Synergistic protective effect of FTY720 and vitamin E against simulated cerebral ischemia *in vitro*

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Abstract. The purpose of the present study was to explore the combination effect of FTY720 and vitamin E on cerebral ischemia. Astrocytes were isolated from newborn Sprague-Dawley rats and were subjected to FTY720, vitamin E, or combination of the two. The astrocyte cultures were then exposed to oxygen-glucose deprivation (OGD) to simulate an ischemic model *in vitro*. Cell viability, lactate dehydrogenase (LDH) leakage and cell apoptosis were detected following 12 h of exposure to OGD. In addition, the levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, total antioxidant capacity, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, chemokine (C-X-C motif) ligand (CXCL)-10, heme oxygenase (HO)-1 and superoxide dismutase (SOD)-1 were measured. Pre-treatment with FTY720 or vitamin E significantly elevated the cell viability and decreased LDH release and number of apoptotic cells. Combination treatment with FTY720 and vitamin E demonstrated a synergistic protective effect on OGD-induced cell viability, toxicity and apoptosis. Pre-treatment with FTY720 markedly reduced the release of IL-1β, TNF-α, IL-6, ICAM-1, VCAM-1 and CXCL-10, and pre-treatment with vitamin E increased the levels of antioxidant, HO-1 and SOD-1. However, pre-treatment with FTY720 combined with vitamin E revealed a synergistic effect. Pre-treatment with FTY720 combined with vitamin E exerts synergistic neuroprotective effects in the simulated cerebral ischemia *in vitro*.

Introduction

Stroke is a heterogeneous disease with high prevalence worldwide. It has been estimated that >200 million patients suffer from stroke annually leading to ~150 million deaths per year (1). Cerebral ischemia accounts for more than 80% of all stroke patients and it is a major cause of morbidity and mortality and is the third cause of disability worldwide (2-4). It is characterized by a severe reduction in cerebral blood flow accompanying with cerebral edema, formation of free radicals and a rapid and substantial local inflammatory reaction (5). Although thrombolytic therapy is now well-established treatment for ischemic stroke, few patients are able to benefit from thrombolytic therapy due to the limited time window (6). Therefore, it is essential to develop effective therapeutic strategies to ameliorate neural damage.

FTY720 and vitamin E have gained interest in the treatment of cerebral ischemia due to their unique pharmacological effects. FTY720, an agonist of sphingosine-1-phosphate (S1P) receptor-1 (S1P1), -3, -4 and -5 (7), exerts immunomodulatory actions by inhibiting the production, egress, trafficking and apoptosis of lymphocytes (8-10). In addition to the immunosuppressive activities, FTY720 has been reported to have the ability of enhancing the integrity of the blood brain barrier (11) and to alleviate infarctions and promote cell regeneration in the central nervous system (CNS) (12). Researchers have recently suggested that FTY720 possesses a neuroprotective effect via S1P1 in ischemic stroke (13,14). In addition, early research has demonstrated that administration of vitamin E serves a protective role in cerebral ischemia due to its both antioxidant activity and non-antioxidant effects (15). However, little information is available concerning the effects of combination of FTY720 and vitamin E on cerebral ischemia.

Therefore, the present study investigated the combined effects of FTY720 and vitamin E on simulated cerebral ischemia, in addition to the underlying mechanisms. This involved pretreating astrocytes with or without FTY720, vitamin E or combination of both. Following exposure to oxygen-glucose deprivation (OGD) to simulate an ischemic model, pre-treatment with FTY720 and vitamin E was found to exert synergistic neuroprotective effects in the simulated cerebral ischemia *in vitro*.

Materials and methods

Primary culture of astrocytes. Astrocytes were isolated from newborn Sprague-Dawley rat pups (1-day-old; weigh 5-6 g; n=10) that were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). The rats were kept under normal animal-house illumination with a 12 h light/dark

