Journal of Cerebral Blood Flow & Metabolism (2011) 31, 2218–2230

www.jcbfm.com

Tocotrienol vitamin E protects against preclinical canine ischemic stroke by inducing arteriogenesis

Cameron Rink[1], Greg Christoforidis[2], Savita Khanna[1], Laura Peterson[1], Yojan Patel[1], Suchin Khanna[1], Amir Abduljalil[3], Okan Irfanoglu[4], Raghu Machiraju[4], Valerie K Bergdall[5] and Chandan K Sen[1]

[1]*Department of Surgery, The Ohio State University Medical Center, Columbus, Ohio, USA;* [2]*Department of Radiology, The University of Chicago Medical Center, Chicago, Illinois, USA;* [3]*Department of Radiology, The Ohio State University Medical Center, Columbus, Ohio, USA;* [4]*Department of Computer Science and Engineering, The Ohio State University, Columbus, Ohio, USA;* [5]*Department of Veterinary Preventive Medicine, The Ohio State University, Columbus, Ohio, USA*

Vitamin E consists of tocopherols and tocotrienols, in which α-tocotrienol is the most potent neuroprotective form that is also effective in protecting against stroke in rodents. As neuroprotective agents alone are insufficient to protect against stroke, we sought to test the effects of tocotrienol on the cerebrovascular circulation during ischemic stroke using a preclinical model that enables fluoroscopy-guided angiography. Mongrel canines (mean weight = 26.3 ± 3.2 kg) were supplemented with tocotrienol-enriched (TE) supplement (200 mg b.i.d, *n* = 11) or vehicle placebo (*n* = 9) for 10 weeks before inducing transient middle cerebral artery (MCA) occlusion. Magnetic resonance imaging was performed 1 hour and 24 hours post reperfusion to assess stroke-induced lesion volume. Tocotrienol-enriched supplementation significantly attenuated ischemic stroke-induced lesion volume (*P* < 0.005). Furthermore, TE prevented loss of white matter fiber tract connectivity after stroke as evident by probabilistic tractography. *Post hoc* analysis of cerebral angiograms during MCA occlusion revealed that TE-supplemented canines had improved cerebrovascular collateral circulation to the ischemic MCA territory (*P* < 0.05). Tocotrienol-enriched supplementation induced arteriogenic tissue inhibitor of metalloprotease 1 and subsequently attenuated the activity of matrix metalloproteinase-2. Outcomes of the current preclinical trial set the stage for a clinical trial testing the effects of TE in patients who have suffered from transient ischemic attack and are therefore at a high risk for stroke.
Journal of Cerebral Blood Flow & Metabolism (2011) **31,** 2218–2230; doi:10.1038/jcbfm.2011.85; published online 15 June 2011

Keywords: antioxidants; angiography; cerebral blood flow; focal ischemia; free radicals

Introduction

Of the 795,000 cases of stroke each year in the United States, ∼25% are repeat stroke events (Lloyd-Jones *et al*, 2010). In addition, 15% of all stroke events are preceded by a transient ischemic attack (TIA), defined as a temporary episode of neurologic dysfunction caused by reduced blood flow to the brain, but without permanent damage to brain tissue (Lloyd-Jones *et al*, 2010). After a TIA, the 90-day risk of stroke is as high as 17.3% (Lloyd-Jones *et al*, 2010). Thus, prophylactic interventions may have a key role in favorably modifying stroke outcomes especially for those who have already suffered from a TIA, and therefore, are facing a major stroke event.

Clinical trials testing the effects of vitamin E in a wide range of major health disorders have come to the general conclusion that vitamin E either is not helpful or could be harmful under certain conditions (Lonn *et al*, 2005; Miller *et al*, 2005). Meta-analyses of over 20 randomized, controlled clinical trials testing vitamin E have now reached conclusions that on one hand serve the basis for readjusting public policies and practices, whereas on the other suffer from a major blind spot, which is not recognized in any of these reports (Schierling *et al*, 2009). Although title claims of such meta-analyses address vitamin E as whole, they fail to recognize that the only form of vitamin E studied in all these trials is α-tocopherol, which represents one-eighth of the natural vitamin E family. Natural vitamin E exists in two forms: tocopherols and tocotrienols. Both tocopherols and

Correspondence: Dr CK Sen, 473 W. 12th Avenue, Columbus, OH 43210, USA.
E-mail: chandan.sen@osumc.edu
Supported in part by NIH NS42617 to CKS, UL1RR025755 to CR and SK, and Carotech Inc.
Received 8 March 2011; revised 26 April 2011; accepted 4 May 2011; published online 15 June 2011

tocotrienols possess a chromanol ring, and within families the isoforms are differentiated as α, β, γ, and δ according to the presence of methyl groups at positions 5, 7, and 8, respectively. Tocopherols are characterized by a saturated side chain, whereas tocotrienols possess an isoprenoid side chain with double bonds at C-3, -7, and -11.

Recent interest in the biological properties of tocotrienol has sharply risen because of the unique biological functions of this form of natural vitamin E not shared by the better known tocopherols, which have failed to live up to expectations in clinical trials (Miller *et al*, 2005; Schierling *et al*, 2009). At nanomolar concentrations, α-tocotrienol (αTCT) but not α-tocopherol, is a potent neuroprotective agent (Khanna *et al*, 2005*b*). On a concentration basis, this represents the most potent of all the biological functions of the entire vitamin E family. Neural cell biology studies have identified unique αTCT-sensitive signaling checkpoints that rescue cells from inducible cell death caused by a range of insults (Sen *et al*, 2004). Importantly, although αTCP is detected in serum from dietary sources, the presence of αTCT in the serum of non-supplemented Americans is negligible because of Western food habits containing trace levels of tocotrienol (Schwartz *et al*, 2008). Statin-mimetic cholesterol lowering properties of αTCT in humans (Parker *et al*, 1993), in addition to neuroprotection, positions them as a strong candidate for stroke therapeutics. Indeed, we have demonstrated that orally supplemented αTCT protects against stroke-induced lesion in the brain of spontaneously hypertensive rats (Khanna *et al*, 2005*b*). As small animal studies are recognized to be of limited reliability to predict success for stroke therapeutics in clinical trials (Kidwell *et al*, 2001), we developed a minimally invasive preclinical canine model (Rink *et al*, 2008) to test the efficacy of a tocotrienol-enriched (TE) supplement in a randomized, blind, placebo (PBO)-controlled setting. Angiography, enabled in our large animal setting, helped elucidate that prophylactic TE supplementation improves collateral blood flow to the stroke-affected territory during stroke. In the clinic, angiographic collateral grading has been used as a predictor of stroke outcome (Christoforidis *et al*, 2005). Molecular mechanisms of postnatal collateral growth and remodeling, termed as arteriogenesis, are distinct from those invoked in angiogenesis and vasculogenesis. Outcomes of the current work provide first evidence of a direct link between tocotrienol supplementation and the expression of pro-arteriogenic factors in perfused collaterals of the stroke-affected hemisphere.

Materials and methods

Randomized, Blind, Placebo-Controlled, Supplementation Regimen

All experimentation was approved by the Institutional Animal Care and Use Committee of The Ohio State University. Twenty mongrel canines (2.4 ± 0.9 yrs, 26.6 ± 2.6 kg) were subjected to gross physical, heartworm, complete blood count, and blood chemistry tests by veterinary faculty of The Ohio State University before study inclusion. No gross physical abnormalities, heartworm, or significant differences in complete blood count or blood chemistry were observed by veterinary staff. Following baseline physicals, canines were randomized into two treatment groups—one receiving TE ($n = 11$, 200 mg mixed tocotrienols, Carotech, Perak, Malaysia), and the other receiving vitamin E-deficient corn oil ($n = 9$, vehicle PBO). Canines were maintained on standard chow (TD2025; Harlan Teklad) for the duration of the supplementation. Tocotrienol-enriched and PBO supplements were delivered orally in gel capsules that were identical in appearance and size. Canines received supplements twice per day, after morning and evening meals, for a period of 10 weeks. Stroke was induced within 12 hours after the last supplement was received. Research and veterinary staff were blinded to capsule contents and treatment groups until all magnetic resonance imaging (MRI) stroke outcome data were independently reviewed by faculty of the Center for Biostatistics at The Ohio State University Medical Center.

