



**Original Contribution**

**EFFICACY OF VITAMIN E, PHOSPHATIDYL CHOLINE, AND PYRUVATE ON BUFFERING NEURONAL DEGENERATION AND OXIDATIVE STRESS IN CULTURED CORTICAL NEURONS AND IN CENTRAL NERVOUS TISSUE OF APOLIPOPROTEIN E-DEFICIENT MICE**

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**Abstract**—Oxidative stress is a pivotal factor in neuronal degeneration. However, vitamin E was only marginally effective in clinical trials. We examined whether or not a mixture of vitamin E (as  $\alpha$ -tocopherol), sodium pyruvate and phosphatidyl choline (PC), a mixture that promotes wound healing in non-neuronal systems, would provide neuroprotection beyond that observed with vitamin E alone. Combined treatment with these agents improved survival and neuritic spouting of murine embryonic cortical neurons in culture, and provided neuroprotection against oxidative damage following treatment with hydrogen peroxide. Dietary treatment with these three agents also compensated for the diminished oxidative buffering capacity of brains of apolipoprotein E-deficient mice, while vitamin E alone failed to do so. These data underscore the possibility that critical nutritional deficiencies may modulate the impact of genetic compromise on neurodegeneration. © 2002 Elsevier Science Inc.

**Keywords**—Oxidative stress, Antioxidants, Vitamin E, Apolipoprotein E, Neurodegeneration, Transgenic mice, Free radicals

**INTRODUCTION**

**AQ:1,2** Oxidative damage is at the core of neuronal degeneration in Alzheimer’s disease (AD; [1,2]). Restoring or maintaining oxidative buffering capacity, and/or quenching reactive oxygen species (ROS), therefore represents one potential therapeutic approach to minimize neurodegeneration. However, a recent clinical study demonstrated only limited efficacy of the antioxidant vitamin E in delaying the severity of some clinical AD symptoms [3]. Despite the use of a substantial dose of vitamin E, these data did not achieve statistical significance [4]. Accordingly, additional treatments, beyond that of vitamin E

alone, may be required to achieve clinical effectiveness [5].

Topical and dietary application of a mixture of  $\alpha$ -tocopherol (vitamin E), sodium pyruvate, and phosphatidyl choline (PC) promotes wound healing in non-neuronal systems [6–8]. This formulation exerts synergistic effects substantially beyond that achieved with individual, or mixtures of any two constituents. We examined herein the relative efficacy of vitamin E alone, and vitamin E along with PC and pyruvate, against oxidative stress in brain by treating cultured cortical neurons; these neurons have been useful for sorting the order of events accompanying Abeta neurotoxicity and its alleviation by antioxidants [9–11]. We also subjected normal and transgenic mice lacking apolipoprotein E to a diet that promotes oxidative stress in the presence and absence of vitamin E, PC, and pyruvate. Apolipoprotein E (ApoE) is a cholesterol transport protein that is important in development and regeneration of the nervous system. The

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ApoE4 allele is associated with a 3–4-fold increase in the prevalence of AD and with a younger mean age of onset [12], although the precise mechanisms underlying this association are not fully resolved. Transgenic mice lacking ApoE demonstrate synaptic loss and cytoskeletal compromise [13]. Since ApoE normally redistributes lipid breakdown products [14], such deficiencies may be crucial under conditions of oxidative stress and resultant membrane compromise [15,16]. In this regard, the ApoE4 allele exhibits deficient antioxidant properties [17,18]. ApoE deficiency may also increase the levels of free iron, which may trigger production of ROS [19]. The relatively increased susceptibility of ApoE knockout mice to oxidative damage [20,21], coupled with the ability of vitamin E to alleviate brain oxidative stress in these mice under certain conditions [21], make these mice an ideal model for investigating the relative efficacy of combined treatment with vitamin E, PC, and pyruvate.

