Neuroprotective effects of lipid emulsion on brain injury in rats

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Directed by Professor Bae Hwan Lee

The Doctoral Dissertation
submitted to the Department of Medical Science
The Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy of Medical Science

Motomasa Tanioka

December 2020

This certifies that the Doctoral Dissertation of Motomasa Tanioka is approved.

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December 2020

ACKNOWLEDGEMENTS

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학위 기간에 생리학교실에서 힘든 순간들도 있었지만, 돌이켜보면 제가 성장하고 배울 수 있는 소중한 시간이었습니다. 늘 중요한 의견을 제공해주시고 정성껏 조언해주신 생리학 교실의 남택상 교수님, 안덕선 교수님, 임중우 교수님, 이영호 교수님, 김동욱 교수님, 김정훈 교수님, 정승수 교수님께 감사드립니다.

문과생이었던 저를 과학의 길로 처음 인도해주신 가천대학교 생명과학과의 김주환 교수님께 감사드립니다. 문과 전공에서 진학해 어색한 저에게 실험실에서 익혀야 할 기초가 많았습니다. 그에 대한 가르침을 주신 곽용호 선생님과 김은정 선생님께 감사의 인사를 드립니다. 연구가 처음에는 미숙하여 막혔을 때 조언해주시고 지도해주신 홍성갑 교수님과 이경희 교수님께 감사드립니다. 실험과 생활에 대해 공유하며 언제나 제게 웃음꽃을 피워 주셨던 사영희 선생님에게 감사합니다. 제게 실험과 과학적 논리에 대해 의논해주시고 가르침을 주신 권민지 그리고 한정수 선배님들께 감사드립니다. 5년 동안 우여곡절을 겪으면서도 서로에게 의지하고 힘들 때 위로를 해주며 졸업 이후에도 끊임없이 도와주는 동기 김민지에게 감사합니다. 좋은 역량을 발휘하고 실험실을 지탱해준 김경민, 최송연 후배에게 감사합니다. 짧은 시간이었지만 함께 실험실 생활을 하며 보냈던 박미선, 박혜지 연구원님들께도 감사합니다.

생리학교실의 학생모임을 짧은 시간 동안 운영하며 친하게 지냈던 이재석, 최상휘, 박상현, 권영인, 곽명지, 조보람 선생님들 덕분에 더욱 즐겁고 유익한 대학원 생활을 하게 되어 감사합니다. 연구의 절반을 진행했을 때 나머지 절반은 숙련도가 비교적 떨어져 난항을 겪고 있을 때 뇌졸중에 대한 모델 형성과 실험을 같이 진행해주신 해부학교실의 박주현 선생님께 큰 감사를 드립니다. 또한 분자생물학적 분석 방법을 밤낮 가리지 않으며 가르쳐 주셨던 약리학교실의 윤은장 선생님께 깊은 감사를 드립니다. 기존 연구에 발표되어 있던 결과에서 벗어나지 못하고 있던 제게 새로운 의견과 돌파구를 제시해주신 구강병리학교실의 김성훈 선생님에게 감사합니다. 전기와 기계에 대해 알려주시고 미로 제작에 큰 도움주신 박병기 선생님에게 감사합니다. 논문에 게재될 이미지 제작과 연구에 대한 적극 지지로 도움을 주신 서아현과 신병철 선생님에게 감사합니다.

힘들 때에도 늘 자리를 지켜주며 함께해왔던 제 소중한 고등학교 친구들 서주원, 이창훈, 김동인, 장동화, 김형선, 함동진, 정재우, 추현균에게, 그리고 대학교 친구들 이승국과 이용희에게 감사의 마음을 전합니다. 대학원 과정중 알게된 심재걸, 김지훈, 백성대, 이준수, 서영현, 김형석에게 감사합니다. 제 멘토이자 삶에 대해 지도를 많이 해주시고 계신 이명준 선배님에게 감사합니다. 조건 없는 응원과 서로에게 의지할 수 있었던 부분이 큰 버팀목이 되었습니다.

마지막으로 위기가 올 때마다, 변함없이 지탱해주시고 격려해주신 어머니, 이모, 그리고 작은삼촌 덕분에 여러 풍파에 대처할 수 있었습니다. 저를 언제나 믿고 지지해주신 어머니, 매주 먼 길 오셔서 챙겨주셨던 이모, 그리고 멀리서 조언해주시고 지원해주신 작은삼촌께 깊은 감사의 말씀을 전합니다. 제 가족이 있어 지금의 제가 있다고 굳게 믿고 있습니다.