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cycle and access to food and water. The animal experiments were performed according to the National Institutes of Health Guide for Care and Use of Laboratory Animals (Bethesda, MA, USA). Briefly, the cortex of newborn rats was isolated, and the meninges were removed under sterile conditions. The tissues were dissociated mechanically in Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), homogenized and incubated in 0.125% trypsin at 37˚C for 20 min. The DMEM medium supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was added to stop trypsinization. The suspension was then filtered, centrifuged at 300 x *g* for 10 min at 4˚C and re-suspended in neurobasal medium (Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% FBS, B-27, 1.0 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (all Sigma-Aldrich; Merck Millipore). The cells ($1x10^6$ cells/ml) were added to 25 cm^2 flasks, incubated in DMEM/F-12 containing 10% FBS at 37˚C in a 5% CO_2 incubator. The culture medium was changed every third day. A total of two weeks later, contaminated microglia and oligodendrocytes were removed by shaking for 5 h, and shaking was repeated again after 5 days to remove microglial cells that were detached from the layer of astrocytes. Following successful removal of microglia, astrocytes were collected using 0.125% trypsin. The purity of astrocytes was ~95-98%, which was identified by analyzing immunofluorescence using anti-glial fibrillary acidic protein (astrocyte marker; Sigma-Aldrich; Merck Millipore). Further analyses were performed on 22-day-old cultures.

Treatment of astrocytes. Astrocytes were randomly assigned to one of five groups: Control group (no medium exposure), medium group (serum-free DMEM exposure), FTY720 group (exposed to serum-free DMEM supplemented with 0.6 μM FTY720; Novartis, Basel, Switzerland), vitamin E group (exposed to serum-free DMEM supplemented with vitamin E (10 μg/ml, Sigma-Aldrich; Merck Millipore), and FTY720 + vitamin E group (exposed to serum-free DMEM supplemented with FTY720 and vitamin E). Following culture for 24 h, the medium in all groups was removed and substituted for glucose-free Earle's Balanced Salt solution (EBSS; Gibco; Thermo Fisher Scientific, Inc.) that was pre-incubated with 95% N_2 and 5% CO_2 for 0.5 h to remove the oxygen in the medium. To induce an OGD condition, the cells were then incubated for 12 h in an airtight box filled with 95% N_2 and 5% CO_2. Meanwhile, the cells in the control group were treated with EBSS supplemented with 10 mmol/l glucose in CO_2 incubator.

Cell viability assay. The cell viability of astrocytes was determined by an MTT assay. Non-treated cells were regarded as the control group. Briefly, 0.5 mg/ml MTT reagent (Sigma-Aldrich; Merck Millipore) was added to each well ($1x10^4$ cells/well) following 12 h of exposure to OGD and incubated for 4 h at 37˚C. Dimethylsulfoxide (10%; 100 μl) was then added to dissolve the blue reaction product formazan by shaking the plates at room temperature for 10 min. The absorbance value at 570 nm was monitored by using a microplate reader (SpectraMax M5; Molecular Devices, LLC, Sunnyvale, CA, USA). Each experiment was carried out in triplicate.

Lactate dehydrogenase (LDH) release assay. LDH release was assessed in order to evaluate the extent of cell injury using a LDH cytotoxicity detection kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. Following exposure to OGD for 12 h, the culture supernatant was harvested and dead cells were removed by centrifugation. The remaining supernatant was used to determine LDH levels. Absorbance at 492 nm was read using a microplate reader. Each experimental was done in triplicate.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. A TUNEL assay was performed to detect the staining of astrocytes cells using a commercially available kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. A total of 12 h after exposure to OGD, the astrocytes were collected, washed with PBS, and examined under a fluorescence microscope.

Apoptosis assay. Cell apoptosis was detected by Annexin V-Cy5 and propidium iodide (PI) staining (BioVision, Inc., Milpitas, CA, USA) followed by fluorescence-activated cell sorting (FACS) analysis. The cells ($2x10^5$/35 mm culture dish) were collected, pelleted and re-suspended in Annexin V-binding buffer containing Annexin V-Cy5 and PI, and incubated at room temperature for 5 min. Thereafter, the cells were evaluated with a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The percentage of total apoptotic cells was (early apoptotic + apoptotic) calculated.

Enzyme-linked immunosorbent assay (ELISA). The levels of secreted tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β in the culture supernatants that were exposed to OGD for 12 h were evaluated by using a commercially available ELISA kit (EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Absorbance at 450 nm was read using a microplate reader.

Measurement of antioxidant levels. Total antioxidant capacity (TAC) levels were assessed using Randox total antioxidant status kit (Randox Laboratories, Ltd., Crumlin, Northern Ireland). Briefly, the cultured astrocytes were collected following 12 h of exposure to OGD, washed twice with PBS, and lysed with 10 mM phosphate buffer. Following centrifugation at 300 x *g* for 10 min at 4˚C, the supernatant was collected to evaluate the levels of TAC.

