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A Potent Antioxidant, Lycopene, Affords Neuroprotection Against Microglia Activation and Focal Cerebral Ischemia in Rats

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Abstract. *We investigated the effects of a potent antioxidant, lycopene, on the free radical-scavenging activity as evaluated by the DPPH test and lipid peroxidation in rat brain homogenates as well as nitric oxide (NO) formation in cultured microglia stimulated by lipopolysaccharide. In addition, we also investigated the therapeutic effect of lycopene in attenuating ischemia/reperfusion brain injury induced by middle cerebral artery (MCA) occlusion in rats. Lycopene (1, 2 and 5 μM) exerted increased DPPH decolorization in the DPPH test, and increased inhibition of iron-catalyzed lipid peroxidation (TBARS formation) in rat brain homogenates in concentration-dependent manners. Furthermore, lycopene (5 and 10 μM) significantly inhibited nitrite production by about 31% and 61% in microglia stimulated by LPS, respectively. Rats which received lycopene at a dosage of 4 mg/kg, but not at 2 mg/kg, showed significant infarct size reductions compared with those which received the solvent control (20% Tween 80). In conclusion, we demonstrate a protective effect of lycopene on ischemic brain injury in vivo. Lycopene, through its antioxidative property, mediates at least a portion of free radical-scavenging activity and inhibits microglia activation, resulting in a reduction in infarct volume in ischemia/reperfusion brain injury.*

Ischemic or hypoxic brain injury often causes irreversible brain damage. The cascade of events leading to neuronal injury and death in ischemia includes the release of cytokines, excitatory amino acids and nitric oxide (NO) or free radicals, as well as damage to mitochondrial respiratory enzymes, induction of programmed cell death and microglia activation (1-3). During cerebral ischemia and subsequent reperfusion, enhanced formation of oxygen free radicals occurs in damaged tissue (2, 3). These oxidant radicals contribute to increased neuronal death by oxidizing proteins, damaging DNA and inducing the lipoperoxidation of cellular membranes (4). In addition, the expression of proinflammatory cytokines during ischemia/reperfusion injury results in up-regulation of inducible nitric oxide synthase (iNOS), thereby producing large amounts of NO under oxidative stress conditions. NO reacts with superoxide to generate peroxynitrite (ONOO-), which is capable of nitrating tyrosine residues of proteins and enzymes (5, 6).

Microglia are a type of neuroglia that support, nurture and protect the neurons which maintain homeostasis of the fluid that bathes neurons (7). Under physiological conditions, residential microglia are quiescent and scattered throughout the CNS. Occasionally microglia are moderately activated to play the classic role as scavengers for the maintenance and restoration of the CNS. Therefore, identification of drugs that down-regulate and/or block the expression of proinflammatory cytokines, NO, or free radicals, should provide the opportunity for treatment of cerebral diseases (8, 9).

Lycopene is the most-potent antioxidant among various common carotenoids (10). Dietary supplementation of lycopene leading to high serum lycopene levels protected men from development of prostate cancer (11). The health benefits of lycopene might extend beyond fighting prostate cancer since accumulating evidence suggests that the antiproliferative properties of lycopene may extend to other types of cancer (12). Furthermore, lycopene may be useful in preventing heart disease. Lycopene apparently inhibits cholesterol synthesis and enhances low-density lipoprotein degradation (13). Available data suggest that the risks of myocardial infarction are reduced in persons with higher adipose tissue concentrations of lycopene (13).

We investigated the antioxidative activity of lycopene as revealed by its protective effects against free radicals and lipid peroxidation and by its inhibitory effect on NO formation in microglia stimulated by LPS. We also investigated the neuroprotective effect of lycopene on ischemic brain damage induced in a focal ischemia/reperfusion model of middle cerebral artery (MCA) in rats. We then utilized these findings

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to characterize the relationship between the antioxidative activities *in vitro* and neuroprotection against ischemia/reperfusion brain damage by lycopene *in vivo*.