#### MATERIALS AND METHODS

Cortical neurons were cultured from day 16 murine embryos as described previously [10]. 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, and supplemented with various combinations of 0.15 mg/ml vitamin E, 0.19 mg/ml Na pyruvate, and 0.20 mg/ml PC. Since the medium already contains 0.11 mg/ml Na pyruvate, cultures supplemented with additional pyruvate therefore contained a total of 0.13 mg/ml pyruvate; all cultures therefore contained at least a basal level of 0.11 mg/ml pyruvate. Additional cultures received a mixture of 50% fresh serum-free medium and 50% serum-free medium that had been in contact with confluent murine neonatal astroglial cells for 24 h (“glial conditioned medium”, or “GCM”; [22]). Cell viability was quantified after 3 d using a commercial “live/dead” assay (Molecular Probes, Eugene, OR, USA) as previously utilized for cultured cortical neurons [23]. The extent of sprouting was also quantified after 72 h; neurons that had elaborated one or more neurites  $\geq 2$  respective somal diameters in length were scored as positive, while live neurons (ascertained by the above assay) lacking neurites or that had elaborated neurites only  $< 2$  somal diameters were scored as negative [22,24].

Additional cultures were treated with 10  $\mu\text{M}$  hydrogen peroxide with and without 0.0625 IU/ml vitamin E, 0.13 mg/ml Na pyruvate, and 0.2 mg/ml separately or in combination for 30 min. Cultures then received dichlorofluorescein diacetate (DCFDA) and ROS were analyzed microscopically as described [10]. Alternatively, peroxide-treated cultures were scraped from the plate, homog-

enized, and analyzed by fluorescent spectrophotometry for thiobarbituric acid-reactive substances (TBARs) as an endpoint index of oxidative damage [25].

Normal C57Bl/6J mice and ApoE<sup>tm1U<sup>ne</sup></sup> homozygous “knockout” mice on a C57Bl/6J background ([26]; obtained from Jackson Laboratories, Bar Harbor, ME, USA) received a vitamin-free, basal diet (“AIN-76”; Purina/Mother Hubbard, Inc., St. Louis, MO, USA [27, 28]) lacking or supplemented with vitamin E (as  $\alpha$ -tocopherol, 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. 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 by standard methodologies previously utilized to analyze CNS in AD [25]. Mice were between 10 and 14 months of age. Data were pooled from four separate experiments, each of which contained 3–4 normal and 3–4 ApoE mice of mixed gender per diet, for a combined total of 12–16 mice for each genotype. The dietary regimen utilized herein specifies replacement of a portion of the water utilized to dissolve the basal diet powder with fish and corn oil to achieve a pro-oxidant state along with iron [27]; inclusion of oil alone did not alter TBARs in CNS; TBARs ( $\mu\text{M}$ ) without oil were  $13.4 \pm 6.0$ , while those with inclusion of the above oil (without iron) were  $12.4 \pm 2.9$  ( $p < .8$ , Student’s *t*-test,  $n = 3$  mice per group). Statistical analyses were conducted by individual comparisons among groups via Student’s *t*-test; values were considered statistically different if  $p \leq .05$ .

#### RESULTS

To ascertain the impact of combined treatment with vitamin E, PC, and pyruvate on neurons, we generated primary cultures of embryonic murine cortical neurons. Embryonic murine cortical neurons were cultured for 72 h in the presence of 10% fetal bovine serum and B27 supplements, after which medium was replaced with medium lacking serum and supplemented B27 formulation lacking antioxidants, along with various combinations of vitamin E, PC, and pyruvate. Our initial trials demonstrated that the concentrations of vitamin E, PC, and pyruvate that were effective at promoting wound healing in non-neuronal systems were toxic to cultured neurons (not shown). However, treatment with reduced concentrations of these constituents (0.0625 IU/ml vitamin E, 0.13 mg/ml Na pyruvate, and 0.20 mg/ml PC) enhanced neuronal survival and sprouting in culture. Supplementation with these concentrations was mark-