C-arm Fluoroscopy-Guided Preclinical Model of Acute Ischemic Stroke

The minimally invasive, endovascular approach to achieve middle cerebral artery occlusion (MCAO) in canines was performed as previously described (Rink *et al*, 2008). Briefly, the anesthetized canine (1.5% to 2.0% isoflurane) underwent bilateral femoral artery access with five French sheaths (ArrowGE Healthsystems, Waukesha, WI, USA) from which 4-Fr and 5-Fr guide catheters (Boston Scientific, Natick, MA, USA) were used to provide access to the basilar artery system and for routine contrast (Omnipaque) visualization of the middle cerebral artery (MCA) territories. Microcatheter techniques were used to access and occlude the MCA from the basilar artery. An embolic coil (3 mm × 20 cm Ultrasoft Matrix2 Platinum Coil, Boston Scientific) was delivered into the M1 segment of either MCA from a microcatheter (SL-10, Boston Scientific), and occlusion was documented using digital subtraction angiograms of the internal carotid and vertebrobasilar circulation every 15 minutes throughout the 1 hour occlusion period. Following 1 hour of MCAO, the embolic coil was retrieved and digital subtraction angiograms used to confirm reperfusion. Angiographic documentation of vessel perforation and hemorrhage was grounds for study exclusion. Physiologic parameters were monitored throughout the procedure, and included blood pressure and blood parameters determined before MCAO, during occlusion, and after reperfusion. Following reperfusion, endovascular devices were withdrawn and arteriotomy sites closed. Under veterinary care, canines were immediately transported to the Wright Center of Innovation at The Ohio State University for 1 hour post reperfusion MRI. Fluoroscopy-guided angiograms documenting the surgical procedure are provided in Supplementary Figure S1.

Magnetic Resonance Imaging

Evaluation of the infarct volume was performed using an 8-channel sensitivity encoding (SENSE) knee coil in a 3T MRI (Achieva, Philips Healthcare, Andover, MA, USA) MRI imaging system. Images were obtained at 1 hour and 24 hours following reperfusion. Sequences included: diffusion tensor imaging (DTI; field of view (FOV) = 140 × 140 mm^2, matrix = 128 × 128, number of excitations (NEX) = 1, repetition time (TR)/echo time (TE) 192-2131/71, Slice thickness = 3 mm, b value = 1,000, total scan time ~4 minutes) and T2 fluid attenuated inversion recovery (FLAIR; FOV = 160 mm, matrix = 512 × 512, NEX = 1, $TR/TE/TI$ (inversion time) = 11,000/125/2,800, slice thickness = 3 mm, total scan time ~8 minutes) and three-dimensional time-of-flight magnetic resonance angiography (FOV = 150 mm, matrix = 512 × 512, TR/TE = 8.6/3.45, flip angle = 20, slice thickness = 1 mm, total scan time ~6 minutes). Diffusion tensor imaging data were transferred to a workstation where mean diffusivity maps were derived from the 1 hour post reperfusion DTI (FSL 4.1.4, Oxford University, Oxford, UK). Magnetic resonance angiography reconfirmed reperfusion to the transiently occluded territory. Infarct volumes were calculated by importing mean diffusivity maps and FLAIR images into Image J (National Institutes of Health). Two blinded observers independently outlined infarct volumes using a semi-automated threshold technique as previously described (Christoforidis *et al*, 2011).

Streamline and Probabilistic White Matter Fiber Tracking

Streamline tractography of the internal capsule was performed using the FACT algorithm with Trackvis software (ver. 0.5.1). Probabilistic tractography (Behrens *et al*, 2003) enables quantitative analysis of DTI-based connectivity as opposed to the streamline tractography. To investigate the therapeutic efficacy of TE to protect white matter connectivity after stroke, a probabilistic tractography framework was employed using the FSL software package (Smith *et al*, 2004). Our probabilistic approached used a single regions of interest (ROI) mask with 10,000 tracts cast from each voxel in the internal capsule ROI (curvature threshold of 0.2). The connectivity images resided in their native space and were not directly comparable. For this reason, tensor images for each sample, for each timestamp, were fed into a tensor field-based elastic registration routine to compute a population average tensor image and the transformations that mapped each data onto this average brain space. This registration was performed using DTI-TK toolkit (ver. 2.0). Transformations were applied to the corresponding tract images in the same coordinate framework, that of the mean tensor image.

Angiographic Evaluation of Cerebrovascular Collateral Recruitment

Digital subtraction angiogram acquisitions obtained just before reperfusion were reviewed to assess cerebrovascular collateral recruitment using an 11-point scale, as previously described (Christoforidis *et al*, 2011). This scale takes into account the anatomic extent and transit time of leptomeningeal collaterals from the posterior and anterior cerebral artery circulations to the affected MCA territory. Digital subtraction angiogram images were reviewed to identify leptomeningeal collateral reconstitution of the anterior, middle, and posterior aspects of the MCA territory. The horizontal portions of the MCA and posterior cerebral artery were used as landmarks dividing the MCA territory into these three regions—anterior, middle, and posterior. Images were compared with the arterial and venous phases of the pre-occlusion arteriograms on the side of the occlusion.

Vitamin E Extraction and Analysis

Vitamin E extraction and analysis of canine brain tissue was performed as previously described using an HPLC-coulometric electrode array detector (Coularray Detector, 12-channel, model 5600, ESA, Chelmsford, MA, USA). This system enables the simultaneous detection of all eight naturally occurring vitamin E family members in a single run (Roy *et al*, 2002).

Laser Microdissection Pressure Catapulting

Following 24 hour MRI, canines were euthanized and brain tissue collected for downstream applications, including laser microdissection pressure catapulting. Continuous coronal slices (3 mm) of canine brain, which include the M1 segment of the MCA were embedded and frozen in OCT compound (Sakura). Embedded brains were sliced into 12 μm thick sections using a cryostat (CM3050 s, Leica Microsystems, Buffalo Grove, IL, USA). Sections were mounted onto RNAse inhibitor-treated thermoplastic (polyethylene napthalate)-covered glass slides (PALM Technologies, Bernried, Germany). Slides were incubated in RNA-later stabilization reagent (Applied Biosystems, Carlsbad, CA, USA) for 4 minutes and quick-stained with anti-VWF antibody (1:50 dilution, 15 minutes) for selective capture of endothelial cells from stroke-affected (ipsilateral) and contralateral control tissue. More than 800,000 μm^2 of capture elements were collected for downstream RNA isolation, cDNA synthesis and real-time PCR. For high-throughput collection, all elements were captured using a PALM MicroLaser, MicroBeam, and RoboStage/RoboMover system. RNA was isolated from captured and catapulted elements using the PicoPure RNA Isolation Kit (Arcturus, Carlsbad, CA, USA) as described (Rink *et al*, 2010).

Real-Time PCR

Expression levels of collateral gene candidates were independently determined at 24 hours from contralateral control and stroke-affected laser microdissection pressure catapulting-captured elements using real-time PCR, as previously described (Rink *et al*, 2010). Briefly, total RNA (>250 ng) was reverse transcribed into cDNA using oligo-dT primer and Superscript III. Reverse transcriptase-generated DNA was quantified by real-time PCR assay using double-stranded DNA-binding dye SYBR Green-I. Relative gene expression was standardized to 18s rRNA. Data are shown as mean ± s.d. Primer sequences are provided in the Online Supplementary Table S1.

Western Blot Analysis

To extract protein from the canine brain, S1 cortex and contralateral control tissue was homogenized on ice in lysis buffer (50 mmol/l Tris-HCL pH 7.6; 1.5 mmol/L NaCl; 0.5 mmol/L CaCl2; 0.01% Brij 35; 1% Triton X-100) and centrifuged at 4°C for 15 minutes at 14,000 g. Protein expression of matrix metalloproteinase-2 (MMP2) in canine cortex was determined by western blot analysis as previously described (Khanna *et al*, 2005*b*) using MMP2 antibody (Enzo Life Sciences, Plymouth Meeting, PA, USA). Proteins were separated on 4% to 12% gels (Invitrogen, Carlsbad, CA, USA) by SDS–PAGE, transferred onto polyvinylidene difluoride membranes, and membranes were incubated with Tris-buffered saline (TBS) containing 5% milk for 12 to 18 hours at 4°C with MMP2 antibody (1:400 dilution). Next, membranes were washed three times with TBS containing 0.1% Tween-20 (TBS Tween-20) and incubated for 1 hour at room temperature in horseradish peroxidase-conjugated secondary donkey anti-rabbit antibody (GE Healthcare Life Sciences, Waukesha, WI, USA, 1:2,000 dilution in TBS Tween-20 containing 5% milk). Immunoblots were developed with ECL PlusTM Western blotting Detection Reagents (GE Healthcare Life Sciences) according to manufacturer's recommendation. To evaluate the loading efficiency, the membranes were probed with anti-β-actin antibody (Sigma-Aldrich, St Louis, MO, USA, 1:5,000, in TBS, 1 hour). Each western blot was scanned and analyzed using National Institutes of Health ImageJ software (ver. 1.44) for the density of the bands.