Materials and Methods

Materials. Lycopene, 2-thiobarbituric acid (TBA), tetramethoxypropane, sulfanilamide, naphthylenediamine, bovine serum albumin (BSA), trypsin, deoxyribonuclease, Tween 80, lipopolysaccharide (LPS; *Escherichia coli*, serotype 0127: B8), 1,1-diphenyl-2-picrylhydrazyl (DPPH), phosphoric acid, 2,3,5-triphenyl tetrazolium chloride (TTC) and trichloroacetic acid (TCA) were purchased from Sigma Chem. (St. Louis, MO, USA). Lycopene was dissolved in 20% Tween 80 with normal saline. In this study, a vehicle solvent control was always included.

Stable free radical-scavenging action. DPPH, a stable nitrogen-centered free radical, was dissolved in ethanol to produce a 100-μM solution. To 1.0 ml of ethanolic DPPH in a cuvette, lycopene or solvent control (20% Tween 80) was added. The decrease in absorption at 517 nm was correlated with the scavenging action of the test compounds (14).

Antioxidant activity in rat brain homogenates. Rat brain homogenates were prepared from the brains of freshly killed Wistar rats and their peroxidation in the presence of iron ions was measured by the TBA method, as described by Hsiao *et al.* (15). In brief, whole brain tissue, excluding the cerebullum, was washed and homogenized in 10 volumes of ice-cold Krebs buffer using a homogenizer (Glas-col 099ck44). The homogenate was centrifuged at low speed (1000 g) for 10 min and the supernatant (adjusted to 2 mg/ml) was immediately used in the lipid peroxidation assays. The reaction mixture with lycopene or solvent control was incubated for 10 min, then stimulated by addition of ferrous ion (200 μM, freshly prepared) and maintained at 37˚C for 30 min. The reactions were terminated by adding 10 μl of ice-cold TCA solution (4% [w/v] in 0.3 N HCl) and 200 μl of thiobarbituric acid-reactive substance (TBARS) reagent (0.5% [w/v] TBA in 50% [v/v] acetic acid). After boiling for 15 min, the samples were cooled and extracted with *n*-1-butanol. The extent of lipid peroxidation was estimated as TBARS and was read at 532 nm in a spectrophotometer (Hitachi, model U3200, Tokyo, Japan). Tetramethoxypropane was used as a standard and the results were expressed as the absorbance at 532 nm per milligram protein of the supernatant of rat brain homogenates. The protein contents of the brain homogenates and other preparations were determined using the Bio-Rad method with BSA as a standard (16).

Cell cultivation. Wistar rats (7 days old) were deeply anesthetized with ether and transcardically perfused with normal saline until the lung and liver were clear of blood. After perfusion, the brain was removed and kept in RPMI-1640 medium (Gibco-BRL). After dissecting the meninges, the brain tissue was minced in ice-cold RPMI-1640 and treated with trypsin (0.25%) and deoxyribonuclease (10 mg/ml) in RPMI-1640 for 2 h at 37˚C. The treated tissues were further minced in 10% FBS and centrifuged at 1000 rpm for 10 min. The tissue pellet was resuspended in RPMI-1640 and then seeded in 75-cm^2 flasks at 37˚C (95% O_2, 5% CO_2).

Microglia were harvested from flasks of mixed glial cultures by shaking for 2 h. Cells were collected by centrifugation and seeded at 5 x 10^5 cells/ml. After incubation for 1 h at 37˚C, non-adherent or weakly adherent cells were removed by gentle shaking and washed out. The cells were further cultured in RPMI-1640 supplemented with 10% FBS for 1 day. Approximately 2 x 10^6 cells were obtained per brain used. Cells (2 x 10^5 cells/well) were preincubated with lycopene (1, 5 and 10 μM) for 15 min followed by the addition of LPS (250 ng/ml) for activation for 24 h. The conditioned media were collected, centrifuged and stored at -70˚C for less than 2 weeks.

