at room temperature of 25–28°C. Values are means $\pm$ SEM, n = 5. Significance of mean difference in percentage from control value: * $p < 0.01$, ** $p < 0.001$. Substrate as chromogenic hydrogen donor detected at 460 nm was *o*-dianisidine at 9 mg/dl in buffered saline of 140 mM NaCl, $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ of 3.0 mM, pH 7.4. Allantoin at 5.6 mg/dl (357 μM) and both L-lysine and L-arginine free base at 0.44 mg/dl (30 μM) and 0.52 mg/dl (30 μM), respectively, failed to decrease the developed absorbance below that of the developed control oxidation in individual experiments.

Table 2. Uric acid inhibition of substrate oxidation induced by human hemoglobin level of 12 mg/dl in reaction with hydrogen peroxide at 20 μM

	Time after H_2O_2	Absorbance increase after H_2O_2	% inhibition
Control	5 min	0.059 ± 0.002	
	10 min	0.084 ± 0.017	
Urate, 6.0 mg/dl (357 μM)	5 min	0.006 ± 0.002	$90.6 \pm 0.90^{***}$
	10 min	0.016 ± 0.005	$80.3 \pm 3.54^{**}$
Urate, 0.5 mg/dl (30 μM)	5 min	0.028 ± 0.004	$50.7 \pm 7.56^*$
	10 min	0.052 ± 0.009	$38.8 \pm 3.25^{**}$

Mixtures of 5.060 ml were incubated at room temperature of 25–28°C. Values are means $\pm$ SEM, n = 4. Significance of mean difference in percentage from control value: * $p < 0.01$, ** $p < 0.005$, *** $p < 0.001$. Substrate as chromogenic hydrogen donor detected at 460 nm was *o*-dianisidine at 9 mg/dl in buffered saline of 140 mM NaCl, $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ of 3.0 mM, pH 7.4.

Allantoin, at the same micromolar concentration that was used at the higher urate level of 357 μM listed in table 1, failed completely to inhibit the substrate oxidations. This was with use of the same concentrations of iron sulfate and hydrogen peroxide. Similarly, 30 μM levels of

Table 3. Ascorbic acid inhibition at concentration of 1.6 mg/dl (89 μM) of substrate oxidation induced by micromolar reaction of ferrous sulfate (9.8 μM) with hydrogen peroxide at 20 μM

	Time after H_2O_2	Absorbance increase after H_2O_2	% inhibition
Control	5 min	0.039 ± 0.007	
	10 min	0.039 ± 0.007	
Ascorbate, 1.6 mg/dl (89 μM)	5 min	0.000 ± 0.000	$100.0 \pm 0.0^*$
	10 min	0.000 ± 0.000	$100.0 \pm 0.0^*$

Mixtures of 5.085 ml were incubated at room temperature of 25–28°C. Values are means $\pm$ SEM, n = 4. Significance of mean difference in percentage from control value: * $p < 0.0001$. Substrate as chromogenic hydrogen donor detected at 460 nm was *o*-dianisidine at 9 mg/dl in buffered saline of 140 mM NaCl, $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ of 3.0 mM, pH 7.4.

L-lysine and L-arginine, employed at concentrations close to the values found in human CSF [16, 17], also failed to inhibit the substrate oxidations (data not shown).

Table 2 lists data which demonstrate that free heme protein may be pro-oxidant at low concentration by reacting with a low concentration of hydrogen peroxide to cause oxidations. At a saline concentration of 12 mg/dl, human hemoglobin reacted with a 20- μM level of hydrogen peroxide to cause oxidation of dianisidine.

Oxidations became greater with interaction for 10 min compared to the absorbance increases from 5 min of interaction with the heme protein (see table 2).

Urate at 6.0 mg/dl (357 μM) inhibited the absorbance increases of dianisidine by a mean value of $90.6 \pm 0.90\%$ ($p < 0.001$) at 5 min of incubation with the peroxide. The color absorbances were greater at 10 min of reaction time with the hydrogen peroxide, with mean percentage inhibition of the oxidations by the urate averaging less at $80.3 \pm 3.54\%$. Apparently, the greater absorbance increases at 10 min resulted because more bivalent iron was freed in the hemoglobin by the action of hydrogen peroxide to cause more iron to be available to react by Fenton-type chemistry with the peroxide to oxidize more dianisidine. At 12-fold lower urate concentration of 0.5 mg/dl (30 μM), uric acid inhibited the oxidations significantly less by percentage, both at 5 and 10 min of exposure to the hydrogen peroxide.