Gelatin Zymography

Matrix metalloproteinase-2 activity was determined by gelatin zymography as described (Beceriklisoy *et al*, 2007). Briefly, 50 μg total protein were combined in a 1:1 ratio with Tris-glycerine SDS-loading buffer (Invitrogen) and samples were separated through electrophoresis on 10% polyacrylamide gels containing 0.1% gelatin (Invitrogen). Gels were incubated in renaturing buffer (Invitrogen) for 30 minutes, and then treated in developing buffer (Invitrogen) for 30 minutes. Gels were incubated for 24 hours at 37°C in fresh developing buffer with gentle agitation. Gels were stained with 20 ml of SimplyBlue SafeStain (Invitrogen), destained, and imaged using Pharos FX plus molecular imager (Bio-Rad, Hercules, CA, USA) and analyzed using National Institutes of Health ImageJ software (ver. 1.44) for the density of the bands.

Statistical Analysis

Statistically treated data are reported as mean ± SD. Difference between means was tested with Student's *t*-test or one-way ANOVA with Tukey's *post hoc* test where appropriate (alpha level = 0.05). SPSS software (v17.0, IBM, Somers, NY, USA) was used for all statistical calculations.

Results

Oral TE Supplementation Attenuates Stroke-Induced Lesion Volume and Edema

Healthy mongrel canines were randomized to treatment groups and orally administered 200 mg TE (containing 61.52 mg αTCT, 112.8 mg γ-tocotrienol, and 25.68 mg δ-tocotrienol; $n = 11$) or vehicle control (PBO containing vitamin E stripped corn oil, $n = 9$) gel capsules twice daily for 10 weeks before experimental stroke. Randomization was supervised by the trial statistician, while research and veterinary personnel were blinded to supplement content and experimental groups until the conclusion of the study. Tocotrienol-enriched supplementation had no significant effect on monitored physiologic parameters before (baseline), during, or immediately after stroke reperfusion (Table 1). Oral TE capsule supplementation significantly increased the concentration of tocotrienols in MCA supplied cerebral cortex as compared with PBO controls (Figure 1A). Tocotrienol-enriched supplementation enriched cortical brain tissue with nearly equal amounts of α- and γ-tocotrienol isoforms (77.4 nmol/g protein and 77.5 nmol/g protein, respectively) and approximately one-third that amount of δ-tocotrienol isoform (22.4 nmol/g protein). Like Western diet, canine chow is deficient in tocotrienols. No appreciable amount of α-, γ-, or δ-tocotrienol was detected in cortex of PBO controls despite using a highly-sensitive electrochemical HPLC approach (Roy *et al*, 2002). The concentration of α- and γ- tocotrienol in TE-supplemented animals was 10-fold less than that of α-tocopherol found in cerebral cortex (Figure 1B). Tocotrienol-enriched supplementation, representing a blend of natural vitamin E enriched from palm oil,

Table 1 Physiological parameters

	PBO	TCT
Age (years)	2.2 ± 0.3	2.8 ± 1.3
Weight (kg)	25.9 ± 2.8	26.5 ± 3.5
Body temperature (°C)		
Baseline	36.0 ± 0.4	36.1 ± 0.5
During MCAO	35.7 ± 0.5	35.4 ± 0.6
Post-reperfusion	35.7 ± 0.5	35.4 ± 0.6
Blood glucose (mg/dL)		
Baseline	137 ± 33	110 ± 32
During MCAO	93 ± 41	120 ± 37
Post-reperfusion	102 ± 29	117 ± 19
Hematocrit (%)		
Baseline	37.4 ± 7.7	36.4 ± 5.6
During MCAO	34.3 ± 7.7	33.3 ± 7.5
Post-reperfusion	33.0 ± 7.0	31.9 ± 3.0
$ETCO_2$ (mm Hg)		
Baseline	39.6 ± 13.2	35.3 ± 7.0
During MCAO	39.2 ± 14.1	36.7 ± 9.4
Post-reperfusion	41.2 ± 17.7	34.7 ± 7.7
Arterial pulse (BPM)		
Baseline	127 ± 14	112 ± 20
During MCAO	128 ± 29	112 ± 14
Post-reperfusion	124 ± 24	111 ± 16
Arterial pH		
Baseline	7.3 ± 0.1	7.3 ± 0.1
During MCAO	7.3 ± 0.1	7.3 ± 0.1
Post-reperfusion	7.3 ± 0.1	7.3 ± 0.1
pCO_2 (mm Hg)		
Baseline	47.0 ± 10.8	48.3 ± 12.2
During MCAO	48.7 ± 14.7	49.4 ± 13.1
Post-reperfusion	53.7 ± 23.0	44.6 ± 8.6
HCO_3 (mEq/L)		
Baseline	20.8 ± 2.3	21.6 ± 1.8
During MCAO	21.2 ± 1.7	21.3 ± 2.2
Post-reperfusion	22.5 ± 2.0	21.8 ± 2.0
pO_2 (mm Hg)		
Baseline	545 ± 32	524 ± 85
During MCAO	513 ± 42	520 ± 65
Post-reperfusion	524 ± 33	539 ± 45
O_2 sat (%)		
Baseline	98.4 ± 0.9	95.6 ± 2.3
During MCAO	98.2 ± 0.8	95.8 ± 2.4
Post-reperfusion	98.2 ± 0.8	97.0 ± 1.8
Systolic blood pressure		
Baseline	107 ± 16	105 ± 15
During MCAO	104 ± 19	108 ± 11
Post-reperfusion	109 ± 16	105 ± 12
Diastolic blood pressure		
Baseline	68 ± 5	74 ± 10
During MCAO	70 ± 12	73 ± 16
Post-reperfusion	75 ± 12	63 ± 13
Mean arterial pressure		
Baseline	82 ± 4	89 ± 12
During MCAO	90 ± 18	85 ± 16
Post-reperfusion	92 ± 16	84 ± 7

BPM, beats per minute; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; PBO, placebo; TCT, tocotrienol.
Canine physiological parameters were assessed at baseline (before embolic occlusion of the MCA), during ischemia, and immediately following reperfusion.

modestly increased the concentration of α-tocopherol in brain tissue as compared with PBO controls; whereas no difference in γ-tocopherol concentration was observed between PBO and TE groups.

Cytotoxic edema is characterized by cellular swelling in the acute phase (<24 hours) of stroke onset. Cerebral ischemia in hyper-metabolic brain tissue causes failure of ATP-dependent ion transporters, resulting in rapid accumulation of intracellular Na^{2+} and an influx of water to maintain osmotic equilibrium. Diffusion tensor imaging enables early detection of cytotoxic edema after acute ischemic stroke. Mean diffusivity maps generated from DTI revealed that TE-supplemented canines had significantly attenuated ($P<0.05$) cytotoxic edema at 1 hour following acute ischemic stroke as compared with PBO controls (Figures 1C and 1D). Although stroke-induced lesion volume more than doubled in PBO canines between the 1 hour and 24 hours (9804.7to 20579.8 mm^3) time points after reperfusion, lesion volume in TE-supplemented canines remained consistently low (3675.3 to 3834.9 mm^3, Figures 1C and 1E). At 1 hour time point, stroke-induced lesion volume of TE-supplemented canines was <40% that of PBO controls; and at 24 hours TE infarct volume was <20% of their PBO counterparts. Three-dimensional volumetric reconstruction of brain from representative PBO and TE FLAIR images at 24 hours provides a clear visual appreciation of the protective effects of TE supplementation (Supplementary Figure S2).

White Matter Fiber Tract Connectivity is Protected In TE-Supplemented Canines after Stroke

White matter fiber pathways represent the brain's communication network. The cytoarchitecture and anatomical connectivity of cerebral white matter with cerebral cortex (gray matter) directly influences brain function (Passingham *et al*, 2002). White matter injury in the context of stroke has a direct effect on sensorimotor impairment and post-stroke functional recovery (Schaechter *et al*, 2008). In brain tissue that possesses a high degree of directional organization, the diffusion of water and its protons aligns with the orientation of white matter fiber tracts. Recent developments in DTI have enabled visualization of white matter fiber tract connectivity after stroke. Fiber tract projections from the region of the internal capsule to the corona radiata were dramatically reorganized in PBO canine brain 24 hours after stroke reperfusion (Figure 2A, Supplementary Figure S3). Specifically, streamline tractography visualization of fiber tracts revealed impaired connectivity between ROI set in the internal capsule and corona radiata. Oral TE supplementation protected fiber tract projections in the stroke-affected hemisphere as compared with PBO control. Probabilistic tractography is a powerful tool for quantitative analysis of white matter connectivity (Behrens *et al*, 2003). We used a probabilistic tractography framework to quantitatively assess the effect of TE supplementation on