Determination of nitrite concentration. To determine NO production, nitrite (a stable oxidative endproduct of NO) accumulation in the media of microglia was measured using a colorimetric method (17) with minor modifications. Briefly, 100 μl of supernatant was incubated with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthyl-ethylenediamine dihydrochloride in 2.5% phosphoric acid). After a 30-min incubation at room temperature, the optical absorbance at 550 nm was measured with a microplate reader (MRX). Nitrite concentrations were calculated by regression with standard solutions of sodium nitrite prepared in the same culture medium.

Transient cerebral ischemia/reperfusion injury. Male Wistar rats (250-300 g) used in this study were obtained from the Laboratory Animal Center of the National Taiwan University. All animal experiments and care were performed according to the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington, DC, USA, 1996). Lycopene (2 and 4 mg/kg) was dissolved in 20% Tween 80 before injection. Control rats were injected with an equivalent volume of the solvent control (20% Tween 80).

The animals were anesthetized in a chamber with a mixture of 95% O_2 and 5% CO_2 containing 3% isoflurane. The rectal temperature was maintained at 37±0.5˚C with a homeothermic blanket. Right MCA was occluded as described by Longa *et al.* (18). Briefly, after making a median incision in the neck skin, the right common carotid artery was exposed and a 4-0 monofilament nylon thread (NC 124L, UNIK) coated with silicon (Surflex F™, GC America) was then inserted from the external into the internal carotid artery until the tip occluded the origin of the MCA. After closure of the operative sites, the animals were allowed to awake from the anesthesia. During another brief period of anesthesia, the filament was gently removed after 1 h of MCA occlusion, and reperfusion through the common carotid artery was confirmed under a dissecting microscope. The animals were then allowed to recover from the anesthesia on a warming pad.

An observer blinded to the identity of the groups assessed neurological deficits at 1 and 24 h after reperfusion (before sacrifice) by the forelimb akinesia (also called postural tail-hang) test, while the spontaneous rotational test was used as a criterion for evaluating the ischemic insult (19). Animals not showing behavioral deficits at the above time points after reperfusion were excluded from the study.

Rats were sacrificed by decapitation after 24 h of reperfusion under chloral hydrate (200 mg/kg, *i.p.*) anesthesia. The brains were quickly removed and placed in ice-cold saline for 5 min and then cut into 2-mm coronal slices using an Adult Rat Brain Matrix (World Precision Instruments). Sections were incubated in 2% TTC dissolved in normal saline for 30 min at 37˚C. Stained brain sections were stored in 10% formaldehyde and refrigerated at 4˚C for further processing and storage. Each slice was drawn using a

computerized image analyzer (Image-Pro plus). The calculated infarction areas were then compiled to obtain the infarct volumes (mm^3) per brain. Infarct volumes were expressed as a percentage of the contralateral hemisphere volume by using the formula, (the area of the intact contralateral [left] hemisphere – the area of the intact region of the ipsilateral [right] hemisphere) to compensate for edema formation in the ipsilateral hemisphere (20).

All animals were divided into three groups: (i) a sham-operated group; (ii) a solvent (20% Tween 80) control group; and (iii) a lycopene-treated group. In the lycopene-treated group, rats were given lycopene (2 and 4 mg/kg; *i.p.*) twice: 15 min before MCA occlusion and 15 min before reperfusion. Rats in the solvent control were administered the same volume of solvent instead of lycopene at the same time points.

Statistical analysis. Experimental results are expressed as the means±SEM and are accompanied by the number of observations. Student's unpaired *t*-test was used to determine significant differences in the study of cerebral ischemia/reperfusion injury. The other experiments were assessed by the method of analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared using the Newman-Keuls method. A *p* value of less than 0.05 was considered statistically significant.

