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Effects of Vitamin E against Aluminum Neurotoxicity in Rats

V. S. Nedzvetsky[1], M. Tuzcu[2], A. Yasar[3], A. A. Tikhomirov[1], and G. Baydas[3]*

[1]*Department of Biophysics and Biochemistry, Faculty of Biology, Dnepropetrovsk National University, ul. Nauki 13, 49050 Dnepropetrovsk, Ukraine; E-mail: nedzvetskyvictor@ukr.net*
[2]*Department of Biology, Faculty of Science, Firat University, Elazig 23119, Turkey; E-mail: mtuzcu@firat.edu.tr*
[3]*Department of Physiology, Faculty of Medicine, Firat University, Elazig 23119, Turkey; fax: +90-24-37-9138; E-mail: ayasar@firat.edu.tr; baydas@hotmail.com; gbaydas@firat.edu.tr*

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Abstract—The present study examined the protective effects of vitamin E against aluminum-induced neurotoxicity in rats. Wistar rats were given daily aluminum via their drinking water containing 1600 mg/liter aluminum chloride for six weeks. Aluminum induced a significant increase in lipid peroxidation (LPO) in hippocampus and frontal cortex. Furthermore, aluminum caused marked elevation in the levels of the glial markers (glial fibrillary acidic protein (GFAP) and S100B) and proinflammatory cytokines (TNF-α and IL-1β) in both brain areas. Vitamin E treatment reduced the contents of glial markers and cytokines and the levels of LPO. In conclusion, this study demonstrates that vitamin E ameliorates glial activation and reduces release of proinflammatory cytokines induced by aluminum.

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When aluminum (Al) enters the body with food, air, water, and drugs, it can accumulate in all body tissues and organs [1]. Aluminum is known to cause toxic effects to a variety of organ systems including brain, bone, kidney, and blood. Clinical and experimental analyses have provided evidence for Al being implicated in the etiology of various neurological disorders, e.g., Alzheimer's disease, amyotrophic lateral sclerosis, dialysis encephalopathy, Parkinson's disease, etc. [2-4]. Although knowledge about clinical aspects of Al intoxication has increased over the last few years, the mechanisms by which Al interacts with neurons *in vivo* and induces its neurotoxicity are poorly understood. The direct neurotoxic actions of Al include apoptosis, excitotoxicity, influences on neurotransmitter storage and release processes, production of second messengers, activation of cerebrovascular endothelial cells, and both astro- and oligodendroglia [5-7]. It was also suggested that Al leads to changes in glutathione levels, lipid peroxidation (LPO) products, intracellular calcium, and membrane fluidity [8].

Glial cells are known to play a very important role in neuronal survival and functioning, and evidence has accumulated for astrocytes having a role in a number of neurodegenerative disorders [9]. It was reported that Al can induce apoptotic degeneration of astrocytes [10], and that this toxicity is critical in determining neuronal degeneration and death. However, the mechanism by which Al affects glial cells still remains controversial [7, 11].

Reactive gliosis (hypertrophy and astrocyte proliferation) is a widespread response to damage of neurons. Expression of glial fibrillary acidic protein (GFAP) is the hallmark of the activation process. Changes in GFAP levels have been proposed as an index of toxicant-induced reactive gliosis [12]. Astrocytes also "switch on" transcription of genes coding for trophic factors and cytokines.

As an antioxidant, vitamin E protects cell membranes and other fatty cellular components by donating electrons to free radicals. In regards to cell membranes, for instance, polyunsaturated fatty acid-enriched membranes are at greater risk of oxidative damage [13]. Vitamin E can break the chain of free radical propagation along such structures, thereby preserving cellular integrity and function [14]. Several lines of evidence suggest that vitamin E has the ability to protect neuronal tissue in several neurodegenerative disorders including Alzheimer's disease [15]. These neuroprotective properties of vitamin

Abbreviations: LPO) lipid peroxidation; ROS) reactive oxygen species.

* To whom correspondence should be addressed.

