

Vitamin E Derivative Alpha-Tocotrienol Failed to Show Neuroprotective Effects after Embolic Stroke in Rats

*¹Mohammad Allahtavakoli, ¹Aliasghar Pourshanazari, ²Behnam Heshmatian

Abstract

Objective(s)

Previous studies have demonstrated that pretreatment with alpha-tocotrienol (α -TCT) can reduce ischemic damage in mice following middle cerebral artery (MCA) occlusion. It is also reported to decrease stroke-dependent brain tissue damage in 12-Lox-deficient mice and spontaneously hypertensive rats. In the present study, the neuroprotective effects of α -TCT and rosiglitazone (RGZ) at 3 hr after cerebral ischemia were investigated.

Materials and Methods

Stroke was induced by embolizing a preformed clot into the MCA. Rats were assigned to vehicle, α -TCT (1 or 10 mg/kg), RGZ and sham-operation.

Results

Compared to the control group, only RGZ decreased infarct volume ($P<0.05$), neurological deficits ($P<0.05$) and sensory impairments ($P<0.01$) but low and high doses of α -TCT did not show any significant neuroprotective effect.

Conclusion

Our data showed that α -TCT was not neuroprotective at 3 hr after the embolic model of stroke. Further studies should be undertaken to clarify the neuroprotective effects of α -TCT after stroke.

Keywords: Alpha-tocotrienol, Cerebral ischemia, Embolic model, Neuroprotection, Vitamin E

1- Department of Physiology, School of Medicine, Rafsanjan University of Medical Sciences, Rafsanjan, Iran

* Corresponding author: Tel: +98-391-5234003, Fax: +98-391-5225209; email: alahtavakoli@razi.tums.ac.ir

2- Department of Physiology, School of Medicine, West Azarbaejan University of Medical Sciences, Oroomiah, Iran

Introduction

Despite several advantages in stroke care and therapy, only a small number of acute stroke patients (3%) receive specific therapy (1). Sustained attempts to increase this percentage include the extension of the 3 hr time window for intravenous thrombolysis (2). In addition to vessel recanalizing strategies, research has focused on the introduction of neuroprotective pharmacological therapies. Potential neuroprotective drugs were evaluated in clinical trials but except thrombolysis therapy, no successful candidates could be identified (3).

Several concomitant factors have likely contributed to these failures, including poor preclinical pharmacodynamic evaluations, especially narrow treatment windows. Evaluation of long-term recovery in both permanent and transient focal stroke models, improves patient selection criteria and trial design based on the preclinical modeling (4). As suggested by STAIR (Stroke Therapy Academic Industry Roundtable), the efficacy of a new neuroprotective drug should be demonstrated in a variety of stroke models performed in different species and by different laboratories. Outcome measures should include both infarct size and behavioral assessments, with attention to the therapeutic time window in relationship to the time period of penumbral survival in the models being used (5).

Clinical and experimental findings have suggested that free radical-induced lipid peroxidation is an important factor in the pathogenesis of ischemic brain damage (6). Various natural antioxidants are present in cerebral tissue, of which the lipid-soluble nutrient vitamin E scavenges lipid peroxy radicals and thereby inhibits lipid peroxidation. However, during ischemia and subsequent reperfusion, because of an overwhelming production of radicals, natural antioxidants cannot suppress the radicals. In these circumstances, administration of Alpha-tocotrienol (α -TCT) have been shown to reduce ischemic damage in mice by middle cerebral artery (MCA) occlusion (7) or stroke-

dependent brain tissue damage in 12-Lox-deficient mice and spontaneously hypertensive rats (8). It has been suggested that at micromolar concentrations, α -TCT protects neural cells by virtue of its antioxidant property and at nanomolar concentrations; it prevents neurodegeneration by regulating specific signaling processes involved in (9). In the studies that reported the neuroprotective effects of α -TCT, functional outcomes were not investigated and the drug administered only before or immediately after ischemia (7, 8). Although functional outcome does not always correlate with infarct size, it is the measure used in clinical trials of neuroprotective agents (10). Due to delayed hospitalization, even in recent clinical trials, fewer than 2% of patients received study agent within 2 hr of onset of event (11). The purpose of this study is to examine the neuroprotective effects of α -TCT at 3 hr after cerebral ischemia induced by embolic model of stroke in rats. The embolic model has been used previously in different experiments to induce experimental stroke in rodents. This model mimics human stroke and is more relevant to the pathophysiological situation in patients. To the best of our knowledge, neither the long-term beneficial effects of α -TCT on ischemic damage nor its effects on functional outcomes after embolic stroke have been examined.