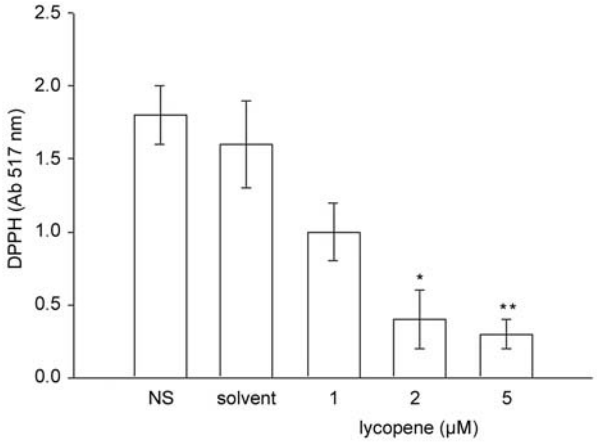

Figure 1. *Effect of lycopene on the free radical-scavenging activity in the 1,2-diphenyl-2-picrylhydrazyl (DPPH) test. The stable free radical-scavenging action was evaluated at concentrations of lycopene (1, 2, and 5 μM) which decreased the absorbance of the stable free radical, DPPH, at 517 nm. Data are presented as the means ± SEM (n=4). *p<0.05 and **p<0.01 as compared with the solvent control (20% Tween 80). NS, normal saline.*

Results

Effect of lycopene on free radical (DPPH)-scavenging activity. The change in absorbance produced by reduced DPPH was used to evaluate the ability of test compounds to act as free radical scavengers. DPPH decolorization was increased by lycopene (1, 2 and 5 μM) in a concentration-dependent manner (Figure 1). This result showed that lycopene was a good scavenger for interacting with the nitrogen-centered stable free radical, DPPH. The solvent control (20% Tween 80) did not significantly affect this reaction (Figure 1).

Effects of lycopene on lipid peroxidation in rat brain homogenates. Lycopene (1, 2 and 5 μM) exerted concentration-dependent inhibition of iron-catalyzed lipid peroxidation (TBARS formation) in rat brain homogenates (Figure 2). At a higher concentration (10 μM), lycopene also inhibited spontaneous lipid peroxidation by about 95% (data not shown). The IC_{50} of lycopene for inhibition of TBARS was about 1.8 μM. Furthermore, lycopene (5 μM) alone did not significantly interfere with the absorption at 532 nm when added to rat brain homogenates that were either intact or already oxidatively modified (data not shown). The solvent control (20% Tween 80) also did not significantly affect this reaction (Figure 2).

Effect of lycopene on nitrite production in LPS-induced microglia activation. According to our preliminary test, activation of microglia by LPS (250 ng/ml) induced a significant and maximal increase in nitrite formation, a stable oxidative endproduct of NO. Therefore, we used this concentration of LPS (250 ng/ml) for this experiment. In this study, the concentration of nitrite production in the cell supernatant was elevated from 0.8 ± 0.2 to 6.4 ± 0.9 μM at 24 h after LPS activation (Figure 3). Lycopene (5 and 10 μM) markedly inhibited nitrite production stimulated by LPS by about 31% and 61%, respectively (Figure 3). Lycopene neither interfered with the Griess reaction nor reacted with native NO. These results demonstrate that lycopene markedly suppresses NO production stimulated by LPS in microglia.

Effect of lycopene in MCA occlusion. Figure 4 shows the control sections from ischemic brain (panel A, white area) and coronal sections from brains pretreated with lycopene (2 mg/kg, panel B) and (4 mg/kg, panel C) prior to ischemic insult. Animals that received an *i.p.* injection of lycopene, at a dosage of 4 mg/kg (n=9), but not at 2 mg/kg (n=9), showed significant infarct size reductions ($p<0.05$) compared with the solvent control (n=18) (Figure 5). Infarct volume in untreated animals was 33.8 ± 2.6%. Preatment with lycopene (4 mg/kg) decreased the infarct volume to 21.9 ± 3.0% (Figure 5).