E have been attributed largely to its free radical scavenger action [16, 17]. However, vitamin E has additional actions that might be pertinent. One such action of vitamin E is suppression of signaling events necessary for glial activation [18]. The goal of the present study was to examine the effects of vitamin E treatment on levels of the oxidative stress markers and glial markers, and to determine if such treatment would inhibit oxidative stress and reactive gliosis, thus providing a protective effect against Al-induced neurotoxicity in rats.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing between 200 and 250 g at the beginning of the study were purchased from the Animal Research Unit, Firat University, Elazig (Turkey). They were kept in a temperature- and light-controlled room with free access to food pellets and tap water. The animals were randomly divided into four groups (n = 7 animals/group) as follows: one group of animals (control group) received intraperitoneal olive oil, the second group received daily vitamin E at a dose of 100 mg/kg body weight diluted in olive oil (Vit E group), the third group (Al group) was given Al orally via drinking water containing 1600 mg/liter aluminum chloride [19], and the fourth group received the same dose of aluminum chloride plus 100 mg/kg per day vitamin E (Al + Vit E group) for six weeks. Food, water intake, and body weight of the animals were monitored throughout the experimental period. All protocols described were reviewed and approved by the Local Institutional Committee for the Ethical Use of Animals.

Sample collection. On completion of the experimental period, the rats were sacrificed by decapitation. The brain was removed and the hippocampus and frontal cortex were dissected for the biochemical studies. Protein was estimated by the method of Lowry et al. [20].

Immunoblotting. Hippocampus and frontal cortex were homogenized (10% w/v) in 10 mM Tris-HCl (pH 7.4), 0.1 mM NaCl, 1 mM EDTA, 2 mM β-mercaptoethanol, and 0.5% Triton X-100 containing proteinase inhibitors (10 μg/ml soybean trypsin inhibitor, 1 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The homogenates were centrifuged at 40,000*g* for 1 h at 4°C, and the supernatants were collected and stored at −70°C.

SDS-PAGE. Sodium dodecyl sulfate (SDS)-polyacrylamide gradient gel electrophoresis was performed as described previously [21]. Samples and standard protein markers were subjected to an SDS-polyacrylamide gradient gel and the separated proteins were transferred to nitrocellulose filters (Schleich & Schuell Inc., USA). Nonspecific binding was prevented by incubation with 1% bovine serum albumin. The blots were then incubated with primary antibodies, anti-GFAP and anti-S100B (Santa Cruz Biotechnology, Inc., USA) at 1 : 2000 dilution. After 1 h incubation, the blots were washed extensively in TBS-Tween (25 mM Tris-HCl, 0.2 mM NaCl, 0.1% Tween-20). The blots were then incubated for 1 h with a secondary antibody, a goat anti-rabbit Ig peroxidase conjugate (Sigma, USA). Specific binding was detected using diaminobenzidine and H_2O_2 as substrates. The relative amounts of immunoreactive bands on Western blots were quantified in arbitrary units by scanning blots using a computerized software program (LabWorks 4.0; UVP, Inc., UK).

Determination of TNF-α and IL-1β. Tissue samples were processed as previously described [22]. Briefly, total protein was mechanically dissociated from tissue using an ultrasonic cell disruptor. Sonicated samples were centrifuged at 20,000*g* at 4°C for 10 min. The supernatants were removed and stored at 4°C until ELISA was performed. The levels of TNF-α and IL-1β in the tissue supernatants from rat brain were measured using commercially available ELISA kits (R&D Systems, USA). This assay employs the quantitative sandwich enzyme immunoassay technique. The ELISA assays were performed according to the manufacturer's instructions.

Lipid peroxidation (LPO) and glutathione (GSH) assay. NADPH-dependent and Fe^{2+}-induced LPO levels were determined as described previously [23, 24] with minor modifications.

Determination of NADPH-dependent LPO levels. Tissue samples were homogenized in 25 mM Tris-HCl (pH 7.4). The reaction mixtures contained 2 ml of homogenate, 0.1 ml 0.5 μM NADPH, 0.1 ml 2 μM ADP, and 0.1 ml 5 μM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$. Reactions were initiated by the addition of NADPH. Incubations were carried out at 37°C in a shaking water bath.

Determination of Fe^{2+}-induced LPO levels. Two milliliters of homogenates, 0.1 ml 0.5 μM ascorbate, and 0.1 ml 4 μM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ were mixed and incubated at 37°C in a shaking water bath. The amount of malondialdehyde (MDA) was measured using the TBA test and taking molar extinction coefficient $\varepsilon_{532} = 1.56 \cdot 10^5\ M^{-1} \cdot cm^{-1}$.