Table 3 lists the data which demonstrate that, at a level of 1.6 mg/dl (89 μM), ascorbic acid inhibited the oxidations to color substance by 100% when the dianisidine was oxidized by micromolar reaction of 9.8 μM of ferrous

Table 4. Ascorbic acid inhibition at concentration of 1.6 mg/dl (89 μ M) of substrate oxidation induced by reaction of human hemoglobin of 12 mg/dl with hydrogen peroxide at 20 μ M

	Time after H ₂ O ₂	Absorbance in- crease after H ₂ O ₂	% inhibition
Control	5 min	0.050 $\pm$ 0.028	
	10 min	0.094 $\pm$ 0.006	
Ascorbate, 1.6 mg/dl (89 μ M)	5 min	0.000 $\pm$ 0.000	100.0 $\pm$ 0.0*
	10 min	0.001 $\pm$ 0.008	99.8 $\pm$ 1.2*

Mixtures of 5.060 ml were incubated at room temperature of 25–28°C. Values are means $\pm$ SEM, n = 4. Significance of mean difference in percentage from control value: * p < 0.0001. Substrate as chromogenic hydrogen donor detected at 460 nm was o-dianisidine at 9 mg/dl in buffered saline of 140 mM NaCl, Na₂HPO₄–KH₂PO₄ of 3.0 mM, pH 7.4.

sulfate with 20 μ M of hydrogen peroxide at physiological pH of 7.4. No absorbance increases resulted after addition of hydrogen peroxide. The complete inhibition which was found at 5 min persisted over the next 5 min (and longer) of exposure to the peroxide at the employed antioxidant ascorbate level of 1.6 mg/dl (89 μ M).

Table 4 shows that ascorbic acid at level of 1.6 mg/dl (89 μ M) also inhibited completely at 5 min the oxidations of dianisidine from the oxidant reaction of 12 mg/dl of hemoglobin in solution with 20 μ M of hydrogen peroxide. The inhibition remained virtually complete at 10 min of continued exposure to the hydrogen peroxide, in spite of much greater absorbance increases or oxidations in the control solutions from the continuing reaction of the hydrogen peroxide with the iron that resided in the hemoglobin (see table 4).

Discussion

The described experiments demonstrate that, at unprotected bivalent iron present at low level or at iron contained in heme protein like hemoglobin at quite low concentration, the iron can react with hydrogen peroxide at low level to produce oxidation of organic molecules very promptly. The demonstrated hemoglobin-hydrogen peroxide reactions confirm the finding of Gutteridge [18] who used concentrations quite higher of hemoglobin and hydrogen peroxide (e.g. 0.67 or 0.8 mM of peroxide) with 2-hour incubations at 37°C.

I postulate, after Goodman [7] and others [8, 9], that the Fenton reaction involving iron is likely involved seminally in the early pathogenesis of AD [6] and also in late-onset MCI, after Markesbery and Lovell [5]. In many cases, the oxidative stress and cerebral injury probably begin a few decades earlier before the clinical manifestations of AD become obvious. Also, my in vitro experiments suggest that antioxidant protection by both sufficient ascorbic acid and uric acid may be effective to prevent Fenton reaction injury in these disorders. The results with these two hydrophilic antioxidants are also suggestive that both are essential in inducing the unusual long lifespan potential in humans among mammals [12–14].

Aerobic metabolic activity produces hydrogen peroxide by superoxide dismutases and select enzymes in many tissues; also by red blood cells which form this peroxide from its generated residual superoxide radical upon release of oxygen from its hemoglobin [19]. Cerebral metabolic activity is high in humans and catalase, an enzyme which converts hydrogen peroxide to water and ground state oxygen, is poor in the human brain [10]. It is relevant that hydrogen peroxide mean plasma levels of 34 $\pm$ 18 μ M have been reported in healthy young adults and mean plasma levels of 38.3 $\pm$ 6.2 μ M have been reported in adults with cardiac arrhythmias before cardioversion [20, 21]. Catalase is normally absent or extremely low in human plasma [10].