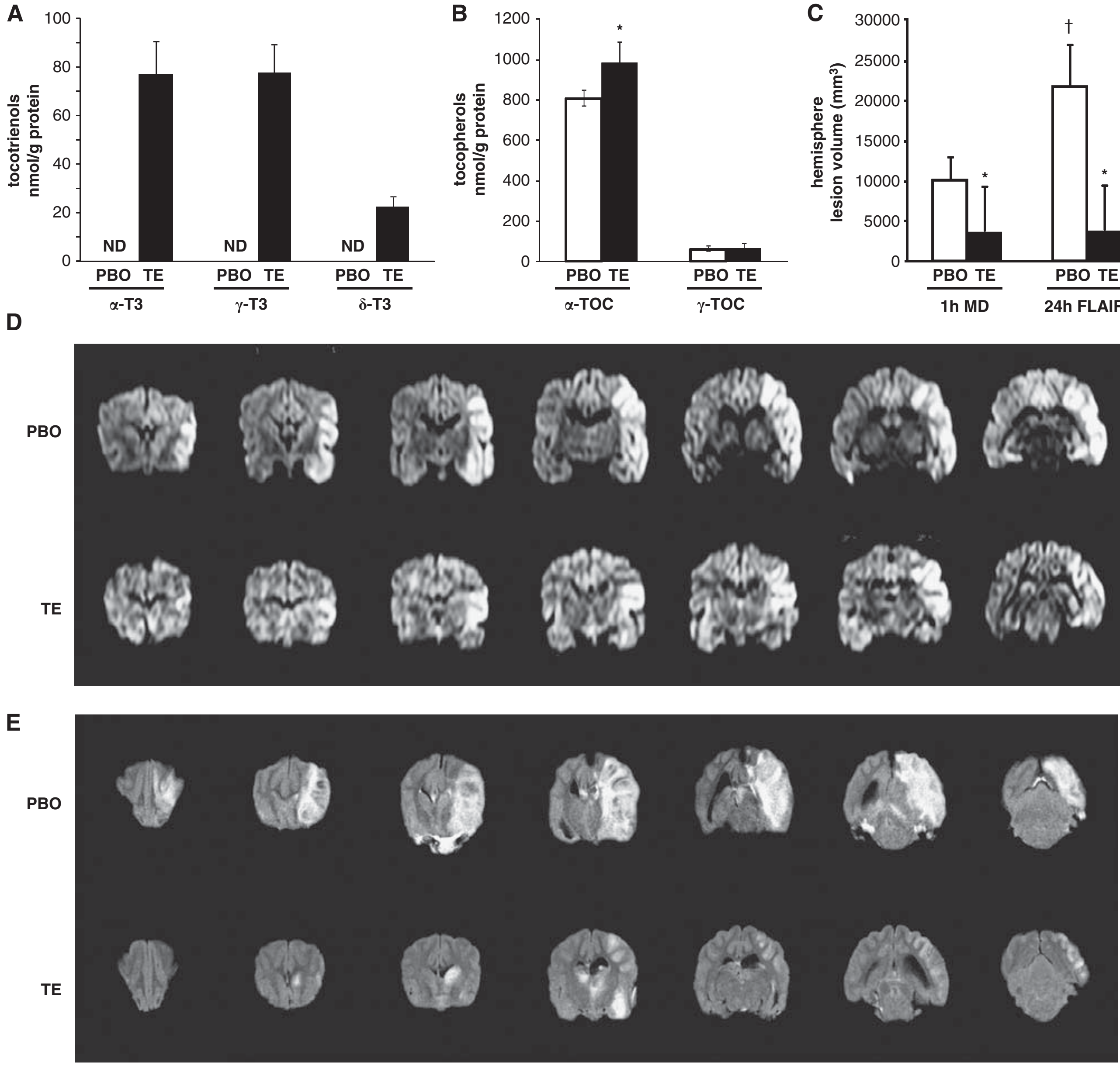

Figure 1 Tocotrienol-enriched (TE) natural vitamin E protects against stroke-induced brain injury. (**A** and **B**) Effect of 10-week oral supplementation on cerebral cortex concentration of tocotrienols and tocopherols. (**A**) No tocotrienols were detected in brain of PBO-supplemented canines. Tocotrienol-enriched supplementation significantly increased α-, γ-, and δ- tocotrienol isomers in cerebral cortex. (**B**) A moderate, but significant ($*P = 0.047$) increase in brain α-tocopherol level was observed as each TE gel capsule contains 61.5 mg of α-tocopherol. (**C**) Stroke-induced infarct volume in response to stroke. MD, mean diffusivity map taken at 1 hour; FLAIR, fluid attenuated inversion recovery taken at 24 hours. Representative coronal slice MR images of canine brain at (**D**) 1 hour demonstrating cytotoxic edema ($*P < 0.05$) and (**E**) 24 hours demonstrating cytotoxic and vasogenic edema following reperfusion ($*P < 0.005$). ND, not detected; PBO, placebo. Three dimensional volumetric reconstruction in color available online as Supplementary Figure S2.

white matter fiber tract connectivity in stroke-affected cortex. To quantitatively assess connectivity, 40,000 tracts were cast from voxels in the internal capsule ROI to the distal corona radiata ROI (Figure 2B). Relative connectivity of fiber tracts between the internal capsule and corona radiata was much higher in representative TE-supplemented canine brain as compared with PBO control. The PBO canine brain had a higher tract variance as a function of distance from the internal capsule seed ROI as compared with the TE counterpart (Figure 2C).

TE Supplementation Improved Cerebrovascular Collateral Circulation During Ischemic Stroke

Collateral arteries of the leptomeningeal space anastomose across border zones of cortical water-