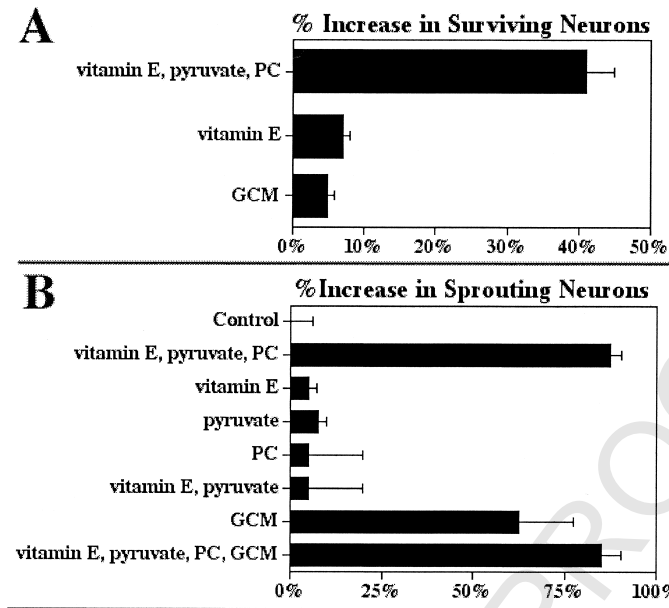


Fig. 1. Vitamin E, PC, and pyruvate enhance survival and sprouting of cultured murine cortical neurons. (A) Embryonic cortical neuronal cultures received, individually and in combinations, 0.0625 IU/ml vitamin E, 0.13 mg/ml Na pyruvate, and 0.20 mg/ml PC, and GCM. Note that combined treatment with vitamin E, PC, and pyruvate was markedly more effective than basal culture medium, vitamin E alone, or GCM at promoting neuronal survival. (B) Neurons were scored as possessing or lacking at least 1 neurite  $\geq 2$  respective somal diameters. Approximately 42% of neurons sprouted neurites when cultured with serum alone. Treatment with vitamin E alone, pyruvate alone, or vitamin E and pyruvate in combination did not improve sprouting beyond this value. By contrast, combined treatment with vitamin E, PC, and pyruvate increased the percentage of neurite-bearing neurons to approximately 75%. GCM increased the percentage of neurite-bearing neurons to 68%.

edly more effective than vitamin E alone or GCM at promoting neuronal survival (Fig. 1A). We next examined the extent of sprouting in surviving neurons. Neurons were scored as possessing or lacking at least 1 neurite  $\geq 2$  respective somal diameters (e.g., [22]). Combined treatment with vitamin E, PC, and pyruvate increased the percentage of neurite-bearing neurons to approximately 75%, as compared to 42% in cultures receiving 10% serum alone. The influence of these agents in combination was similar to that of GCM, which GCM increased the percentage of neurite-bearing neurons to 68%. Treatment with vitamin E alone, pyruvate alone, or a combination of vitamin E and pyruvate did not improve sprouting beyond that observed in the presence of serum alone (Fig. 1B).

We next examined whether or not combined treatment with vitamin E, PC, and pyruvate could protect cultured neurons against oxidative stress by treatment with 10  $\mu$ M hydrogen peroxide in the presence and absence of these compounds individually or in combination. Treatment with vitamin E, PC, and pyruvate in combination prevented the increase in ROS that accompanied hydrogen peroxide treatment (Fig. 2). Treatment with individual agents did not reduce ROS under these conditions (Fig. 2). 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. Peroxide-treated cultures receiving peroxide along with vitamin E, PC, and pyruvate displayed a  $35.1 \pm 13.8\%$  reduction in TBARs versus those treated with peroxide alone ( $p < .02$ ). Cultures receiving peroxide along with vitamin E did not display a reproducible reduction in TBARS versus those in cultures receiving peroxide alone; samples receiving vitamin E contained  $10.8 \pm 31.7\%$  less TBARs than did those receiving peroxidase alone ( $p < .60$ ). Note that the standard deviation of these values is so large that there was no reproducible decrease following treatment with vitamin E alone.