Discussion

Animals models of focal cerebral ischemia use MCA occlusion-induced brain damage as observed in many human ischemic stroke patients (21). Cerebral ischemia restricted to the distribution of the MCA gives rise to focal metabolic disturbances that result in infarction, neuronal necrosis and brain edema (22). Our results confirmed that lycopene significantly reduced the cerebral infarct volume following

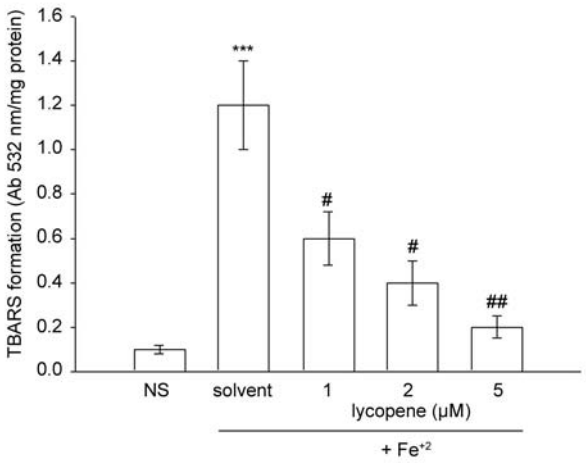

Figure 2. *Effect of lycopene on lipid peroxidation in rat brain homogenates. Brain homogenates were preincubated with solvent control (20% Tween 80) or various concentrations of lycopene (1, 2 and 5 μM) for 10 min followed by the addition of 200 μM Fe^{2+}. Results are presented as the absorbance at 532 nm/mg protein in brain homogenates. Data are presented as the means ± SEM (n=4). ***p<0.001 as compared with the normal saline (NS, resting group); #p<0.05 and ##p<0.01 as compared with the solvent control (20% Tween 80).*

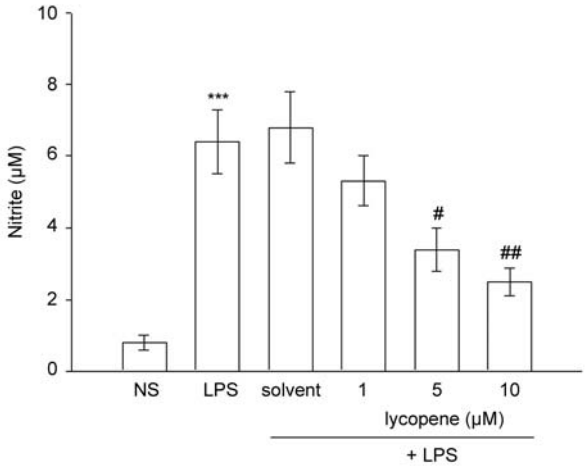

Figure 3. *Effect of lycopene on nitrite production in LPS-stimulated microglia. Microglia (2 x 10^5 cells/well) were treated with solvent control (20% Tween 80) or various concentrations of lycopene (1, 5 and 10 μM) for 15 min followed by the addition of LPS (250 ng/ml) for 24 h. Cell-free supernatants were assayed for nitrite production as described in "Materials and Methods". Data are presented as the means ± SEM (n=4). *p<0.001 as compared with the normal saline (NS, resting group); #p<0.05 and ##p<0.01 as compared with the LPS group.*

focal cerebral ischmeia and was effective in improving neurobehavioral deficits (data not shown). The rationale for the design of this pretreatment was based on the fact that multiple deleterious processes in different cell types of an organelle are initiated during ischemia/reperfusion injury which ultimately synergistically contribute to irreversible injury. Lycopene has been demonstrated to be the most potent antioxidant with the ranking: lycopene > α-tocopherol > α-carotene > β-cryptoxanthin > zeananthin = α-carotene > lutein (23). Dietary supplementation of lycopene resulted in a significant increase in serum lycopene level and diminished amounts of serum TBARS. The consumption of 25 g of tomato puree (containing 7 mg of lycopene) for 14 consecutive days increased plasma and lymphocyte carotenoid concentrations and this was related to an improvement in lymphocyte resistance to an oxidative stress (24). Therefore, lycopene absorbed from tomato products may act as an *in vivo* antioxidant (23).