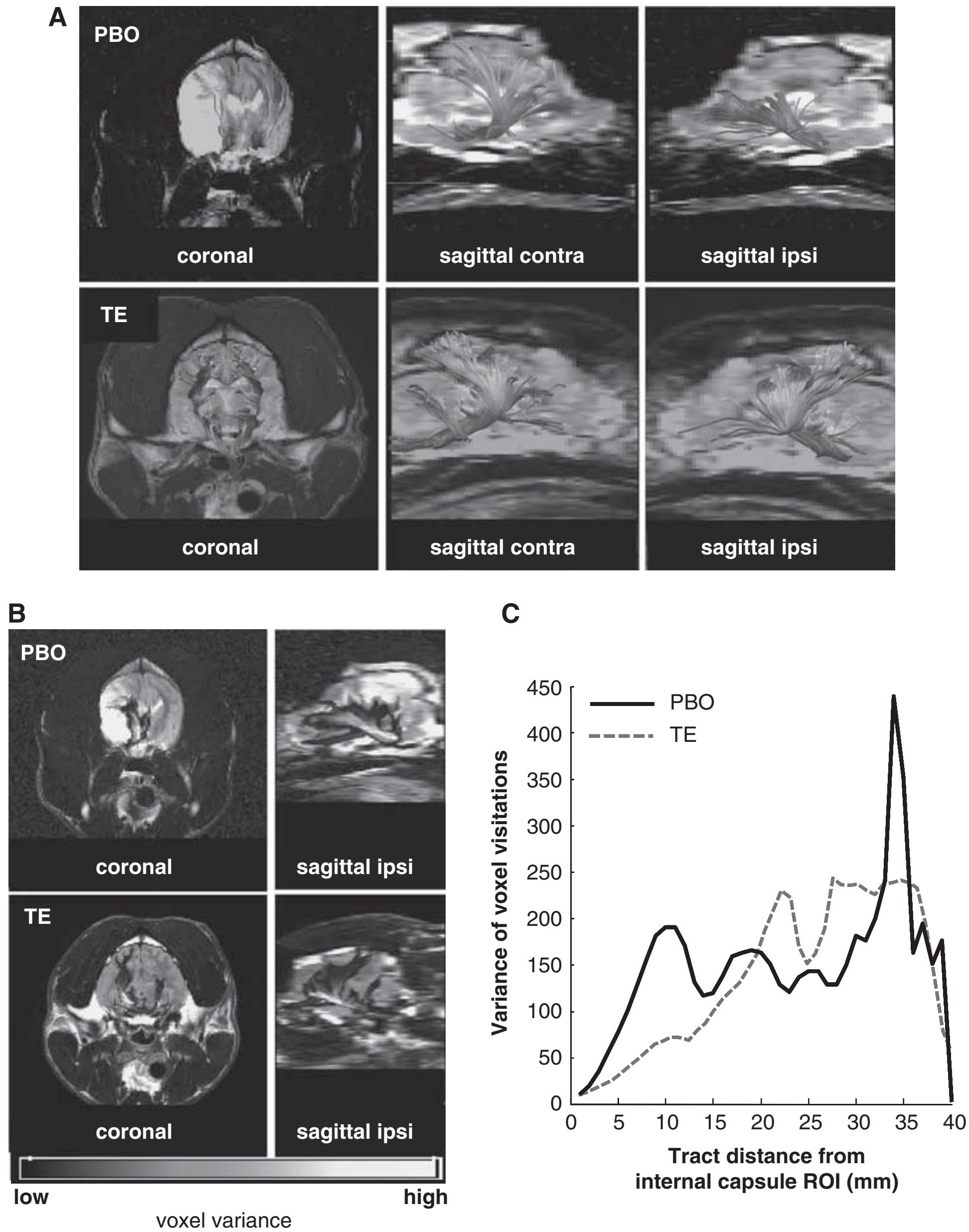

Figure 2 Tocotrienol-enriched (TE) attenuates white matter injury following acute ischemic stroke. (**A**) Streamline tractography of placebo (PBO) and TE white matter fiber tracts was performed with two regions of interest (ROI) masks to visualize tracts connecting the corona radiata to the internal capsule at 24 hours. Fiber tracts were overlaid on T2-weighted structural scan (512×512 matrix) to visualize in context of contralateral (right) and ipsilateral (left) hemispheres in the coronal orientation. Sagittal views of contralateral and ipsilateral hemispheres demonstrate the protective effect of TE supplementation. (**B**) Probabilistic tractography reveals connectivity of white matter fiber tracts projecting from the seed region of the internal capsule to the corona radiata in representative canines. Color shift from black→ red→ yellow→ white denotes a higher degree of relative connectivity between regions in the stroke-affected hemisphere of PBO- and TE-supplemented canines. (**C**) Variance of probabilistic tracts as a function of the distance from the internal capsule seed region. Contra, contralateral; ipsi, ipsilateral. Three dimensional color video available online as Supplementary Figure S3.

sheds in humans and large mammals alike underscoring the translational significance of our approach. This arterial network facilitates an alternative means to circulate blood, via retrograde filling, to tissue in instances when injury or occlusion to primary cortical branches disrupts cerebrovascular blood flow. Improving collateral circulation and blood perfusion to the stroke-affected territory is a therapeutic target of recognized value in the clinic (Brozici *et al*, 2003). In many cases, a focal circulatory abnormality created by arterial occlusion can be adequately compensated through cerebrovascular collateral circulation. Our preclinical canine stroke model benefits from angiographic assessment of collateral circulation during MCAO (Christoforidis *et al*, 2011; Rink *et al*, 2008). *Post hoc* analysis of

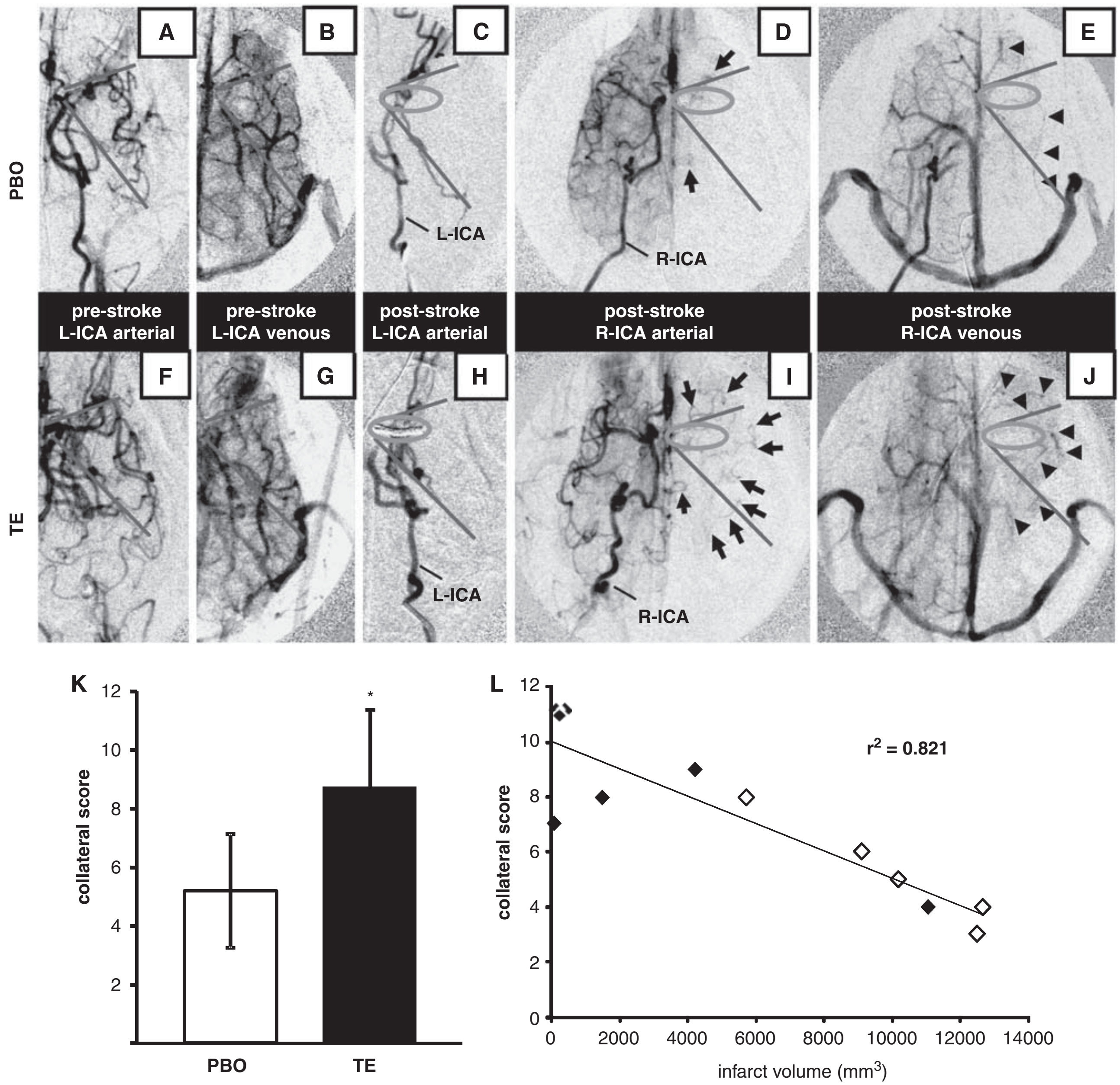

Figure 3 Tocotrienol-enriched (TE) supplement improves cerebrovascular collateral circulation during acute ischemic stroke. Cerebrovascular collaterals were identified by digital subtraction angiography (DSA) in placebo (PBO)- (**A–E**) and TE- (**F–J**) treated canines. To visualize collaterals of the stroke-affected MCA territory (green lines), pre-stroke arterial (**A**, **F**) and venous (**B**, **G**) DSA of left internal carotid artery (L-ICA) were compared with post-stroke arterial (**D**, **I**) and venous (**E**, **J**) DSA of right internal carotid artery (R-ICA). Post-stroke L-ICA DSA during the arterial phase (**C**, **H**) demonstrates effective MCA occlusion by embolic coil (marked by red oval). During the post-stroke arterial phase, greater collateral perfusion (black arrow) was observed in MCA territory of TE-supplemented canines as compared with PBO controls (**I** versus **D**). Likewise, more venous flow and contrast 'blush' (black triangle) was observed in stroke-affected hemisphere of TE-supplemented canines (**J** versus **E**). Mean collateral score for PBO- and TE-supplemented canines was determined according to an 11-point scale (methods). (**K**) Collateral score during stroke was significantly higher in TE-supplemented canines as compared with PBO controls. *$P < 0.05$. (**L**) Collateral score correlation with infarct volume (coefficient of determination, $r^2 = 0.821$), open diamonds represent PBO, closed diamonds represent TE canines.

cerebral angiograms during ischemic stroke revealed that canines receiving oral TE supplementation had improved cerebrovascular collateral circulation as compared with PBO controls (Figure 3). Pre- and post-MCAO internal carotid artery angiograms (Figures 3A–J) enable objective scoring of stroke-affected hemisphere collaterals according to a clinically relevant 11-point scale. Middle cerebral artery-territory collateral score was significantly higher in TE-supplemented canines as compared with PBO controls ($PBO = 5.2 \pm 1.9$, $TE = 8.1 \pm 2.9$; Figure 3K). A higher collateral score, and therefore better perfusion in the stroke-affected hemisphere, tightly correlated with smaller stroke-induced lesion size at 24 hours ($r^2 = 0.821$, Figure 3L).