Having ascertained that combined treatment with vitamin E, PC, and pyruvate can exert beneficial effects on murine neurons in culture, we next examined whether or not treatment with these three compounds provided improved neuroprotection over vitamin E alone in situ. Normal C57Bl/6J mice and ApoE<sup>tm1U<sup>ne</sup></sup> homozygous “knockout” mice on a C57Bl/6J background received a diet lacking vitamin E. Additional mice were subjected to oxidative stress by inclusion of iron in their diet. Groups of iron-challenged mice received vitamin E alone or vitamin E along with PC and pyruvate. After 1 month of these various dietary regimens, homogenates of total CNS tissue were examined for TBARs as an index of oxidative damage. CNS of normal and ApoE knockout

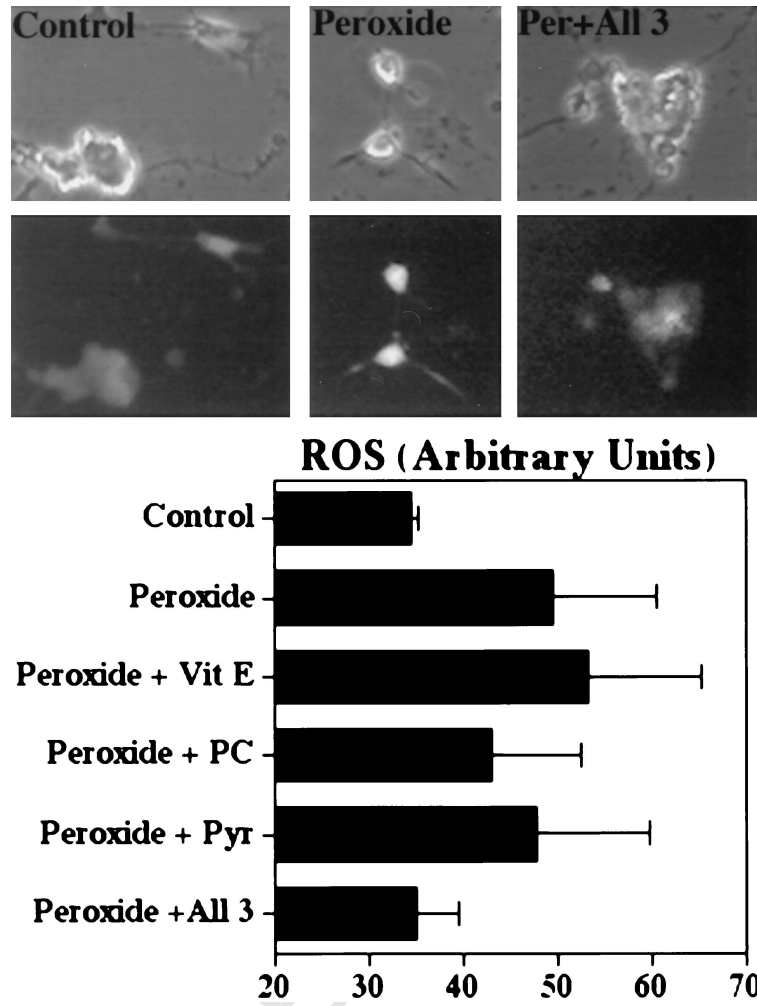


Fig. 2. Vitamin E, PC, and pyruvate protect against oxidative stress in cultured neurons. Embryonic cortical neuronal cultures received 10  $\mu$ M hydrogen peroxide with and without 0.0625 IU/ml vitamin E, 0.13 mg/ml Na pyruvate, and 0.20 mg/ml separately or in combination for 30 min, after which cultures received DCFD, as described in Materials and Methods. Micrographs present phase-contrast and corresponding fluorescent images. The accompanying graph presents the mean  $\pm$  standard error of the mean fluorescence intensity for 15–30 individual cells under each condition. Values for peroxide-treated cultures, and those receiving vitamin E, PC, or pyruvate individually differed statistically from untreated controls ( $p < .05$ ); peroxide-treated cultures receiving all three were statistically identical to controls ( $p > .05$ ). Note that combined treatment with vitamin E, PC, and pyruvate attenuated the increase in ROS following hydrogen peroxide treatment, while individual agents did not similarly attenuate this increase.