Western blot analysis. Following 12 h of exposure to OGD, astrocyte cultures were collected and washed with ice-cold PBS. The proteins were extracted from the cells using RIPA lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA). The protein concentrations were determined using a BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Equal amounts of the protein samples (20 μg) were resolved on 10-12% SDS-PAGE, and transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were then blocked with 5% nonfat dry milk in TBS with 0.1% Tween (TBST) for 1 h at room temperature. The membranes were incubated overnight at 4˚C with rabbit anti-polyclonal intercellular adhesion molecule (ICAM)-1

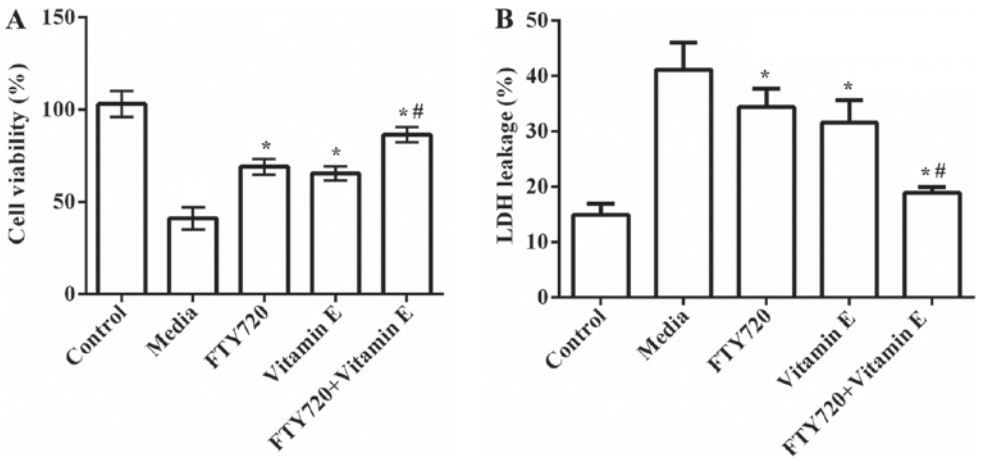

Figure 1. Combined effects of FTY720 and vitamin E on cell viability and LDH release. (A) The cell viability is markedly increased by pre-treatment with FTY720 or vitamin E. The cell viability is markedly higher in the FTY720 combined with vitamin E group than that in the FTY720 group or vitamin E group. (B) Pre-treatment with FTY720 or vitamin E markedly attenuates LDH leakage when compared to the media group, and while pre-treatment with FTY720 combined with vitamin E represents a more protective effect. *P<0.05 vs. media group. #P<0.05 vs. FTY720 or vitamin E group LDH, lactate dehydrogenase.

(catalog no. ab124760; 1:1,000), vascular cell adhesion molecule (VCAM)-1 (catalog no. ab215380; 1:1,000), chemokine (C-X-C motif) ligand (CXCL)-10 (catalog no. ab7206; 1:1,000), heme oxygenase (HO)-1 (catalog no. ab68477; 1:1,000) or superoxide dismutase (SOD)-1 (catalog no. ab16831; 1:1,000) antibody. All antibodies were obtained from Abcam (Cambridge, MA, USA). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (catalog no. ab191866; 1:5,000; Abcam) for 2 h at room temperature. The positive bands were visualized with enhanced chemiluminescence western blotting substrate (Pierce; Thermo Fisher Scientific, Inc.) and autoradiography film. β-actin was used as an internal control.

Statistical analysis. All data were expressed as the mean ± standard deviation. The statistical significance of differences among different groups was evaluated using SPSS software (version, 18.0; SPSS Inc., Chicago, IL, USA) with one-way analysis of variance. The Tukey-Kramer's post hoc test was performed for the multiple comparisons. P<0.05 was used to indicate a statistically significant difference.