In this study, two *in vitro* antioxidative tests were used to assess the activity of lycopene. Among cell-free systems, brain homogenates are usually chosen to evaluate antioxidant effects on lipid peroxidation (25). Rat brain homogenates exposed to ferrous ion exhibit lipid peroxidation in air by a mechanism whose induction step may primarily involve site-bound iron-mediated decomposition of lipid hydroperoxides to yield alkoxy or peroxyl radicals, leading to the chain reaction of lipid peroxidation (25). In this system, lycopene effectively inhibits lipid peroxidation. The DPPH test provided direct information about the reactivity of lycopene with a stable free radical. In the DPPH test, lycopene acted as a potent and direct free radical scavenger.

Morioka *et al.* (26) observed that microglial activation began prior to neuronal degeneration in an animal model of transient brain ischemia. It is also known that microglia play a key role in mediating inflammatory processes in the CNS, which are associated with various neurodegenerative diseases. In *in vitro* experiments using cultured microglia, LPS, a glycolipid derived from the membrane surface of gram-negative bacteria (endotoxin), has generally been used for cell activation. LPS can trigger a series of inflammatory reactions in phagocytes such as microglia. With LPS stimulation, microglia are activated to drastically change their cellular functions, producing various types of inflammatory cytokines, chemokines and NO. NO is beneficial as a messenger or modulator, but in conditions such as oxidative stress, it is potentially toxic. Induction of inducible nitric oxide synthase (iNOS) under inflammatory conditions leads to the production of large amounts of NO for longer periods of time. NO has been seen to inactivate antioxidative enzymes such as glutathione peroxidase and catalase (27). There is increasing evidence that NO is involved in mechanisms of cerebral ischemic injury (28). Altered NO synthesis has been implicated in the pathophysiological changes of ischemia/reperfusion injury in several key organs. For example, nephrotoxicity or neurotoxicity in animal models of kidney ischemia and brain