mice exhibited no significant difference in TBARs prior to oxidative challenge ( $10.9 \pm 6 \mu$ M versus  $15.5 \pm 1 \mu$ M for normal and ApoE knockout mice, respectively;  $n = 3$  mice each;  $p < .27$ , Student's *t*-test). Dietary challenge with iron in the absence of vitamin E increased TBARs in CNS of both normal and ApoE mice by approximately 50% (Fig. 3). Vitamin E substantially diminished this increase in CNS of normal mice, but was unable to do so in CNS of ApoE knockout mice (Fig. 3). Combined treatment with vitamin E, PC, and pyruvate was markedly more effective at quenching oxidative damage than was vitamin E alone. Supplementation with vitamin E, PC, and pyruvate reduced TBARs in CNS of iron-challenged normal and ApoE knockout mice sub-

stantially below those observed for CNS of these mice prior to iron challenge (Fig. 3). The markedly differential TBARs observed among various diets demonstrate that TBARs are not derived simply by the presence of this level of dietary iron.

Unlike its limited efficacy in CNS tissue, vitamin E alone was as effective in mice as combined treatment with vitamin E, PC, and pyruvate at quenching TBARs in liver tissue of both normal and ApoE knockout mice (Fig. 3). The markedly differential influence of these dietary regiments on CNS and liver tissue also make it unlikely that residual plasma within CNS vasculature contributed to the differential TBARs observed in normal and ApoE knockout CNS.

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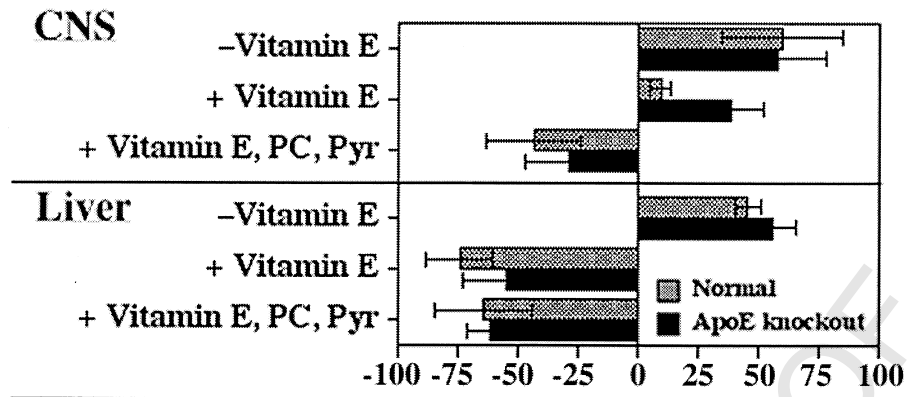


Fig. 3. Oxidative damage in normal and ApoE-knockout mice following dietary challenge with iron in the presence and absence of vitamin E. Normal and ApoE “knockout” mice ( $n = 4$  of each strain per diet) received a basal diet (“AIN-76”; Purina) with and without vitamin E (as tocopherol acetate, 50 IU/kg), Na pyruvate: (30 g/500 g total diet) PC (5g/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. Values represent the percent change (mean  $\pm$  standard error of the mean) observed for iron-challenged normal or ApoE mice, respectively, versus those receiving no vitamin E and no iron. Values for CNS of normal mice receiving vitamin E differed significantly from those not receiving vitamin E ( $p < .05$ ); values for CNS of ApoE knockout mice receiving vitamin E did not differ significantly from those not receiving vitamin E. Values for normal and ApoE knockout mice receiving vitamin E, PC, or pyruvate differed statistically from those receiving vitamin E as well as those not receiving vitamin E ( $p < .05$ ). Values for liver of normal and ApoE knockout mice receiving vitamin E differed significantly from those not receiving vitamin E ( $p < .05$ ); values for liver of ApoE knockout mice receiving vitamin E did not differ significantly from those not receiving vitamin E ( $p < .05$ ). Values for normal and ApoE knockout mice receiving vitamin E, PC, or pyruvate did not differ statistically from those receiving vitamin E.