Tocotrienol-Enriched Supplement Induced Expression of Arteriogenic Genes in Cerebral Cortex Collaterals

Arteriogenesis refers to a positive outward remodeling of pre-existing collateral arteries into larger vessels, which bypass sites of occlusion (Buschmann and Schaper 2000; Hillmeister *et al*, 2008). To determine whether TE supplementation invoked molecular mechanisms of cerebral arteriogenesis, arterioles from the stroke-affected (ipsilateral) and contralateral control cerebral cortex were selectively isolated laser microdissection pressure catapulting (Figures 4A–D). Known gene targets of cerebral arteriogenesis include members of the chloride intracellular channel, tissue inhibitor of metalloprotease 1 (TIMP1), and vascular endothelial growth factor (Chalothorn *et al*, 2007; Chalothorn *et al*, 2009; Hillmeister *et al*, 2008). Increased gene expression of chloride intracellular channel 1 and TIMP1 was observed in stroke-affected cortex of TE-supplemented canines as compared with PBO controls (Figures 4E and 4G). Of particular note, TE supplementation-dependent increase in TIMP1 expression was not limited to stroke-affected endothelial cells at the ipsilateral site. Tocotrienol-enriched supplementation induced TIMP1 in arterioles captured from contralateral control tissue (Figure 4G). These effects were specific as other arteriogenic candidate genes such as *chloride intracellular channel 4* and *vascular endothelial growth factor* were not affected by TE supplementation (Figures 4F and 4H). Tissue inhibitor of metalloprotease 1 binds to active MMP2 in a 1:1 stoichiometric ratio, providing localized control of MMP activity. Independent of MMP2 protein expression (Figures 5A and 5B), TE supplementation significantly attenuated MMP2 activity in the stroke-affected cerebral cortex (Figures 5C and 5D).

Discussion

Emblematic of the high morbidity and mortality associated with stroke are the failures of potential

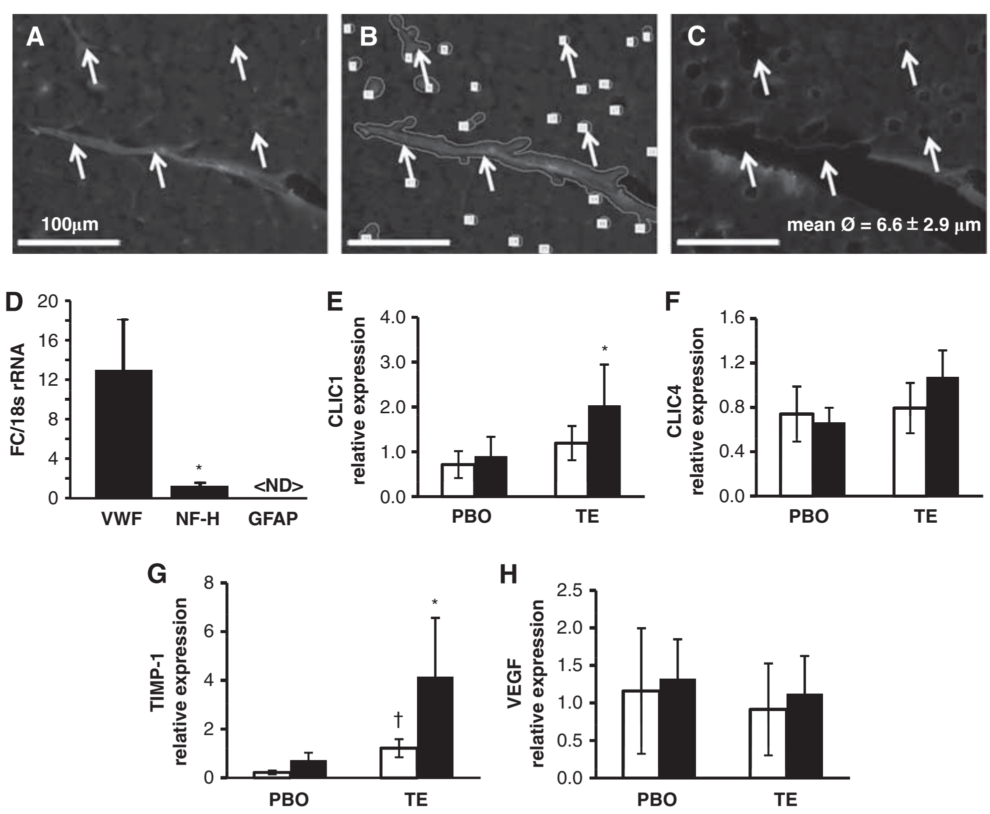

Figure 4 Tocotrienol-enriched (TE) supplement increases expression of arteriogenic markers in laser capture isolated cortex arterioles. (**A–C**) Arterioles (arrows, mean diameter $6.6 \pm 2.9\ \mu m$) were selectively captured from contralateral control and ipsilateral stroke-affected cerebral cortex 24 hours after stroke onset. (**D**) To verify specificity of captured elements, gene expression of vessel marker (VWF), neuron marker (NF-H), and glial marker (GFAP) was checked with real-time PCR. $*P < 0.05$ VWF versus NF-H, ND, not detected. (**E–H**) Expression of arteriogenic genes was validated using real-time PCR in contralateral (white) and ipsilateral (black) arterioles. $*P < 0.05$ in TE-supplemented control versus stroke. $^{\dagger}P < 0.05$ in placebo (PBO) versus TE control tissue. (**E**) Chloride intracellular channel 1 (CLIC1). (**F**) Chloride intracellular channel 4 (CLIC4). (**G**) Tissue inhibitor of metalloproteinase 1 (TIMP1). (**H**) Vascular endothelial growth factor (VEGF).

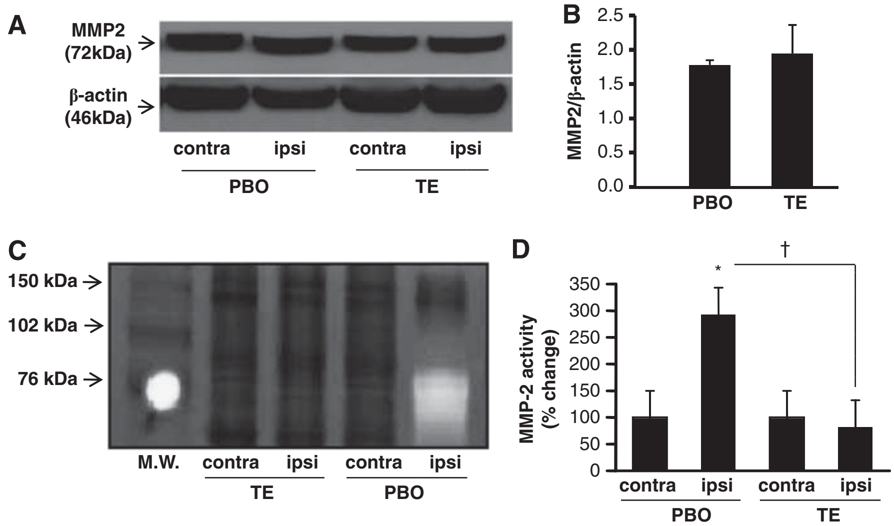

Figure 5 Tocotrienol-enriched (TE) supplement inhibits matrix metalloproteinase-2 (MMP2) activity in stroke-affected cerebral cortex. No difference in MMP2 protein expression was observed by western blot (**A**) and densitometric analysis (**B**) of contralateral (contra) and ipsilateral (ipsi) somatosensory cortex of placebo (PBO)- and TE-supplemented canines 24 hours after stroke. Gelatin zymography (**C**) and densitometry (**D**) demonstrates significantly higher MMP-2 activity in stroke-affected hemisphere of PBO, not TE canines. $^{*}P < 0.05$ PBO cont versus stroke, $^{\dagger}P < 0.05$ PBO stroke versus TE stroke.

stroke therapeutics, which showed benefit in small animal rodent stroke models but failed to translate into clinical success (Kidwell *et al*, 2001). As a result, rodent stroke models have been criticized for the anatomical disparity between small and large mammalian brains, large variability in infarct volumes, and inaccurate methods of inducing and confirming arterial occlusion (Gerriets *et al*, 2004). These considerations develop a compelling rationale to engage a translational relevant preclinical approach to test the therapeutic efficacy of TE natural vitamin E (Rink *et al*, 2008). As compared with the lissencephalic brain of rodents, the size and anatomical feature set of the canine brain closely mimics that of humans. Canines have a highly evolved gyrencephalic neocortex with a white to gray matter ratio that closely approximates primates, and like humans, collateral circulation in the MCA territory has been documented in canines (Symon, 1960). Furthermore, the current experimental model benefits from C-arm fluoroscopy visualization of MCAO. As opposed to the widely used rodent intraluminal thread model of MCAO, this method permits repeated real-time documentation of the stroke event, improving the overall reproducibility of the procedure and enabling objective assessment of collateral circulation during cerebral ischemia (Christoforidis *et al*, 2011). In this work, the latter proved to be pivotal in identifying the effects of TE on perfusion of the stroke-affected brain tissue. This observation, enabled by the translational approach adopted, was the first to elucidate the cerebrovascular effects of tocotrienol. Until this point, the current literature documents significant protective effects of stroke *in vivo* but explains it exclusively on the basis of TE's neuroprotective properties. α-Tocotrienol-specific mechanisms of neuroprotection depend on three key cytosolic targets involved in glutamate excitotoxicity and neurodegeneration: c-Src kinase, 12-lipoxygenase, and phospholipase A_2 (Khanna *et al*, 2005*b*; Khanna *et al*, 2010; Sen *et al*, 2000). Neuroprotectants alone, however, are thought to be insufficient in providing meaningful protection against stroke (Rogalewski *et al*, 2006). Multimodal therapies that target both neuro and vascular pathophysiology are desirable. This work is the first to demonstrate a prophylactic intervention to improve collateral circulation during acute ischemic stroke. The favorable effects of TE on collateral perfusion of the stroke site taken together with its known neuroprotective properties provide two powerful mechanisms that support the case for TE in stroke therapeutics.