Results

Combined effects of FTY720 and vitamin E on cell viability on LDH release. To explore the combined effect of FTY720 and vitamin E on cerebral ischemia, the cultured rat astrocytes were subjected to medium, FTY720, vitamin E, or FTY720 combined with vitamin E. A total of 24 h later, the cultured astrocytes were exposed to OGD for 12 h to simulate cerebral ischemia. The combined effect of FTY720 and vitamin E on cell viability and LDH release was initially investigated. As indicated in Fig. 1A, the results demonstrated that the cell viability was significantly decreased by exposure to OGD when compared to the control group (P=0.032). However, the cell viability was markedly increased by pre-treatment with FTY720 or vitamin E compared to non-treatment (P=0.023 or P=0.036). No significant differences were observed between the FTY720 group and vitamin E group. In addition, the cell viability was markedly higher in the FTY720 combined with vitamin E group than that in the FTY720 group or vitamin E group (P=0.029 or P=0.018).

Following this, the combined effect of FTY720 and vitamin E on LDH release was investigated, which is another indicator of cell toxicity (16). As presented in Fig. 1B, LDH leakage was significantly increased when the cells were exposed to 12 h OGD compared to the control group (P=0.034). Pre-treatment with FTY720 (P=0.031) or vitamin E (P=0.019) significantly attenuated OGD-induced LDH leakage, and while pre-treatment with FTY720 combined with vitamin E represented the best protective effect (P=0.013). These results indicated that pre-treatment with FTY720 combined with vitamin E presented a synergistic protective effect on OGD-induced cell viability and toxicity of astrocytes.

Combined effects of FTY720 and vitamin E on astrocyte death. A total of 12 h following exposure to OGD, apoptotic cell death was detected by TUNEL staining and an apoptosis assay. As demonstrated in Fig. 2A and B, there was a significantly higher number of TUNEL-positive cells in media exposed to OGD when compared with the control group (P=0.016). Pre-treatment with FTY720 or vitamin E significantly reduced the number of TUNEL-positive cells (P=0.024 or P=0.017), and pre-treatment with FTY720 combined with vitamin E presented the fewest number of TUNEL-positive cells (P=0.012). The results suggested that pre-treatment with FTY720 and vitamin E showed a synergistic protective effect on OGD-induced astrocyte death.

Combined effects of FTY720 and vitamin E on IL-1β, TNF-α, IL-6 and antioxidant release. Exposure of astrocytes to OGD for 12 h lead to increased IL-1β (P=0.034), TNF-α (P=0.029) and IL-6 release (P=0.042), but significantly decreased TAC (P=0.038). Pre-treatment with FTY720 markedly reduced the release of IL-1β (P=0.042), TNF-α (P=0.038) and IL-6 (P=0.036), however had no effect on the release of antioxidant level, confirming that FTY720 protects against cerebral ischemia by reduction of the levels of pro-inflammatory cytokines. However, pre-treatment with vitamin E markedly increased the