## DISCUSSION

We demonstrate herein that combined treatment with vitamin E, PC, and pyruvate, previously shown to exert synergistic effects on wound healing in non-neuronal systems [6–8], could provide protection beyond that provided by vitamin E alone for neurons in culture and CNS tissue in situ. These findings also demonstrate that ApoE knockout mice subjected to vitamin deficiency are less capable of buffering the consequences of oxidative challenge from dietary iron than are normal mice (see also [19–21]). The ability of vitamin E alone to protect liver but not CNS of ApoE mice from oxidative damage further suggests that CNS tissue, perhaps because of the blood-brain barrier, may be relatively less accessible than other organs to certain systemic treatments, and therefore may require additional agents and/or higher concentrations than other organs.

The mechanism underlying the increased efficacy of cotreatment with PC and pyruvate has not been determined. While vitamin E can prevent oxidative damage to the plasma membrane, the lipophilic nature of vitamin E restricts its ability to quench cytosolic products derived from lipid peroxidation [17,29,30]. One likely possibility is that pyruvate, which is cell permeant [31], quenches intracellular oxidative species that may be inaccessible to vitamin E (e.g., [32]). Similar findings were reported following comparison of the efficacy of vitamin E and N-acetyl cysteine (NAC) on Abeta-induced oxidative stress [9]. Both vitamin E and NAC were effective if

applied simultaneously with Abeta; however, the efficacy of vitamin E was diminished if applied subsequently to Abeta, while that of NAC was not, suggesting that NAC, a cell-permeant antioxidant and glutathione precursor [33], was able to attenuate cytosolic ROS. In addition, PC may provide stability to the plasma membrane, which may be crucial for neuronal survival following oxidative membrane damage [7]. PC provides a source of fatty acids for membrane stabilization and repair, and, in doing so, obviates metabolic steps that themselves generate ROS [6,7]; this putative function is particularly important with regard to neurons, since a major portion of the oxidative stress imparted to neurons is targeted towards lipid peroxidation [34–38]. It remains possible that metabolites of pyruvate and/or PC are responsible for their additional protective effect. Notably, our data are derived from total CNS tissue, not just “at-risk” areas for oxidative damage and AD neurodegeneration; accordingly, the damage in key areas such as hippocampus and cortex are likely to be even more elevated.

Prior studies underscore the importance of vitamin E in maintaining neuronal health in culture (e.g., [10,11]) and in situ. Prolonged vitamin E deficiency (9–12 months) is known to promote oxidative damage in CNS tissue of Apo E knockout [20,21]. In addition, vitamin E supplementation can counteract spatial learning deficits in ApoE knockout mice [21]. Our findings indicate, however, that vitamin E alone may be insufficient to

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provide neuroprotection under certain conditions. This physiological relevance of this latter consideration is underscored by the limited efficacy of vitamin E on the progression of AD in clinical trials [3].

Compensation for the complete absence of ApoE function by supplementation with vitamin E, PC, and pyruvate allows the speculation that a similar dietary regimen may compensate at least in part for the deficient antioxidant properties inherent in the ApoE4 allele [17, 18]. These data also suggest that dietary deficiencies in compounds that normally promote neuronal oxidative buffering capacity may have particular deleterious consequences on neuronal homeostasis when coupled with genetic compromise in oxidative buffering capacity. Additional latent genetic deficiencies related to AD, as well as other neuronal disorders involving oxidative stress, may manifest only when coupled with dietary compromise. These data may provide insight into why ApoE4, although strongly linked to AD, exhibits only approximately 60% penetrance [12]. Further investigations of the interplay between nutrition and genetic compromise may provide insight into the etiology and pathogenesis of chronic neurodegenerative conditions including AD.

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