연구를 하는 중에는 외롭게 느껴지며 혼자서 모든 걸 해결해야 했던 것처럼 느껴졌으나, 뒤돌아보니 제 곁에는 언제나 도움을 주시는 분들께서 계셨다는 것을 알게 되었습니다. 이 과정은 결코 혼자 완성할 수 없었고 어느새 많은 도움과 응원을 바탕으로 종착점에 도달하게 되었습니다. 앞으로도 저는 제 주위를 신경 쓰며 감사함을 마음속 깊이 새기며 더 나은 사람으로서 발전하고 싶습니다. 도와주신 모든 분께 다시 한번 감사드립니다.

2020년 12월

단위오까 모토마사

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ABSTRACT

Neuroprotective effects of lipid emulsion on brain injury in rats

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Lipid emulsion has recently shown to attenuate cell death caused by excitotoxic conditions in the heart. There are key similarities between neurons and cardiomyocytes such as excitability and conductibility, which yield vulnerability to excitotoxic conditions. However, investigations on the protective effects of lipid emulsion in the central nervous system are still lacking. Therefore, this study was conducted to observe the neuroprotective effects of lipid emulsion against excitotoxicity when injected into the brain.

The first study aimed to determine the neuroprotective effects of lipid emulsion in an in vivo rat model of kainic acid-induced excitotoxicity through intrahippocampal microinjections. Kainic acid-injected Sprague Dawley rats were observed using the Racine scale to measure the degree of seizures. Rats that did not experience seizures equivalent to stage 3 or higher were excluded from the study. After the seizures have been terminated by benzodiazepine, rats were subjected to intrahippocampal administration of vehicle or lipid emulsion of different dosages. The survival rates of each experimental group were recorded and analyzed using the log-rank test. Then, they were subjected to the passive avoidance test and elevated plus maze for behavioral assessment. Brains were also cryosectioned for morphological analysis through cresyl violet staining and Fluorojade C staining. Rats were also sacrificed at 24 hrs and 72 hrs after kainic acid injections for molecular study including immunoblotting and qPCR. There were no significant differences in

survival rates however the increase in survival of the 1% lipid emulsion-treated group was notable. Anxiety and memory functions were significantly preserved in 1% lipid emulsion-treated rats. Neurodegeneration was significantly reduced mainly in the CA1 region with increased cell survival. Lipid emulsion was dose-dependent on the protein expression of β-catenin and the phosphorylation of GSK3-β and Akt. *Wnt1* mRNA expression was elevated in lipid emulsion-treated rats compared to vehicle. Therefore, the administration of lipid emulsion exhibited significant protection against kainic-acid induced excitotoxicity affecting the canonical Wnt signaling pathway.

The second study aimed to determine the neuroprotective effects of lipid emulsion in an in vivo rat model of ischemic reperfusion injury through middle cerebral artery occlusion (MCAO). Under sodium pentobarbital anesthesia, rats were subjected to MCAO surgery and were administered with lipid emulsion through intra-arterial injection during reperfusion. The experimental animals were assessed for neurological deficit wherein the brains were extracted at 24 hrs after reperfusion for triphenyltetrazolium chloride (TTC) staining, immunoblotting, and qPCR. Neuroprotection was found to be dosage-dependent, and the rats treated with 20% lipid emulsion had significantly decreased infarction volumes and lower Bederson scores. Phosphorylation of Akt and GSK3-β were increased in the 20% lipid-emulsion treated group. The Wnt-associated signals showed marked increase with a concomitant decrease in signals of inflammatory markers in the group treated with 20% lipid emulsion. The protective effects of lipid emulsion and survival-related expression of genes such as Akt, GSK-3β, Wnt1, and β-catenin were reversed by the intra-peritoneal administration of XAV939 through the inhibition of the Wnt/β-catenin signaling pathway.

The results suggest that lipid emulsion has neuroprotective effects against ischemic reperfusion injury in the brain through the modulation of the Wnt signaling pathway and may provide potential insights for the development of therapeutic targets.