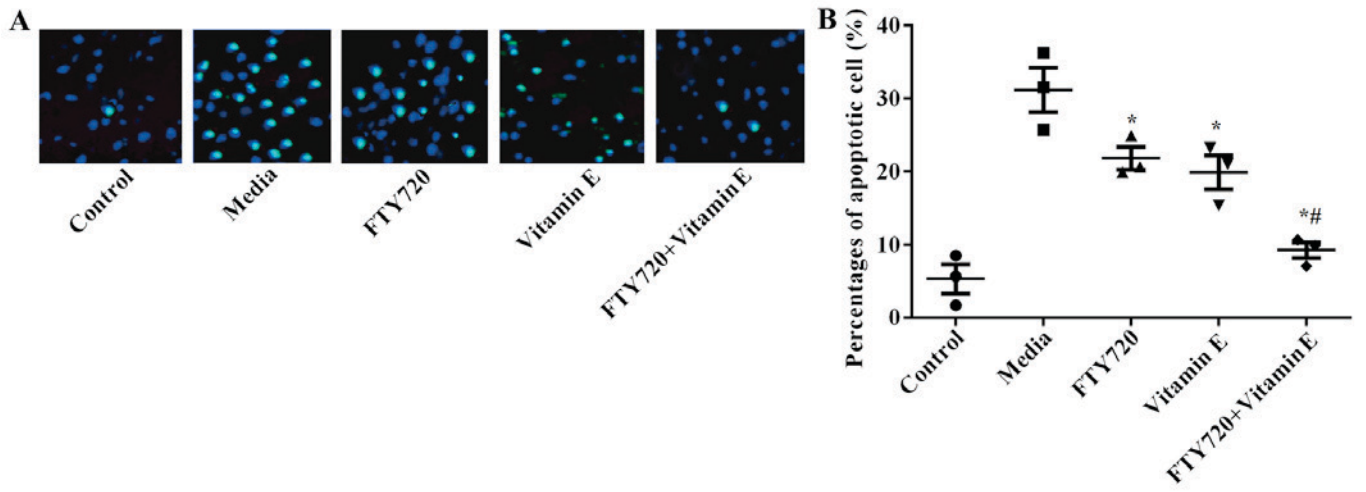

Figure 2. (A) Combined effects of FTY720 and vitamin E on astrocyte death. (B) Pre-treatment with FTY720 or vitamin E significantly reduces the number of TUNEL-positive cells compared with the media group, and pre-treatment with FTY720 combined with vitamin E has the fewest number of TUNEL-positive cells. *P<0.05 vs. media group; #P<0.05 vs. FTY720 or vitamin E group. TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

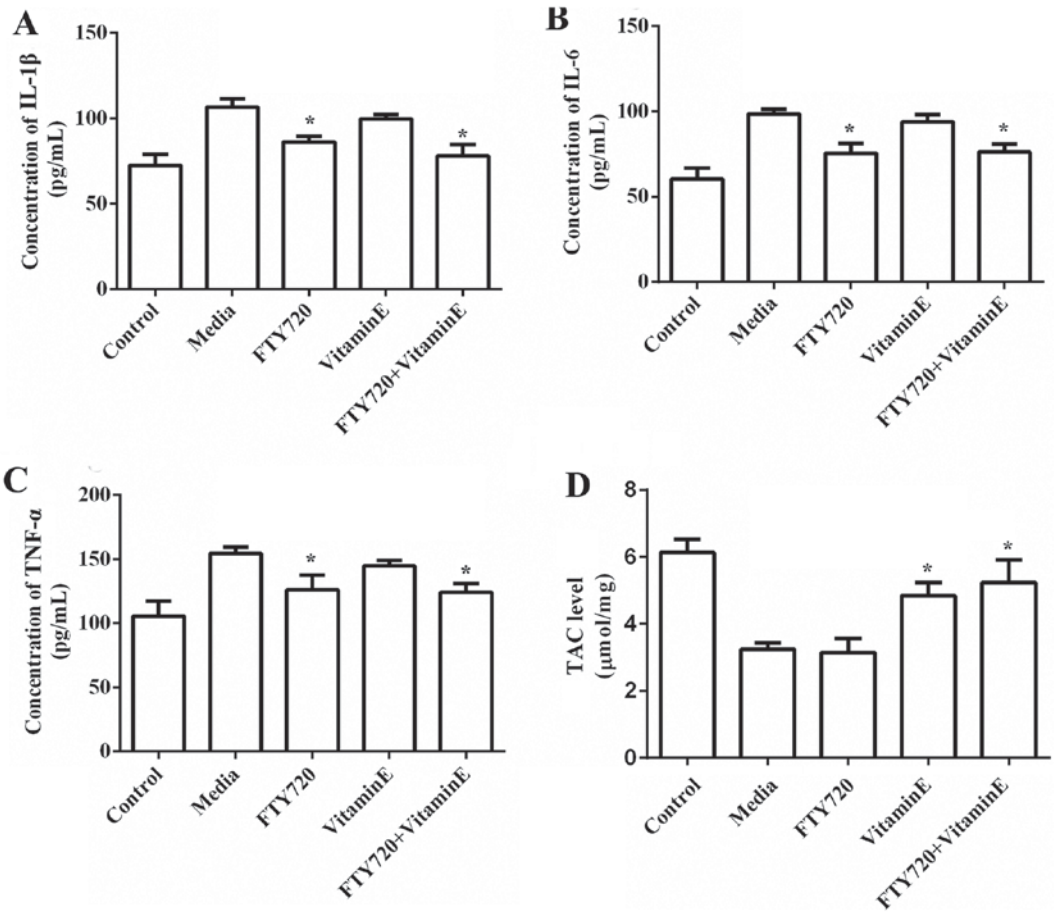

Figure 3. Combined effects of FTY720 and vitamin E on (A) IL-1β, (B) IL-6, (C) TNF-α, and (D) TAC. Pre-treatment with FTY720 markedly reduces the release of IL-1β, TNF-α, and IL-6, and while pre-treatment with vitamin E increases the release of antioxidants. Combination of FTY720 and vitamin E shows a synergistic effect. *P<0.05 vs. media group. IL, interleukin; TNF, tumor necrosis factor; TAC, total antioxidant capacity.

release of antioxidant level (P=0.022) but had no effect on the release of IL-1β, TNF-α and IL-6, confirming that vitamin E protects against cerebral ischemia by decrease of the levels of antioxidants. The combination of FTY720 and vitamin E significantly reduced the levels of IL-1β, TNF-α and IL-6, however simultaneously elevated the levels of antioxidant, presenting a synergistic protective effect (Fig. 3A-D).