GSH levels were determined using a GSH-400 kit (Oxis International, Inc., USA). The method is based on a chemical reaction that proceeds in two steps. The first step leads to the formation of substitution products (thioethers) between a 4-chloro-1-methyl-7-trifluoromethyl-quinolinium methylsulfate and all mercaptans. The second step is an elimination reaction, which takes place under alkaline conditions. The reaction is mediated by 30% NaOH that transform the thioether obtained with GSH into a chromophoric thione having maximal absorbance at 400 nm.

Statistical analysis. Results are expressed as means ± SD; significance of differences between the groups compared was evaluated with ANOVA followed by a post-hoc Bonferroni test. The level of significance of $p < 0.05$ was used.

RESULTS

In the present study, we have determined the levels of LPO in brain homogenates using two different methodologies. We found that LPO levels were up to 2-fold lower in the NADPH-dependent system than in the Fe^{2+}-dependent system in hippocampal and cortical homogenates from control rats (table). Treatment with Al significantly increased LPO in both systems but, as seen in the table, the enhancement of LPO induced by Al was higher in the NADP-dependent system. Treatment with vitamin E significantly reduced LPO levels in hippocampus and frontal cortex both in the Fe^{2+}-dependent and the NADPH-dependent systems. Neither Al nor vitamin E treatment changed the levels of GSH in brain homogenates (table).

The levels of TNF-α were markedly elevated in hippocampus and frontal cortex of Al treated rats ($p < 0.001$). Daily administration of vitamin E significantly reduced TNF-α levels in both hippocampus and frontal cortex ($p < 0.01$ and $p < 0.05$, respectively). Similarly, Al treatment led to a marked elevation in the concentration of IL-1β both in hippocampus and cortex ($p < 0.01$), and treatment with vitamin E reduced its levels significantly in both brain areas ($p < 0.05$; table).

Long-term Al treatment resulted in distinct elevation in GFAP content in hippocampus and frontal cortex of rats ($p < 0.05$). Vitamin E administration decreased GFAP content in cortex ($p < 0.05$) but not in hippocampus of Al treated rats (Fig. 1). Furthermore, Al treatment significantly elevated S100B levels both in hippocampus ($p < 0.001$) and frontal cortex ($p < 0.05$; Fig. 2). Daily administration of vitamin E effectively reduced S100B levels in hippocampus and frontal cortex ($p < 0.001$ and $p < 0.01$, respectively).

DISCUSSION

Although normal uptake of Al by the brain is very slow, it cannot be eliminated from the brain and it therefore accumulates. Previous studies have shown that animals exposed to Al have more than 4-fold increased Al concentrations in brain. Thus, accumulation of Al has been recognized as a contributing factor in several neurological disorders [25]. However, the mechanism by which Al causes neuronal damage is not fully understood.

Since free radical generation has been implicated in Al toxicosis leading to neural damage [19, 26, 27], the present study focused on the evaluation of the molecular

Levels of TNF-α, IL-1β, LPO, and GSH in hippocampus and cortex of control, vitamin E only, Al only, or Al + vitamin E treated rats

Parameter	Control	Vit E	Al	Vit E + Al
			Hippocampus	
TNF-α, pg/100 μg protein	0.33 ± 0.05	0.32 ± 0.04	0.57 ± 0.08***	0.39 ± 0.06^{++}
IL-1β, pg/100 μg protein	0.17 ± 0.03	0.16 ± 0.02	0.27 ± 0.04**	0.20 ± 0.03^{+}
NADP-dependent LPO, nmol/mg protein	13.0 ± 2.0	5.0 ± 1.0***	36.0 ± 5.0***	11.0 ± 2.0^{+++}
Fe^{2+}-dependent LPO, nmol/mg protein	27.2 ± 3.2	18.4 ± 1.6**	32.8 ± 3.6*	13.2 ± 1.6^{+++}
GSH, nmol/mg protein	3.19 ± 0.37	3.51 ± 0.42	2.97 ± 0.31	3.19 ± 0.37
			Cortex	
TNF-α, pg/100 μg protein	0.32 ± 0.05	0.30 ± 0.04	0.51 ± 0.08***	0.41 ± 0.06^{+}
IL-1β, pg/100 μg protein	0.22 ± 0.03	0.20 ± 0.03	0.30 ± 0.04**	0.24 ± 0.03^{+}
NADP-dependent LPO, nmol/mg protein	12.0 ± 2.0	5.0 ± 1.0***	31.5 ± 4.5***	9.0 ± 1.5^{+++}
Fe^{2+}-dependent LPO, nmol/mg protein	24.8 ± 3.2	18.8 ± 2.4*	30.0 ± 3.6*	15.2 ± 2.0^{++}
GSH, nmol/mg protein	3.29 ± 0.42	3.45 ± 0.42	3.08 ± 0.37	3.19 ± 0.32