Key words : Lipid emulsion, Kainic acid, Epilepsy, Stroke, Wnt, Brain

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I. INTRODUCTION

Excitotoxicity is considered a major mechanism underscoring neurodegenerative disorders or neural injuries involving functional loss and death of neurons in the central nervous system (CNS).[1,2] Excitotoxic conditions have been implicated in acute and chronic neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and epilepsy.[3] In animal models of excitotoxic neurodegeneration, chemical convulsants such as kainic acid (KA) have been utilized to mimic pathological conditions observed in patients.[4,5] KA is a neurotoxic analogue of glutamate that binds to kainate receptors, resulting in overstimulation of neurons at high doses.[6] KA-induced excitotoxicity in rodents results in deficient cognitive functions[7], elevated anxiety levels,[8] and disruptive morphological changes in different areas of the brain.[9,10] In particular, the hippocampus has been established as a site of damage following KA administration, which elicits cognitive dysfunction.[11,12] Numerous therapeutic approaches have been investigated to terminate convulsive seizures induced by KA; however, the pursuit for appropriate remedies against neural damage induced by excitotoxic conditions is an ongoing endeavor.

Ischemic reperfusion injury in the brain encompasses abnormal production of oxygen radicals that may exacerbate initial ischemic injuries.[13] Such injury can occur in the current clinical therapies for stroke, such as thrombectomy. These surgeries are effective to ameliorate

ischemic damage in the brain by the elimination of blockages.[14] However, it is currently arduous to prevent further excitotoxic damage caused by reperfusion. Again, secondary damage caused by reperfusion is intractable and could render leaving the reversible areas, such as the penumbra, vulnerable to oxidative damage.[15]

Previous studies reported that the expression of Dickkopf-related protein 1 (Dkk-1), an antagonist of the canonical Wnt signaling pathway that promotes glycogen synthase kinase3-β (GSK3-β) activity, was elevated in biopsies of patients[16] and animal models[17,18] undergoing neurodegeneration. Proteasomes subsequently degrade β-catenin, a downstream survival marker of GSK3-β, through ubiquitination which often leads to cell death. Wnt is a canonical lipid-modified signaling glycoprotein that regulates the phosphorylation of GSK3-β.[19] The brain tissue of KA-administered rats expressed higher antagonistic activity of Wnt,[20] indicating that the Wnt signaling pathway may have an important role in neurodegenerative excitotoxicity of the brain.

Regarding oxidative stress, previous studies have implicated the importance of the canonical Wnt signaling pathway in the case of ischemic reperfusion injury.[21,22] The activity of GSK-3β leads to the degradation of downstream survival markers such as β-catenin through ubiquitination. Furthermore, canonical Wnt signals, which are lipid-modified glycoproteins, have been reported to inactivate GSK-3β through phosphorylation and thereby promote cell survival.[23] A recent study reported that motor exercise stimulated the canonical Wnt/ β-catenin pathway for recovering from focal cerebral ischemic reperfusion injury in juvenile rats. Motor activity regulated canonical Wnt/ β-catenin pathway and promoted neurogenesis and myelin repair.[24] Another study reported that electroacupuncture in multiple acupoints of a paralyzed limb stimulated the proliferation of neural progenitor cells through the activation of the Wnt/ β-catenin signaling pathway and suppression of GSK-3β.[25]

In 1962, lipid emulsion (LE) was approved for clinical use as a component of parenteral nutrition. Composition of lipid emulsion (Intralipid™ 20%, Fresenius Kabi, Uppsala, Sweden) is 20% soybean oil, 1.2% egg yolk phospholipids, 2.25% glycerin, and water for injection. The major component fatty acids of soybean oil are linoleic (44-62%), oleic (19-30%), palmitic (7-14%), linolenic (4-11%) and stearic (1.4-5.5%). In a previous study, LE effectively protected the heart from ischemic reperfusion injury.[26] The protective mechanisms underscoring LE treatment included the phosphorylation of protein kinase B (Akt)[27] and

glycogen synthase kinase-3β (GSK3-β), hence promoting cell survival.[28,29] Moreover, increased expression levels of such signals were also reported for neuronal survival in the CNS,[30] which may correlate to possible neuroprotective actions of LE in the brain. Cardioprotective properties have been reported from CNS studies are sometimes diagnostic tools or therapeutic targets for neuroprotection.[31,32] Extensive researches regarding LE's cardioprotection were conducted over the past decades, but investigations on the protective effects of LE in the CNS are still lacking. Similitude of these organs shed light to speculations on the protective effects of LE in the brain. Despite the accumulating conjectures about the effect of LE on neuroprotection, studies have not yet assessed its protective effects in the brain. This study aimed to elucidate the protective effects of LE when administered into the excitotoxic brain.