Combined effects of FTY720 and vitamin E on ICAM-1, VCAM-1, CXCL-10, HO-1 and SOD-1. Results of western

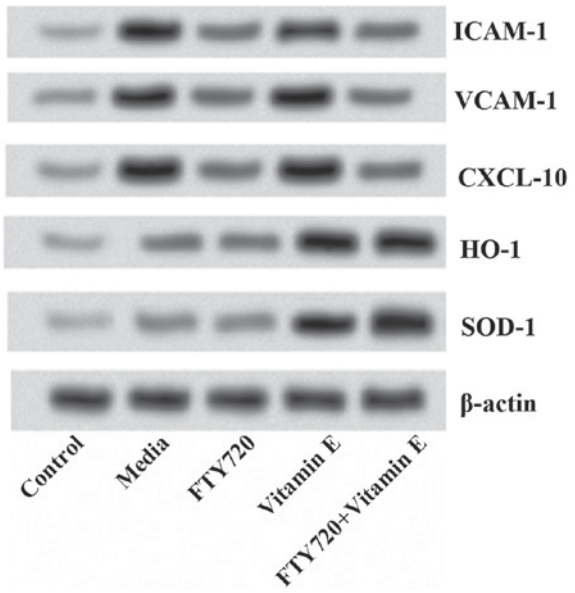

Figure 4. Combined effects of FTY720 and vitamin E on ICAM-1, VCAM-1, CXCL-10, HO-1 and SOD-1. Pre-treatment with FTY720 significantly reduces the protein levels of ICAM-1, VCAM-1 and CXCL-10, while pre-treatment with vitamin E markedly increases the levels of HO-1 and SOD-1. Additionally, pre-treatment with FTY720 combined with vitamin E indicates a synergistic effect. ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; CXCL, chemokine (C-X-C motif) ligand; HO, heme oxygenase; SOD, superoxide dismutase.

blot analysis indicated that exposure of astrocytes to OGD for 12 h markedly increased the levels of ICAM-1, VCAM-1 and CXCL-10, but markedly decreased the levels of HO-1 and SOD-1. Pre-treatment with FTY720 markedly reduced the protein levels of ICAM-1, VCAM-1 and CXCL-10, however appeared to have no effect on the levels of HO-1 and SOD-1. Meanwhile, pre-treatment with vitamin E markedly increased the levels of HO-1 and SOD-1, however, had no effect on the levels of ICAM-1, VCAM-1 and CXCL-10. Additionally, pre-treatment with FTY720 combined with vitamin E statistically reduced all the levels of ICAM-1, VCAM-1, CXCL-10, and simultaneously increased the levels of HO-1 and SOD-1, revealing a synergistic protective effect of FTY720 and vitamin E on cerebral ischemia (Fig. 4).

Discussion

Not only do astrocytes serve significant roles in regular neuronal function, but they are also involved in the pathology of stroke (17,18). Following ischemic stroke, astrocytes may exhibit the ability of proliferation and differentiation (astrogliosis). Therefore, astrocytes were used in the present study and treated with or without FTY720, vitamin E, or combination of FTY720 and vitamin E. The results indicated that combination of FTY720 and vitamin E exerted a synergistic protective effect on astrocyte viability, toxicity and apoptosis induced by OGD. In addition, it was identified that FTY720 markedly reduced the levels of pro-inflammatory cytokines TNF-α, IL-1β and IL-6, cell adherence molecules ICAM-1 and VCAM-1, and chemokine CXCL-10, whereas vitamin E increased the levels of antioxidant enzymes, HO-1 and SOD-1. The current results suggested that FTY720 combined with vitamin E possesses a synergistic neuroprotective effects in the simulated cerebral ischemia *in vitro*.