Materials and Methods

All procedures used in this study were carried out in accordance with the Prevention of Cruelty to Animals Act 1986 under the guidelines of the NH and MRC Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia. Adult male hooded wistar rats weighting 300 to 350 g were maintained on a 12 hr light/dark cycle with food and water available *ad libitum*. Eight animals were used in each group. Rats were assigned to vehicle (Dimethylsulfoxide, DMSO), α -TCT (1 or 10 mg/kg) (100%; Carotech Selangor, Malaysia), rosiglitazone (RGZ; Alexis biochemicals, Lausanne, Switzerland) and sham-operation. α -TCT and

RGZ (3 mg/kg) were dissolved in DMSO and sterile 0.9% saline (up to 10% v/v) and injected (ip) 3 hr after the middle cerebral artery (MCA) embolization.

Focal embolic stroke model

Clot formation

One day before ischemic onset, arterial blood was withdrawn from donor rats into PE-50 tubes, stored at room temperature for 2 hr, and then kept at 4 °C for 22 hr.

Physiological parameters monitoring

Rats were anesthetized with isoflurane (5% induction and 2% maintenance) (Aerrane, Fort Dodge, IA, USA). Body temperature was maintained normothermic (37 °C) by using a heating pad placed under the animal, and temperature was monitored by means of a rectal temperature probe. Arterial blood samples were obtained via the catheter placed in the tail artery, for continuous blood pressure monitoring. A blood gas, oximetry, electrolyte and metabolic system machine (Radiometer Medical A/S, Copenhagen, Denmark) was used to monitor arterial Pa_O₂, Pa_CO₂, pH and glucose. Blood was analyzed exactly 5 min before and after embolization.

Cerebral blood flow monitoring

Relative regional cerebral blood flow was monitored with laser Doppler monitor (LD-CBF). A coronal incision was made across the scalp, the temporalis muscle was reflected and a burr hole (1 mm posterior to the bregma and just lateral to the linea temporalis) was created (12). The Doppler probe was placed extradurally. The Doppler probes were fixed to the burr holes with a 2 mm cranial bolt (Integra Scientific) to eliminate motion and ensure stability. Recordings were taken at a frequency of 2 Hz for a minimum of 10 min prior to embolization, continuously throughout the surgery and for a minimum of 5 min after clot injection. According to the method described by Dinapoli *et al* (2006), accurate laser Doppler placement over the MCA perfusion area was verified by pinching temporarily the

common carotid artery, before induction of the stroke (12).

Focal cerebral ischemia

The focal cerebral ischemia was induced by embolizing a preformed clot into the MCA as reported previously (12, 13). A longitudinal incision of 1.5 cm in length was made in the midline of the ventral cervical skin. The right common carotid artery, right internal carotid artery, and right external carotid artery were exposed. The distal portion of the external carotid artery was ligated and cut. Coagulated blood was subsequently sectioned into 20 mm segments, washed with saline and transferred to a modified PE-50 catheter with a tip diameter of 0.3 mm for injection. The modified PE-50 tube with the 20 mm clot (5 µl) was connected to a 50 µl Hamilton lock syringe, and advanced 19 mm in the internal carotid artery until its tip was away from the origin of the MCA. The preformed clot was then injected, and the catheter was removed. A minimum initial reduction of 70% in the laser Doppler reading was considered a successful occlusion of middle cerebral artery perfusion territory (12). Animals without 70% reduction in the laser Doppler reading, were deemed to have had failed successful clot placement and were excluded from any further study. For sham-operated animals, 5 µl saline was injected. The wound was closed and the animal returned to its cage. The duration of surgery did not exceed 30 min in any case.

Measurement of infarct volumes

The quantification of infarct volume has been previously described in detail (12, 13). All animals were sacrificed 48 hr after the onset of MCA occlusion. The brains were removed from the skull, frozen at -70 °C for 5 min and using a brain matrix sliced into 2 mm intervals. A total of 6 coronal sections were collected, and the sections were stained with a 2% 2, 3, 5- triphenyltetrazolium chloride solution (Sigma, UK) at 37 °C and fixed by immersion in a 10% phosphate-buffered formalin solution. The stained brain sections were placed directly on the scanning screen of a color flatbed scanner (Canon, Japan). The

person who analyzed the images was unaware of the treatments animals received, using a commercial image processing software program. The total volume of each hemisphere and infarction was determined by integration of the distance of the 6 sections. The infarct volume was calculated with the following formula:

Infarct volume= [volume of left hemisphere-(volume of right hemisphere-measured infarct volume)]/volume of left hemisphere].