The cerebrovascular collateral circulation refers to a subsidiary network of small vascular channels that can stabilize cerebral blood flow when principal conduits are obstructed, as in ischemic stroke (Liebeskind, 2005). These small collateral pathways can occur through leptomeningeal arterioles that overlap and anastomose distal branches of the anterior and posterior cerebral arteries with the MCA. Indeed, the risk and severity of stroke-mediated pathology is worse in patients with poor collateral circulation (Christoforidis *et al*, 2005). The mechanistic process in which pre-existing arterioles are recruited to bypass the site of occlusion is termed arteriogenesis. Arteriogenesis invokes a rapid proliferative and remodeling response that is distinct from passive dilatation, developmental vasculogenesis, or neovascular angiogenesis (Buschmann and Schaper, 1999). Induction of arteriogenic collateral growth in the brain occurs as early as 24 hours following vessel occlusion (Schierling *et al*, 2009) and the onset of adaptive arteriogenesis is marked by early-phase expression of protease inhibitor TIMP1 in growing collaterals of the brain (Hillmeister *et al*, 2008). In this work, TE supplementation significantly increased TIMP1 expression in both contralateral control and stroke-affected arterioles of the cerebral cortex. The observation that TE induces TIMP1 expression in the blood vessel of contralateral hemisphere points to the hypothesis that long-term orally supplemented TE may prime the cerebral vasculature, enabling adaptive arteriogenesis in response to focal cerebral ischemia. Indeed, controlling extracellular matrix degradation and advancing vascular remodeling by the activation of cell proliferation represents an important role of TIMP1 in arteriogenesis (Hillmeister *et al*, 2008). This work provides first evidence of TE supplementation regulating TIMP1 expression and subsequently invoking cerebrovascular arteriogenesis. It is reported that the tocotrienol-rich fraction of palm oil improves endothelium-dependent relaxation in isolated aortic rings of diabetic and hypertensive rats (Muharis *et al*, 2010). Thus, in addition to the pro-arteriogenic property TE may improve cerebrovascular circulation at the stroke site by inducing arterial dilatation.

DTI enables repeated, non-invasive assessment of white matter cytoarchitecture and connectivity due to unrestricted parallel (anisotropic) diffusion of water molecules along axonal fiber tracts. This MRI-based technique has emerged as a clinically relevant tool for the prognostic diagnosis of neurologic deficit and assessment of rehabilitation potential in stroke patients (Kunimatsu *et al*, 2003). Proceeding from the cortex, white matter fiber tracts of the corona radiata, or 'radiating crown', converge and pass between the lenticular nucleus and thalamus in the form of a band called the internal capsule. The fiber tracts of the corona radiata and internal capsule contain corticospinal nerve bundles that are responsible for sensorimotor neurotransmission between somatosensory cortex and motor neurons (Higano *et al*, 2001). This work used streamline and probabilistic tractography to assess stroke-mediated injury and loss of white matter connectivity between internal capsule and the corona radiata after stroke. White matter of TE-treated animals, not PBO, maintained the cytoarchitectural connection between internal capsule and corona radiata, suggesting that TE protected anatomical connectivity, and therefore biological function, from stroke injury. Taken together with the marked improvement in functional outcomes following MCAO in TE-supplemented mice, data strongly suggest that prophylactic TE supplementation attenuates the severity of stroke-associated sensorimotor injury.

In addition to tractography, DTI also enables assessment of stroke-induced lesion. During the acute phase of cerebral ischemia (0 to 24 hours post reperfusion), a decline in apparent diffusion coefficient maps generated from DTI is associated with cytotoxic edema causing irreversible brain injury (Ducreux *et al*, 2001). Using DTI imaging immediately after stroke reperfusion, we found that TE supplementation attenuated stroke-induced cytotoxic edema within the first hour following reperfusion. Although cytotoxic edema evolves over minutes to hours, vasogenic edema occurs over hours to days and is associated with blood–brain barrier disruption (Heo *et al*, 2005). Magnetic resonance imaging performed at 24 hours used a T2-weighted FLAIR sequence that captures both cytotoxic and vasogenic components of stroke-induced edema. Lesion volume in TE-supplemented animals did not significantly increase between 1 and 24 hours MRI, suggesting that TE largely prevented blood brain barrier disruption and subsequent vasogenic edema.

As a nutrient tocotrienols have been safely consumed by humans, especially in the Far East, for many years. Furthermore, tocotrienols have been Generally Recognized As Safe (GRN No. 307) certified by the US FDA as ingredients in food. In nature, tocopherols and tocotrienols are found in abundance throughout the plant kingdom. Tocopherols are the primary source of vitamin E in photosynthetic plant tissue, whereas tocotrienols are enriched in endosperm of cereals, grains, and palm seed (Sen *et al*, 2010). A growing body of studies support that different members of the natural vitamin E family may have unique biological properties relevant to health and disease (Aggarwal *et al*, 2010). For example, anti-tumorigenic properties of γ-tocotrienol, not shared by α-tocopherol, have been described in both breast (Park *et al*, 2010) and prostate (Kumar *et al*, 2006) cancer. Furthermore, tocotrienol transport to tissue, including brain, has been reported in the absence of tocopherol transfer protein (TTP), the transport system with high affinity for α-tocopherol (Khanna *et al*, 2005*a*). Indeed, loss of fertility in $TTP^{-/-}$ mice could be rescued by TE supplementation (Khanna *et al*, 2005*a*). At a time when meta-analyses of clinical trials testing the effect of tocopherols in a variety of disease setting draw major conclusions relevant to public health policies and practices, this work illuminates a blind spot reminding that title claims on vitamin E should be limited to the specific form of vitamin E studied.

This work demonstrates that prophylactic supplementation of natural vitamin E tocotrienols reduces brain injury after stroke in a preclinical setting. Given the observed effect of TE in improving collateral circulation during cerebral ischemia and the established hypo-cholesterolemic effects of tocotrienol supplementation (Parker *et al*, 1993), the current work lays the foundation to test the effects of prophylactic TE supplementation on reducing stroke incidence. Outcomes of the current study clearly support clinical assessment of TE in a high-risk stroke population, such as TIA patients. With more than 200,000 Americans each year, the TIA patient population is well suited for testing the efficacy of TE in a clinical trial setting.

Disclosure/conflict of interest

The authors declare no conflict of interest.

References

Aggarwal BB, Sundaram C, Prasad S, Kannappan R (2010) Tocotrienols, the vitamin E of the 21st century: its potential against cancer and other chronic diseases. *Biochem Pharmacol* 80:1613–31

Beceriklisoy HB, Walter I, Schafer-Somi S, Miller I, Kanca H, Izgur H, Aslan S (2007) Matrix metalloproteinase (MMP)-2 and MMP-9 activity in the canine uterus before and during placentation. *Reprod Domest Anim* 42:654–9

Behrens TE, Johansen-Berg H, Woolrich MW, Smith SM, Wheeler-Kingshott CA, Boulby PA, Barker GJ, Sillery EL, Sheehan K, Ciccarelli O, Thompson AJ, Brady JM, Matthews PM (2003) Non-invasive mapping of connections between human thalamus and cortex using diffusion imaging. *Nat Neurosci* 6:750–7

Brozici M, van der Zwan A, Hillen B (2003) Anatomy and functionality of leptomeningeal anastomoses: a review. *Stroke* 34:2750–62

Buschmann I, Schaper W (1999) Arteriogenesis versus angiogenesis: two mechanisms of vessel growth. *News Physiol Sci* 14:121–5

Buschmann I, Schaper W (2000) The pathophysiology of the collateral circulation (arteriogenesis). *J Pathol* 190:338–42

Chalothorn D, Clayton JA, Zhang H, Pomp D, Faber JE (2007) Collateral density, remodeling, and VEGF-A expression differ widely between mouse strains. *Physiol Genomics* 30:179–91

Chalothorn D, Zhang H, Smith JE, Edwards JC, Faber JE (2009) Chloride intracellular channel-4 is a determinant of native collateral formation in skeletal muscle and brain. *Circ Res* 105:89–98