Therefore, in this study, the neuroprotective effects of LE was investigated in an *in vivo* rat model of KA-induced excitotoxicity. Examination of neuroprotection was conducted by measuring the preservation of hippocampal function through behavioral tests after acute damage of hippocampal neurons. The changes in mRNA expression of *Wnt1*, *Wnt3*, and *Wnt5a* were assessed, which are representative genes of canonical and non-canonical Wnt signaling pathways to further investigate the signaling affected by neuroprotection. Additionally, changes in protein expression levels of downstream markers of the canonical Wnt signaling pathway in relation to cell survival were observed. Data on neurodegeneration and morphological changes of the hippocampus were analyzed.

The neuroprotective roles of LE were also examined in an *in vivo* rat model of the middle cerebral artery occlusion and reperfusion. The assessment of neurological deficits was conducted using the modified Bederson score[33] and extracted the brain to measure the severity of infarctions in experimental groups. The changes in protein and mRNA expression of distinct genes related to cell survival, Wnt/β-catenin signaling pathway, and inflammation were assessed. XAV939, a Wnt/β-catenin signaling pathway inhibitor, reversed the protective effects. Based on the results, LE provided neuroprotection against ischemic reperfusion injury in the brain by regulating the Wnt/β-catenin signaling pathway. Based on behavioral studies, molecular analysis, and morphological examinations, LE provided neuroprotection in the brain.

II. STUDY 1: Neuroprotective effects of LE in KA-induced excitotoxicity

1. Purpose of the study

KA is a neurotoxic analogue of glutamate that binds to kainate receptors, resulting in overstimulation of neurons at high doses.[6] KA-induced excitotoxicity in rodents results in deficient cognitive functions,[7] elevated anxiety levels,[8] and disruptive morphological changes in different areas of the brain.[9,10] In particular, the hippocampus has been established as a site of damage following KA administration, which elicits cognitive dysfunction.[11,12] Wnt is a canonical lipid-modified signaling glycoprotein that regulates the phosphorylation of GSK3-β.[19] Proteasomes subsequently degrade β-catenin, a downstream survival marker of GSK3-β, through ubiquitination, which often leads to cell death. The brain tissue of KA-administered rats expressed higher antagonistic activity of Wnt,[20] indicating that the Wnt signaling pathway may have an important role in neurodegenerative excitotoxicity of the brain. Study 1 aimed to determine the neuroprotective effects of LE in an *in vivo* rat model of KA-induced excitotoxicity through intrahippocampal microinjections. The protective properties of LE with regards to the Wnt signaling pathway were investigated.

2. Materials and methods

A. Animals

Adult Sprague Dawley rats weighing 200-250 g (Koatec, Pyeongtaek, South Korea) were used for experiments in this study. Animals were housed in groups of three per cage under 12-hour light/dark cycles, with free access to food and water. Animals were subjected to 7 days of acclimation upon arrival at the Association and Accreditation of Laboratory Animal Care (AAALAC)-accredited Yonsei University College of Medicine Animal Care Facilities. All experimental procedures were performed according to the National Institutes of Health Guide for Care, and were approved by the Institutional Animal Care and Use Committee of Yonsei University Health System (permit no.: 2016-0100).

B. Stereotaxic surgery and cannula implantation

Rats were anesthetized by intraperitoneal (i.p.) injection of 50 mg/kg sodium pentobarbital (Hanlim Pharmaceutical, Seoul, South Korea) and were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) for surgical procedures. Stainless steel guide cannulas (22-gauge, 4 mm long, Plastics One, Roanoke, CA, USA) were bilaterally implanted into the CA1 region of the hippocampus (from bregma: anterior/posterior, -3.3 mm; medial/lateral, ±2.4 mm; dorsal/ventral, -3.0 mm). Cannulas were secured with dental acrylic cement with stainless steel screw anchors fixed to the skull. Obturators were placed in the guide cannulas after surgery, and rats were returned to their home cages for 1 wk of recovery. Injection sites are illustrated in Figure 1a and 1b and were verified through cresyl violet staining (Figure 1c).