Notes: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus control; $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$, and $^{+++}$ $p < 0.001$ versus Al group.

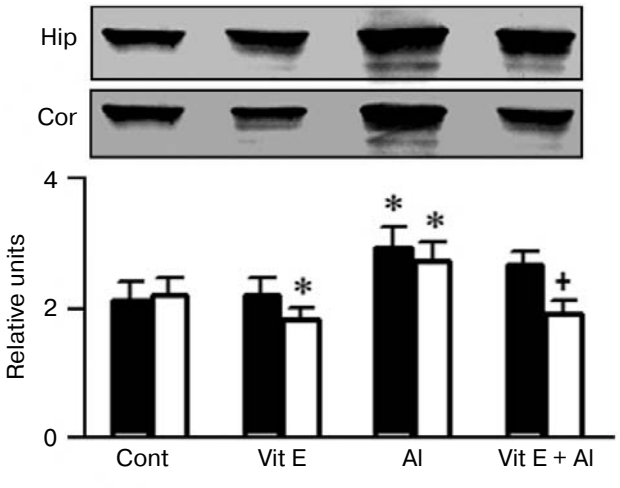

Fig. 1. Representative western blots for GFAP from hippocampus (Hip) and frontal cortex (Cor) obtained from rats (n = 7 animals/group) treated with vitamin E only, Al only, or both substances (Vit E + Al) for six weeks. The relative amounts of the immunoreactive bands on Western blots were quantified in arbitrary units by scanning the blots using a computerized software program. Black and white columns designate hippocampus and cortex, respectively (* $p < 0.05$, versus control; $^{+}$ $p < 0.05$, versus Al group).

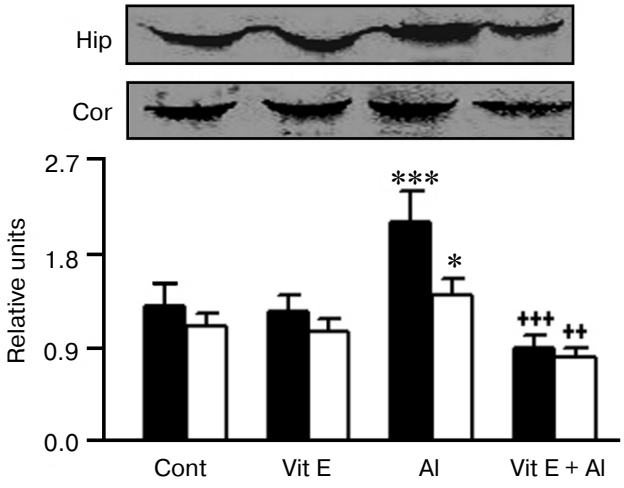

Fig. 2. Representative western blots of S100B protein. The relative amounts of immunoreactive bands on Western blots were quantified in arbitrary units by scanning the blots using a computerized software program. Mean levels of S100B in hippocampus (Hip) and frontal cortex (Cor) of rats treated with vitamin E only, Al only, or both substances (Vit E + Al) for six weeks. Black and white columns designate hippocampus and cortex, respectively (*** $p < 0.001$ and * $p < 0.05$, versus control; $^{+++}$ $p < 0.001$ and $^{++}$ $p < 0.01$, versus Al group).

changes during Al exposure and the therapeutic efficacy of vitamin E on Al-induced reactive gliosis in rats. The results presented showed a significant increase in the levels of LPO in hippocampus and frontal cortex of Al-treated rats, and this suggests the participation of free-radical induced oxidative cell injury in mediating the toxicity of Al [28, 29]. Recent investigations have shown that Al is a pro-oxidant agent, and antioxidants can protect against oxidative damage in Al-exposed rats [30, 31].