Evaluation of behavior

Neurological deficits were recorded before embolization and 2, 24 and 48 hr after it. Any animal with neurological sign before studying was removed from the study. Neurological deficits were determined with a modified 6-point scale (14) as follow: 0, no observable deficit; 1, forelimb flexion; 2, forelimb flexion plus decreased resistance to lateral push; 3, unidirectional circling; 4, unidirectional circling plus decreased level of consciousness; 5, no spontaneous walking and lying in the cage on left side or death. For evaluation of sensory impairment, the adhesive removal test was used (15). All rats were trained for the adhesive removal test daily for 3 days before MCA embolization. Then the animals were tested before surgery and 24 and 48 hr post-operatively. During the adhesive removal test, one piece of an adhesive paper (10×5 mm²) was placed on the left distal radial regions of forelimb as a tactile stimulus. The time it takes the rat to touch and remove each stimulus from the limb was recorded during 3 trials and averaged. Rats with impaired sensory function allow the paper to stay on the forelimb for longer periods of time. An observer blind to the experimental groups carried out all behavioral analysis.

Measurement of α -TCT concentration in the brain

To verify whether α -TCT crosses the blood brain barrier and reaches the ischemic area by ip injecting (1 or 10 mg/kg), its concentration in the cerebellum of the animals were measured at 24 and 48 hr after stroke by

HPLC as described before (16). The Waters Alliance 2695 Separations Module connected with a guard cartridge (Jour Guard C18) to an analytical column. Detection: Model 2475 fluorescence detector. Stationary phase: C18 stationary phase coupled with spectrophotometric and fluorometric detection. Mobile phase: acetonitrile-methanol (65:35 by volume), and the flow rate was 1 ml/min. (n=3 for α -TCT at 24 hr, n=3 for α -TCT at 48 hr, and n=2 for control group at 48 hr). Cerebellums of the animals were extracted as reported previously (17).

Statistical Analysis

Infarct volumes, α -TCT concentrations, sensory impairments and physiological parameters are presented as mean±SEM and analyzed with one-way ANOVA followed by Tukey's test. Neurological deficits are reported as medians and interquartile ranges (25 th and 75 th percentiles) and was analyzed with Kruskal-Wallis test. Differences were considered significant when $P < 0.05$.

Results

We did not observe any infarction, neurological deficits or sensory impairment in the sham-operated animals.

Evaluation of physiological parameters during surgery

Physiological parameters including blood gases, pH, heart rate, blood glucose and arterial blood pressure were determined during the surgery. The results are shown in Table 1. There was no difference between groups considering these parameters.

Effect of α -TCT and RGZ on infarct volume

Embolization of a preformed clot resulted in an infarction in the ipsilateral hemisphere, mainly located in the middle cerebral artery irrigated region. The effect of α -TCT (1 and 10 mg/kg, ip) administration on cerebral ischemia onset was determined and compared with control and RGZ (3 mg/kg, ip). RGZ is an agonist of the nuclear receptor and transcription factor peroxisome

proliferator-activated receptors (PPAR)- γ that has been decreased infarct volume and neurological deficits in human (18) and animal stroke models (19, 20). The results are shown in Figure 1.

Table 1. Comparison of physiological parameters at 5 min before or after embolic stroke in different groups.

Variables		Control	α -TCT	RGZ
Glucose (mg/dl)	Before:	220 \pm 14	216 \pm 19	214 \pm 19
	After:	214 \pm 18	218 \pm 13	215 \pm 17
pH	Before:	7.37 \pm 0.02	7.40 \pm 0.02	7.412 \pm 0.03
	After:	7.41 \pm 0.02	7.39 \pm 0.03	4.42 \pm 0.04
P _{O2} (mmHg)	Before:	95 \pm 8	94 \pm 9	98 \pm 6
	After:	100 \pm 3	97 \pm 4	101 \pm 3
P _{CO2} (mmHg)	Before:	35.5 \pm 1.4	34.2 \pm 1.2	33.8 \pm 1.5
	After:	38.3 \pm 0.7	35.3 \pm 1.5	32.9 \pm 1.9
*MAP (mmHg)	Before:	91 \pm 4	95 \pm 6	93 \pm 3
	After:	94 \pm 3	90 \pm 7	92 \pm 5
Heart rate (beats/min.)	Before:	362 \pm 34	358 \pm 46	380 \pm 34
	After:	352 \pm 42	368 \pm 40	367 \pm 43