FTY720 is a novel immunomodulatory drug approved by the Food and Drug Administration to treat multiple sclerosis (19). FTY720 has been previously identified to reduce the trafficking of T cells, B cells, natural killer cells, and other S1PR-bearing cells into the CNS, leading to the reduction of relapses and brain volume loss (12,14). FTY720 has emerged as a promising therapeutic modality, which has demonstrated the beneficial effects on experimental models of stroke (13,20-23). The protective effect of FTY720 on stroke is predominantly involved in induction of lymphocytopenia, and concomitant reduction of microvascular thrombosis (23). The interactions between lymphocytes and endothelial cells enhance the dysfunction of microvascular, possibly leading to secondary infarct growth. In addition to FTY720, vitamin E has long been identified as a major lipid-soluble antioxidant protecting against cerebral ischemia. Kraft *et al* (23) suggested that vitamin E prevents cerebral ischemia neuronal apoptosis by lowering radical damage to hippocampal neurons. However, rare studies are focused on the combination effects of FTY720 and vitamin E on cerebral ischemia. Therefore, combination effects were studied in our present study. As indicated in our results, we observed that pre-treatment with either FTY720 or vitamin E both significantly elevated the astrocytes viability, and markedly decreased astrocytes cytotoxicity (LDH release) and number of apoptotic cells, demonstrating the protective roles of FTY720 or vitamin E in cerebral ischemia. Our results were in line with previous studies which showed FTY720 or vitamin E prevented against cerebral ischemia. These effects, however, were obviously enhanced by combination of FTY720 and vitamin E, indicating a synergistic protective effect. The underlying mechanisms were then investigated.

It has been well established that local and systemic inflammatory responses involving neutrophils, lymphocytes, macrophages and capillary endothelial cells are responsible for the development of ischemic stroke (24-26). Inflammatory molecules, such as adhesion molecules, chemokines and cytokines are involved in the inflammatory process. ICAM-1 and VCAM-1 are members of adhesion molecules, which are expressed on endothelial cells during inflammation (27,28). Furthermore, the inflammatory process is promoted by various pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β, produced by several subtypes of T cells (29). Chemokines, including CXCL-10, are secreted by the activated cells in ischemic regions to attract the inflammatory leukocytes into the region of infarction (30). Elevation of these inflammatory molecules clearly demonstrates detrimental effects on viable brain tissue (31). Therefore, administration of specific antagonists that reduce the release of inflammatory molecules is beneficial for preventing the development and deterioration of cerebral ischemia. In the current study, pre-treatment with FTY720 statistically decreased the levels of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), adhesion molecules (ICAM-1 and VCAM-1) and chemokines (CXCL-10). However, these effects were not observed by pre-treatment with vitamin E, suggesting that reduction of the expression of inflammatory mediators caused by FTY720, but not by vitamin E, prevents against cerebral ischemia.

On the other hand, one of the pathological mechanisms of cerebral ischemia is oxidative/nitrative stress (32). Oxidative/nitrosative stress results in cellular macromolecular destruction and cell death (33). HO is a microsomal enzyme that degrades heme from heme-containing proteins, leading to the production of carbon monoxide, iron and biliverdin (34). The characteristics of HO make it as an crucial antioxidant enzyme in the nervous system (35). HO-1 is a rate-limiting enzyme in heme catabolism, which has potent antioxidant and anti-apoptosis effects (36). Recently, some studies have suggested that overproduction of HO-1 is neuroprotective against cerebral ischemia injury (37,38). In addition, SOD-1 is a well-known antioxidant enzyme, responsible for detoxifying intracellular reactive oxygen species (ROS), thereby protecting cells from oxidative damage. It has been reported that overexpression of SOD-1 in transgenic rats or mice protects neurons from death after focal cerebral ischemia (39). Zhang *et al* (15) found that vitamin E protects against cerebral ischemia by inducing the expression of subunit of HIF-1 and its target genes, including HO-1. Administration of vitamin E slowed the onset and the progression of amyotrophic lateral sclerosis in SOD-1 transgenic mice (40). Similarly, in the present study, astrocytes pretreated with vitamin E presented higher levels of HO-1 and SOD-1; however, these effects were not observed by pre-treatment with FTY720, indicating an antioxidant effect of vitamin E but not FTY720.

In conclusion, the current study suggested that pre-treatment with FTY720 combined with vitamin E reveals a synergistic effect on cerebral ischemia. This provides novel therapeutic strategies for cerebral ischemia. However, animal experiments should be performed to confirm the results.

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