This investigation was conducted because: (1) ascorbic acid and uric acid are two major hydrophilic antioxidants in human plasma and many extracellular fluids and (2) iron toxicity in the human cortex may be an early and seminal event in the pathogenesis of AD. The experiments used ascorbic acid and uric acid at concentrations readily attainable in human plasma by ingredient supplementation orally [12, 22, 23]. The experiments showed that ascorbic acid in the presence of bivalent iron is antioxidant, in contrast to being pro-oxidant [10]. In addition, it should be pointed out that ‘free’ iron in samples of CSF from a range of patients ranged from 3.5 to 24 μ M with a mean value of 9.4 μ M [24]. Also, one may note that the population study of Mikkelsen et al. [25] reported a mean serum uric acid value for males of 4.9 mg/dl with a standard deviation of 1.4 mg/dl. The normal mean value for females is much lower. This fact suggests that the lower serum urate levels in females may be a cause for an apparent greater prevalence of AD in females for unknown reasons.

AD patients are reported to have lower plasma levels of uric acid, present more correctly as its monoanion urate [26, 27]. Of significance, AD patients have an ab-

normally increased renal fractional excretion of urate [26].

Both urate and ascorbate mean plasma levels are reported much lower in AD patients and in patients with MCI, with mean values $<200 \mu\text{M}$ (3.36 mg/dl) for urate and $<26 \mu\text{M}$ (0.46 mg/dl) for ascorbate [28].

Healthy ascorbate values are considered to be in the vicinity of $54 \pm 37 \mu\text{M}$ in plasma and in the vicinity of $133 \pm 59 \mu\text{M}$ in CSF [29]. CSF urate mean value for elderly subjects was reported to be $5.52 \mu\text{M}$ (0.09 mg/dl), but significantly lower at $4.39 \mu\text{M}$ (0.07 mg/dl) in 10 patients with AD-type dementia and their mean serum urate value was lower [30].

Ascorbic acid intakes $>95 \text{ mg/day}$ have been reported to have borderline significance of association with AD [31]. However, no study has yet been done apparently to determine if a combined supplemental intake of ascorbic acid and a precursor of uric acid like inosine or hypoxanthine [23, 32] may be effective in preventing the symptomatology of AD or late-onset MCI. The renal excretion of urate in man increases at plasma urate levels $>3\text{--}5 \text{ mg/dl}$ because of greater saturation of the tubular reabsorptive system for urate [33].

Higher urate plasma levels can be attained readily by daily inosine supplementation as shown by Spitsin et al. [23] during a trial in multiple sclerosis patients or by hypoxanthine, a more immediate metabolic precursor to uric acid. At 0.1 mmol/kg of body weight, hypoxanthine orally raised serum uric acid levels by $2.4 \pm 0.2 \text{ mg/dl}$

(mean $\pm$ SE) in normal adults [32]. In subjects with higher plasma uric acid levels in gout, CSF levels averaged as high as 1.79 mg/dl at average plasma level of 7.36 mg/dl [34].

CSF levels of urate in man are generally only about 6–15% of the concomitant usual plasma or serum urate levels of about 3–5 mg/dl [34]. Levels are attainable higher as measured in patients with gout [34]. Plasma ascorbic acid concentrations in the vicinity of 1.6 mg/dl ($89 \mu\text{M}$) as used in this in vitro investigation are readily attainable by employing less than megadoses of ascorbic acid daily [12]. In AD patients and in healthy subjects, plasma and CSF concentrations of ascorbic acid correlate highly and the levels also correlate with daily dosages of ascorbic acid [35]. The renal plasma level for substantial ascorbate excretion is generally >1.0 and $<2.0 \text{ mg/dl}$ [33]. Combined supplementation with ascorbic acid may be warranted in a trial to render uric acid becoming innocuous biologically as an antioxidant, since ascorbate can scavenge and reduce urate anion free radical which forms in the potent antioxidant action of uric acid [36, 37]. Damage to enzymes by urate free radical in vitro can be prevented by conjoint use of ascorbate [36, 37]. Urate may act critically in the repair of oxidative damage to nucleobase in DNA [37]. Purines like uric acid are known to be essentially planar molecules which make them stack closely in the interior of double-stranded helixes of DNA [38].

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