Physiological parameters were measured at 5 min before or after embolic stroke. Data are presented as mean \pm SEM. The groups were DMSO (0.1 ml/kg, as control), rosiglitazone (RGZ) (3 mg/kg) and alpha-tocotrienol (α -TCT). N=6 in the α -TCT group (3 animals in each dose of 1 and 10 mg/kg) and n=5 in the other groups. Differences between the groups were not significant. *MAP: Mean arterial pressure.

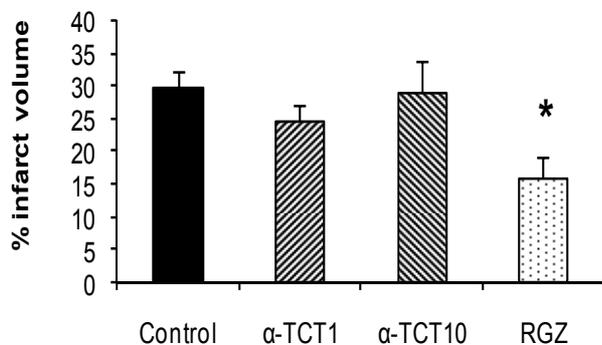


Figure 1. Effect of α -TCT (alpha-tocotrienol) and RGZ (rosiglitazone) on infarct volume at 3 hr after stroke. The groups were control (DMSO 0.1 ml/kg), (RGZ 3 mg/kg) and α -TCT (1 or 10 mg/kg). One-way ANOVA followed by the Tukey-test revealed that infarct volume in RGZ treated group was significantly different from the control group ($P<0.05$). Data are presented as means \pm SEM. * $P<0.05$ compared to the control group. N=8 in each group.

The infarct volume 48 hr after the embolic injury for the control, RGZ, low and high doses of α -TCT groups were 29.42 \pm 2.61%, 15.87 \pm 3.1%, 24.86 \pm 2.14%, and 28.97 \pm 4.79%, respectively. Compared to the control group,

only RGZ decreased infarct volume by 46% ($P<0.05$) but low and high doses of α -TCT did not show any significant neuroprotective effect.

Evaluation of behavior

In this part of experiments, neurological deficits and sensory impairments were recorded before, and 2, 24 and 48 hr after embolization. Changes of neurological deficits scores in different groups are shown in Table 2. Compared to the control group, α -TCT with low and high doses did not improve neurological deficits when administered 3 hr after embolic stroke. Administration of RGZ 3 hr after MCA embolization decreased neurological deficits at 48 hr after embolic stroke ($P<0.05$). The effects of two doses of α -TCT and RGZ on sensory impairment are showed in Figure 2. While RGZ treated animals removed sticky tabs from their affected forelimb more quickly than control group ($P<0.01$) at 24 hr and 48 hr after stroke, neither doses of α -TCT did not show any significant effect.

Table 2. Comparison of neurological deficits scores in different groups.

	Control	α -TCT1	α -TCT10	RGZ
2 hr	3 (2-4) n=8	3 (2-3) n=7	3 (3-4) n=8	3 (2-3.75) n=8
24 hr	3 (2-4) n=7	3 (2-4) n=7	3 (3-5) n=6	2 (2-2) n=7
48 hr	3 (3-4) n=6	4 (2-4) n=6	4 (2-5) n=6	1.5 (1-2)* n=7

Neurological deficits were determined at 2, 24 and 48 hr after the embolic stroke. Data are expressed as medians and interquartile ranges; 25th and 75th percentiles are shown in the parentheses. The drugs studied were DMSO (0.1 ml/kg, as control), rosiglitazone (RGZ, 3 mg/kg), α -TCT (alpha-tocotrienol) 1 or 10 mg/kg. * $P<0.05$ compared to the control group.

Measurement of α -TCT concentration in the cerebellum of animals

For verifying whether α -TCT crosses the blood brain barrier and delivers the ischemic area by ip injecting, its concentration in the cerebellum of the animals were measured as described in the method section. Concentration of α -TCT in the cerebellum of α -TCT treated (10 mg/kg) rats at 24 and 48 hr after stroke and the control group at 48 hr were determined.