Administration of vitamin E along with Al reduced the products of LPO induced by Al exposure in the studied brain regions. Vitamin E is the major lipid-soluble, chain-breaking antioxidant in biological systems. The antioxidant properties of vitamin E have been extensively studied, as has the use of this compound as a cell protector and as a potential disease-preventing agent [32-34].

We studied here the Al-induced activation of glial cells using GFAP and S100B as markers. Glial activation is known to occur as a response to pathogenic insults and may contribute to immune responses in neurodegenerative disease [35]. In the present study, we show that administration of Al significantly increases the GFAP and S100B levels both in cortex and in the hippocampus. Similar evidence for GFAP activation in different brain areas after Al exposure has been provided by others [36, 37]. In contrast to our data on glial activation, some authors reported that GFAP content decreased in rat cortex following Al exposure [38].

The changes observed in the reactive glial cells can reflect an increased protein synthesis activity as a compensatory mechanism for the repair of damaged tissue [39]. However, recent studies have shown that activated glial cells may exert a cytotoxic function by releasing reactive oxygen species (ROS), nitric oxide, proteinases, or inflammatory cytokines [40, 41]. Cytokines produced by glial cells can activate microglia in an autocrine manner and, further, may activate astrocytes that in turn become a source of successive cytokines of potentially neurotoxic function [42, 43]. Thus, Al might induce neurotoxicity as an indirect effector of glial cells.

In the present study, we showed that Al exposure significantly increased the levels of TNF-α and IL-1β both in hippocampus and frontal cortex. The increase in the level of TNF-α observed in this study can lead to the activation of glial cells. The proinflammatory cytokine TNF-α can be synthesized and released in the brain by astrocytes, microglial, and some neurons [44, 45]. TNF-α and IL-1β have been demonstrated to be elevated in various models of nervous system injury and are thought to contribute to the pattern and severity of the response [46]. In agreement with the present results, previous studies indicate that Al can induce the production of pro-inflammatory cytokine [47].

The levels of activated NF-κB, an immune related factor, were found to be significantly increased in the brains of mice treated with Al [48]. Activated NF-κB has been shown to increase TNF-α synthesis [49]. These findings indicate that Al may cause neurodegeneration by two

potentially interrelated mechanisms, immune-mediated and increased oxidative stress-mediated neuronal death.

In vitamin E-administered rats, we observed a significant reduction in Al-induced increase in the levels of TNF-α and IL-1β in hippocampus and cortex. Furthermore, we showed here that vitamin E reduced the levels of GFAP in frontal cortex. Our results demonstrate that administration of vitamin E inhibits Al-induced activation of glial cells and pro-inflammatory cytokine expression in brain areas. Consistent with the present findings, we recently have shown that administration of vitamin E can prevent reactive gliosis induced by uncontrolled diabetes mellitus [50]. The reduction in the level of TNF-α and IL-1β with vitamin E co-administered with Al exposed rats may be ascribed to the inhibition of activation and translocation of NF-κB by this antioxidant [51]. Furthermore, glial cells activated *in vitro* produce toxic reactive oxygen radicals, substances that may directly damage neurons. Increase in the production of ROS leads to activation of NF-κB by causing the release of the inhibitory subunit and increase in the synthesis of TNF-α [49]. Several lines of evidence suggest that vitamin E protects neural tissue against oxidative stress by scavenging free radicals [16, 34]. It appears that there are two main neuroprotective effects of vitamin E against Al neurotoxicity: first, it scavenges ROS induced by Al exposure to inhibit glial activation; second, vitamin E inhibits the activation and generation of many neurotoxic factors such as pro-oxidant cytokines generated by activated glial cells.

The present study offers a novel cellular mechanism underlying the beneficial effects of vitamin E against Al-induced degeneration of the central nervous system. The importance of our findings is that, in addition to the beneficial effects of providing direct antioxidant protection to neurons, vitamin E may provide neuroprotection through the suppression of intracellular signaling steps involved in activation of glial cells.

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