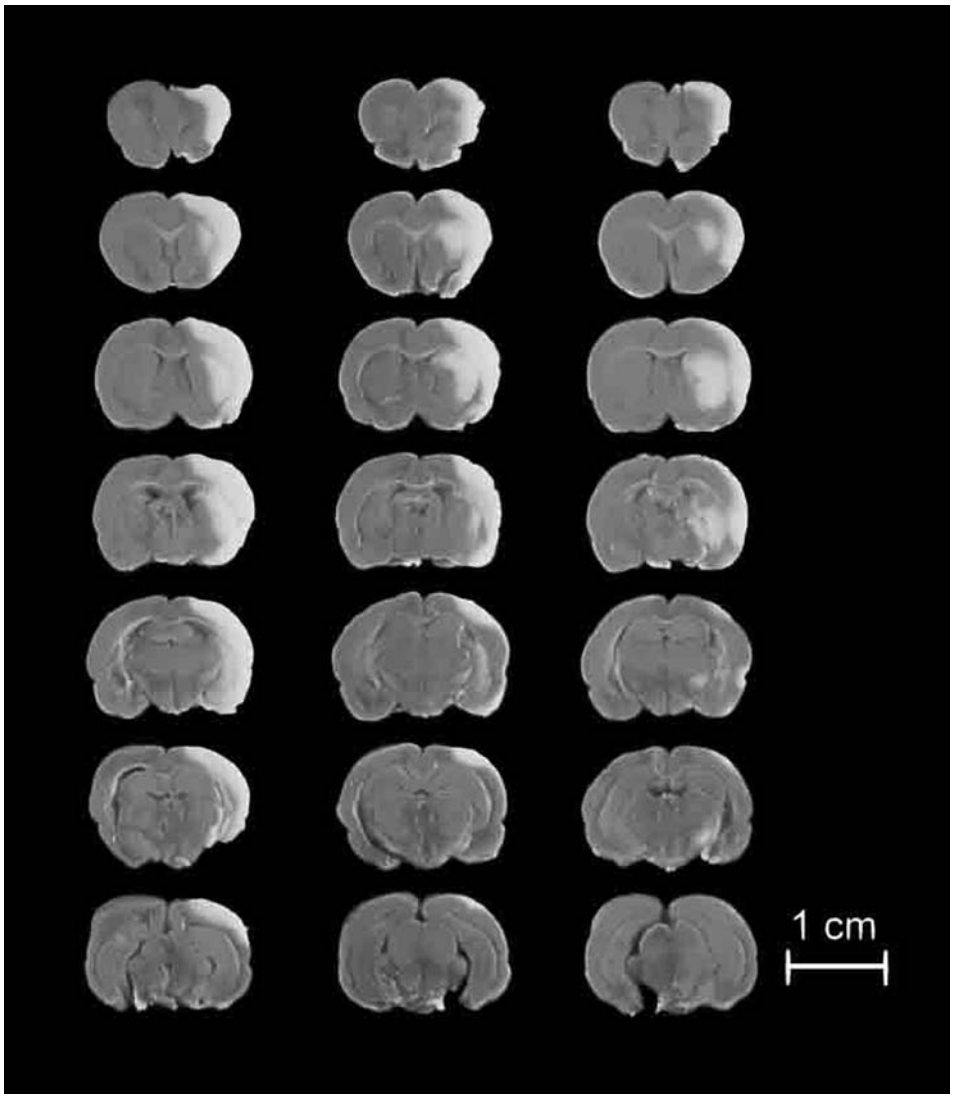

Figure 4. *Effect of lycopene in ischemia/reperfusion brain injury induced by middle cerebral artery occlusion in rats. 2,3,5-Triphenyl tetrazolium chloride-stained coronal brain sections are from representative animals that received intraperitoneal solvent control (A, 20% Tween 80), 2 mg/kg lycopene (B) and 4 mg/kg lycopene (C).*

focal ischemia is mediated at least partially by NO, since this toxicity is blocked by antisense to iNOS and an inhibitor of NOS, respectively (28, 29).

In conclusion, the data presented here demonstrate, for the first time, the protective effect of lycopene on ischemic brain injury *in vivo*. Lycopene may through its antioxidative property mediate, at least partially, free radical-scavenging activity as well as inhibition of NO production, resulting in the reduction of infarct volume in ischemia/reperfusion brain injury. It would be of interest to further study the antioxidative activities of lycopene in various radical-mediated pathological events, particularly for *in vivo* situations, to evaluate its possible use in therapeutics.

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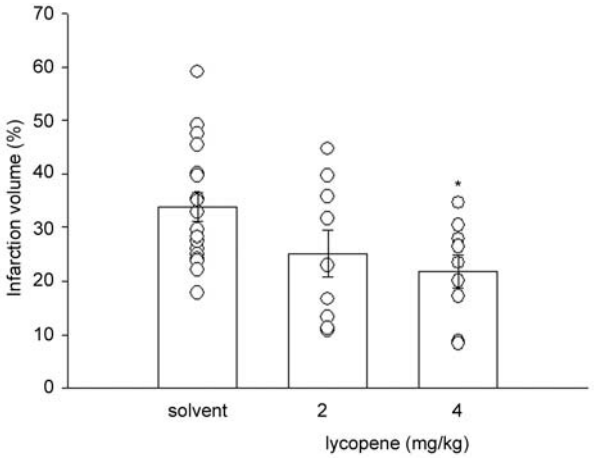

Figure 5. *Dose-response curves of lycopene in ischemia/reperfusion brain injury induced by middle cerebral artery occlusion in rats. Rats received a solvent control (20% Tween 80, n=18), 2 mg/kg lycopene (n=9), or 4 mg/kg lycopene (n=9) followed by ischemic/reperfusion brain injury as described in "Materials and Methods". Infarct volumes are expressed as a percentage of the contralateral hemisphere and data are presented as a superimposed scatterplot showing the infarction volume for each animal in the group as well as the means ± SEM. *p<0.05 as compared with the solvent control (20% Tween 80).*

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