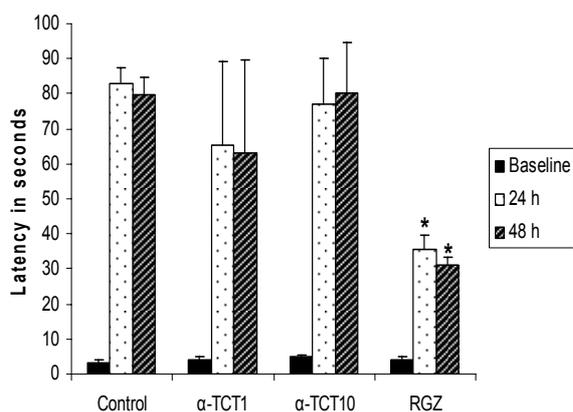


Figure 2. Effect of α -TCT (alpha-tocotrienol) and RGZ (rosiglitazone) administration on sensory impairment (latency in removing the adhesive paper) at 3 hr after stroke. The groups were control (DMSO 0.1 ml/kg), α -TCT (1 or 10 mg/kg) and RGZ (3 mg/kg). One-way ANOVA followed by the Tukey-test revealed that sensory impairment in RGZ treated group was significantly decreased when compared to the control group at 24 and 48 hr after stroke ($P<0.01$). Data are presented as means \pm SEM. * $P<0.05$ compared to the control group. n=6 for α -TCT (10 mg/kg) and n=7 for the other groups at 24 hr after stroke. n=7 for RGZ and =6 for the remaining groups at 48 hr after stroke.

The α -TCT concentrations are expressed in Figure 3. One-way ANOVA followed by the Tukey-test revealed that concentration of α -TCT in the drug treated animals at 24 hr after embolization was significantly higher than the control group ($P<0.01$).

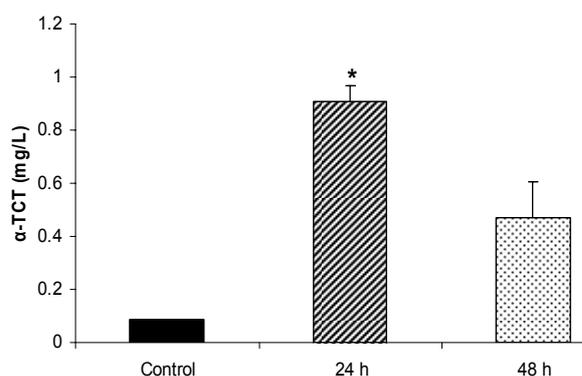


Figure 3. Concentration of α -TCT (mg/l) in the cerebellum of animals at 24 or 48 hr after stroke when the drug injected 3 hr after embolic cerebral ischemia. In the control group, concentration of α -TCT was determined at 48 hr after stroke. α -TCT concentration in the control (n=2), 24 hr (n=3) or 48 hr (n=3) group after stroke were 0.087 \pm 0.001, 0.91 \pm 0.055, and 0.466 \pm 0.14, respectively. One-way ANOVA followed by the Tukey-test revealed that concentration of α -TCT in the drug treated animals at 24 hr after embolization significantly was different from the control group. * $P<0.01$ compared to the control group.

Discussion

In the present study, we examined the neuroprotective effect of α -TCT, on ischemic brain injury using experimental embolic stroke model in rat. The natural vitamin E family includes eight chemically distinct molecules: α -, β -, γ - and δ -tocopherol; and α -, β -, γ - and δ -tocotrienol. Tocochromanols contain a polar chromanol head group with a long isoprenoid side chain. Depending on the nature of the isoprenoid chain, tocopherols (containing a phytyl chain) or tocotrienols (geranylgeranyl chain) can be distinguished (21).

Our results did not show any decrease in infarct volume, neurological deficits or sensory impairments by administering either low or high doses of α -TCT (1 or 10 mg/kg) at 3 hr after cerebral ischemic onset. On the other hand, thiazolidinedione RGZ (3 mg/kg) an agonist of the nuclear receptor and transcription factor peroxisome proliferator-activated receptors γ (PPAR- γ) significantly decreased the infarct volume and improved the functional outcomes when administered at the same time. PPAR- γ activation enhances insulin sensitivity and reduces serum glucose in diabetic patients without significant alterations in serum glucose of non-diabetic animals or humans (22) and its neuroprotection have been well documented. For example, it has recently been reported that thiazolidinediones as the agonists of PPAR- γ , decreased infarct volume and neurological deficits following transient (23), permanent (24) and embolic (19) models of stroke.