Christoforidis GA, Mohammad Y, Kehagias D, Avutu B, Slivka AP (2005) Angiographic assessment of pial collaterals as a prognostic indicator following intra-arterial thrombolysis for acute ischemic stroke. *AJNR Am J Neuroradiol* 26:1789–97

Christoforidis GA, Rink C, Kontzialis MS, Mohammad Y, Koch RM, Abduljalil AM, Bergdall VK, Roy S, Khanna S, Slivka AP, Knopp MV, Sen CK (2011) An endovascular canine middle cerebral artery occlusion model for the study of leptomeningeal collateral recruitment. *Invest Radiol* 46:34–40

Ducreux D, Oppenheim C, Vandamme X, Dormont D, Samson Y, Rancurel G, Cosnard G, Marsault C (2001) Diffusion-weighted imaging patterns of brain damage associated with cerebral venous thrombosis. *Am J Neuroradiol* 22:261–8

Gerriets T, Stolz E, Walberer M, Muller C, Rottger C, Kluge A, Kaps M, Fisher M, Bachmann G (2004) Complications and pitfalls in rat stroke models for middle cerebral artery occlusion: a comparison between the suture and the macrosphere model using magnetic resonance angiography. *Stroke* 35:2372–7

Heo JH, Han SW, Lee SK (2005) Free radicals as triggers of brain edema formation after stroke. *Free Radic Biol Med* 39:51–70

Higano S, Zhong J, Shrier DA, Shibata DK, Takase Y, Wang H, Numaguchi Y (2001) Diffusion anisotropy of the internal capsule and the corona radiata in association with stroke and tumors as measured by diffusion-weighted MR imaging. *Am J Neuroradiol* 22:456–63

Hillmeister P, Lehmann KE, Bondke A, Witt H, Duelsner A, Gruber C, Busch HJ, Jankowski J, Ruiz-Noppinger P, Hossmann KA, Buschmann IR (2008) Induction of cerebral arteriogenesis leads to early-phase expression of protease inhibitors in growing collaterals of the brain. *J Cereb Blood Flow Metab* 28:1811–23

Khanna S, Patel V, Rink C, Roy S, Sen CK (2005a) Delivery of orally supplemented alpha-tocotrienol to vital organs of rats and tocopherol-transport protein deficient mice. *Free Radic Biol Med* 39:1310–9

Khanna S, Roy S, Slivka A, Craft TK, Chaki S, Rink C, Notestine MA, DeVries AC, Parinandi NL, Sen CK (2005b) Neuroprotective properties of the natural vitamin E alpha-tocotrienol. *Stroke* 36:2258–64

Khanna S, Parinandi NL, Kotha SR, Roy S, Rink C, Bibus D, Sen CK (2010) Nanomolar vitamin E alpha-tocotrienol inhibits glutamate-induced activation of phospholipase A2 and causes neuroprotection. *J neurochem* 112:1249–60

Kidwell CS, Liebeskind DS, Starkman S, Saver JL (2001) Trends in acute ischemic stroke trials through the 20th century. *Stroke* 32:1349–59

Kumar KS, Raghavan M, Hieber K, Ege C, Mog S, Parra N, Hildabrand A, Singh V, Srinivasan V, Toles R, Karikari P, Petrovics G, Seed T, Srivastava S, Papas A (2006) Preferential radiation sensitization of prostate cancer in nude mice by nutraceutical antioxidant gamma-tocotrienol. *Life Sci* 78:2099–104

Kunimatsu A, Aoki S, Masutani Y, Abe O, Mori H, Ohtomo K (2003) Three-dimensional white matter tractography by diffusion tensor imaging in ischaemic stroke involving the corticospinal tract. *Neuroradiology* 45:532–5

Liebeskind DS (2005) Neuroprotection from the collateral perspective. *IDrugs* 8:222–8

Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, Ferguson TB, Ford E, Furie K, Gillespie C, Go A, Greenlund K, Haase N, Hailpern S, Ho PM, Howard V, Kissela B, Kittner S, Lackland D, Lisabeth L, Marelli A, McDermott MM, Meigs J, Mozaffarian D, Mussolino M, Nichol G, Roger VL, Rosamond W, Sacco R, Sorlie P, Roger VL, Thom T, Wasserthiel-Smoller S, Wong ND, Wylie-Rosett J (2010) Heart disease and stroke statistics—2010 update: a report from the American Heart Association. *Circulation* 121:e46–215

Lonn E, Bosch J, Yusuf S, Sheridan P, Pogue J, Arnold JM, Ross C, Arnold A, Sleight P, Probstfield J, Dagenais GR (2005) Effects of long-term vitamin E supplementation on cardiovascular events and cancer: a randomized controlled trial. *Jama* 293:1338–47

Miller III ER, Pastor-Barriuso R, Dalal D, Riemersma RA, Appel LJ, Guallar E (2005) Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. *Ann Intern Med* 142:37–46

Muharis SP, Top AG, Murugan D, Mustafa MR (2010) Palm oil tocotrienol fractions restore endothelium dependent relaxation in aortic rings of streptozotocin-induced diabetic and spontaneously hypertensive rats. *Nutr Res* 30:209–16

Park SK, Sanders BG, Kline K (2010) Tocotrienols induce apoptosis in breast cancer cell lines via an endoplasmic reticulum stress-dependent increase in extrinsic death receptor signaling. *Breast Cancer Res Treat* 124:361–75

Parker RA, Pearce BC, Clark RW, Gordon DA, Wright JJ (1993) Tocotrienols regulate cholesterol production in mammalian cells by post-transcriptional suppression of

3-hydroxy-3-methylglutaryl-coenzyme A reductase. *J Biol Chem* 268:11230–8

Passingham RE, Stephan KE, Kotter R (2002) The anatomical basis of functional localization in the cortex. *Nat Rev Neurosci* 3:606–16

Rink C, Christoforidis G, Abduljalil A, Kontzialis M, Bergdall V, Roy S, Khanna S, Slivka A, Knopp M, Sen CK (2008) Minimally invasive neuroradiologic model of preclinical transient middle cerebral artery occlusion in canines. *Proc Natl Acad Sci USA* 105:14100–5

Rink C, Roy S, Khan M, Ananth P, Kuppusamy P, Sen CK, Khanna S (2010) Oxygen-sensitive outcomes and gene expression in acute ischemic stroke. *J Cere Blood Flow Metab* 30:1275–87

Rogalewski A, Schneider A, Ringelstein EB, Schabitz WR (2006) Toward a multimodal neuroprotective treatment of stroke. *Stroke* 37:1129–36

Roy S, Venojarvi M, Khanna S, Sen CK (2002) Simultaneous detection of tocopherols and tocotrienols in biological samples using HPLC-coulometric electrode array. *Methods Enzymol* 352:326–32

Schaechter JD, Perdue KL, Wang R (2008) Structural damage to the corticospinal tract correlates with bilateral sensorimotor cortex reorganization in stroke patients. *Neuroimage* 39:1370–82

Schierling W, Troidl K, Mueller C, Troidl C, Wustrack H, Bachmann G, Kasprzak PM, Schaper W, Schmitz-Rixen T (2009) Increased intravascular flow rate triggers cerebral arteriogenesis. *J Cereb Blood Flow Metab* 29:726–37

Schwartz H, Ollilainen V, Piironen V, Lampi AM (2008) Tocopherol, tocotrienol and plant sterol contents of vegetable oils and industrial fats. *J Food Comp Anal* 21:152–61

Sen CK, Rink C, Khanna S (2010) Palm oil-derived natural vitamin E alpha-tocotrienol in brain health and disease. *J Am Coll Nutr* 29:314S–23S

Sen CK, Khanna S, Roy S, Packer L (2000) Molecular basis of vitamin E action. Tocotrienol potently inhibits glutamate-induced pp60(c-Src) kinase activation and death of HT4 neuronal cells. *J Biol Chem* 275:13049–55

Sen CK, Khanna S, Roy S (2004) Tocotrienol: the natural vitamin E to defend the nervous system? *Ann N Y Acad Sci* 1031:127–42

Smith SM, Jenkinson M, Woolrich MW, Beckmann CF, Behrens TE, Johansen-Berg H, Bannister PR, De Luca M, Drobnjak I, Flitney DE, Niazy RK, Saunders J, Vickers J, Zhang Y, De Stefano N, Brady JM, Matthews PM (2004) Advances in functional and structural MR image analysis and implementation as FSL. *Neuroimage* 23(Suppl 1):S208–19

Symon L (1960) Observations on the leptomeningeal collateral circulation in dogs. *J Physiol* 154:1–14.2

Supplementary Information accompanies the paper on the Journal of Cerebral Blood Flow & Metabolism website (http://www.nature.com/jcbfm)