Glutamate toxicity is a major contributor to neurodegeneration after stroke. It includes excitotoxicity and an oxidative stress component also known as oxytosis (25). It has been suggested that α -TCT is the most potent neuroprotective form of vitamin E in glutamate-induced degeneration of HT4 hippocampal neurons (26). At nanomolar concentrations, α -TCT in contrast to α -tocopherol, protected against glutamate-induced neuronal death by suppressing inducible pp60 csrc kinase activation (26) and inducible 12- lipoxygenase activation (8). These effects of tocotrienol were independent of antioxidant property (26). Moreover, when injected immediately after stroke, α -TCT

decreased the size of the cerebral infarcts 1 day after stroke while γ -TCT and δ -TCT did not (7).

The above reported findings urged us to investigate neuroprotective effects of α -TCT 3 hr after embolic stroke because in none of the previous reports long-term beneficial effects of α -TCT had been studied. The main aim in stroke studies is finding a potential drug with a wider protection because treatment of stroke often does not begin until several hr after the initial insult. One reason that why our results are not consistent with the other investigators may be due to the time of administration. Indeed, the failure of many clinical trials is likely due to the short treatment window in which the compounds can be administered (27). Therefore, gone are the days when drugs that confer neuroprotection when given before or a short period after cerebral ischemia can be considered relevant for therapy of ischemic stroke.

The length of time during which neuroprotectants can be expected to be effective is likely to vary from drug to drug and depends on an individual drug's specific mechanism of action. Glutamate-induced toxicity is a very early event during the acute phase of ischemic stroke, which means that glutamate antagonists are likely to be effective as neuroprotectants for only a short time (perhaps as little as 1 to 2 hr) (28). Therefore, at the time that we injected α -TCT (3 hr after stroke), neurotoxic cascades of glutamate might have been happened and so this compound was not protective.

Oxygen free radicals cause destruction of cellular membranes. Accordingly, the free oxygen radical scavengers might have a potential neuroprotective role. So, we expected that α -TCT might be neuroprotective through its radical scavenging properties as well as two radical scavenger citicoline and NXY-059 which has been shown that they have neuroprotective effects. Preclinical studies in animal stroke models showed that citicoline, a radical scavenger, improved neurological outcome and infarct size when used immediately after stroke. However, the clinical phase III trials with varying dosing schedules did not show efficacy (29). NXY-059 is a

nitronone-based free-radical-trapping agent which has been proven to reduce the infarct size in the transient and permanent MCA occlusion rat stroke models when administered up to 8 hr after onset of ischemia. It was also shown to improve the neurologic outcome (measured by performance scores) 3 weeks and 10 weeks after the stroke, and to reduce the infarct size in a marmoset stroke model when administration was delayed for up to 4 hr after stroke onset. A safety trial tested NXY-059 in stroke patients and this agent was found to be safe in doses that were neuroprotective in the animal models (29, 30). The reason why our results are not consistent with the above studies and α -TCT did not show neuroprotective effects through its radical scavenging properties is uncertain. Perhaps antioxidative effect of α -TCT is not as high as citicoline and NXY-059, or different models of stroke may play a role which remains to be determined in future.

We thought that the drug with ip injection might not be delivered to the brain. Therefore, the α -TCT concentration were measured in the cerebellum of some animals and we have observed that ip injection resulted in significant delivery of α -TCT to the brain and at 24 hr after injecting, its level was increased even to 10 times of the base level (Figure 3). Recently, it has been suggested that the safe dose of various tocotrienols for human consumption is 200–1000 mg/d (31). Therefore, the doses used in current study were enough to show the potential neuroprotection.

Based on our results and the reports of other investigators (25-27), α -TCT might be remaining as a good neuroprotective when administered only before predictable cerebral ischemia. Although the failure of α -TCT seems to be a major limitation in the present study, this natural form of vitamin E cannot be countered by a clinically relevant administration because long-term beneficial effects of brain protective therapies have been recommended as the endpoint of experimental stroke studies as well as clinical stroke studies (5).

Conclusion

Our data showed that α -TCT was not neuroprotective at 3 hr after the embolic model of cerebral ischemia. Further studies should be undertaken to optimize the treatment regimen and to clarify the neuroprotective effects of α -TCT after stroke.

Acknowledgment

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