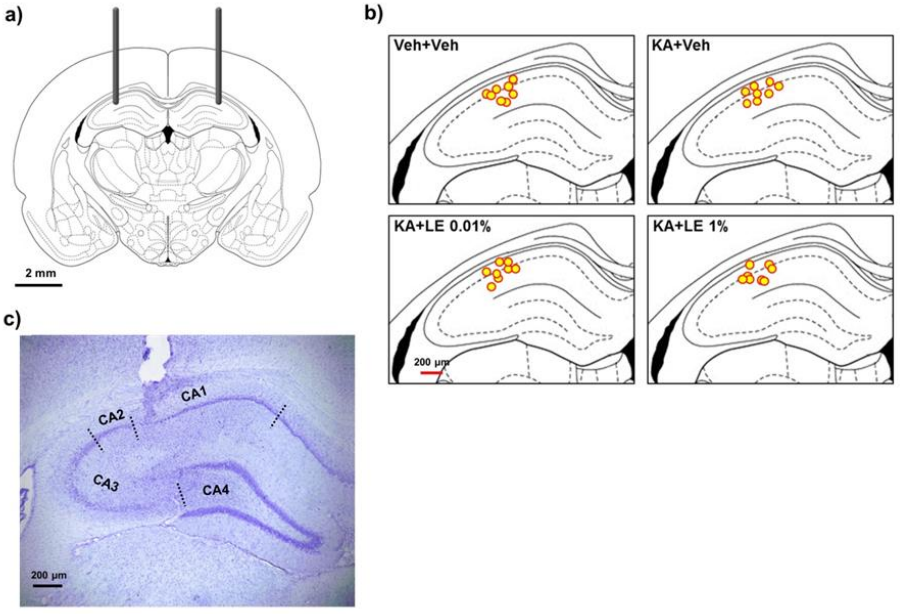

Figure 1. Illustration of stereotaxic implantation of guide cannulas. (**a**) Bilateral implantation of guide cannulas into the CA1 region of the rat hippocampus. Scale bar= 2 mm (**b**) Localization of injection sites after stereotaxic implantation. Scale bar= 200 μm (**c**) Site of injection visualized in a cresyl violet-stained section. The marked areas as CA1 and CA3 were used for the quantification of cell viability in Figure 6. Scale bar= 200 μm (**a-b**) Drawings of the rat brain have been traced from Paxinos and Watson.[34]. KA=kainic acid, LE=lipid emulsion, CA=cornu ammonis.

C. Drug treatment

KA (K0250, Sigma Aldrich, St. Louis, MO, USA) was dissolved in sterile 0.9% NaCl to a final concentration of 0.8 μg/μL. KA was microinjected bilaterally into the hippocampus through internal cannulas (26-gauge, 5 mm long, Plastics One) at a volume of 1 μl over 1 min. The injectors remained in place for an additional 1 min before being replaced by obturators. LE (Intralipid™ 20%, Fresenius Kabi) was dissolved in sterile 0.9% NaCl to final concentrations of 0.01% and 1% LE.

The potential doses of LE were selected from a pilot study *in vitro* using organotypic hippocampal slice cultures (Figure 2). Dosage screening was necessary to avoid possible lipotoxicity induced by LE in the brain. Propidium iodide staining was used to observe cell death by lipotoxicity of LE against hippocampal neurons in slice cultures. Lipotoxocity was observed in dosages higher than 1%. Therefore, we avoided using LE of doses above 1% for direct injections to the hippocampus.

For the study assessing LE against KA-induced seizures, vehicle, LE 0.01%, and LE 1% were intrahippocampally administered 5 mins before or after KA injection via internal cannulas at a rate of 1 μL over 1 min. Seizure activities were evaluated by using the Racine scale of seizures.[35,36] Seizures were terminated by intramuscular injection of 10 mg/kg diazepam (Dong Wha Pharmaceutical, Seoul, South Korea) 90 mins after the initial KA injection.

For the study evaluating neuroprotective effects of LE against KA-induced excitotoxicity, vehicle, LE 0.01%, and LE 1% were intrahippocampally administered 90 mins after KA injection via internal cannulas at a rate of 1 μL over 1 min. Additional injections of vehicle, LE 0.01%, and LE 1% were administered repetitively 24 hrs and 48 hrs after the initial KA injection time points for the experimental groups and sacrificed at 72 hrs. Seizures were terminated by intramuscular injection of 10 mg/kg diazepam (Dong Wha Pharmaceutical) 90 mins after the initial KA injection. Only rats that experienced a seizure level of 3 (forelimb clonus), 4 (rearing), or 5 (falling after rearing) of the Racine scale[35,36] were included.

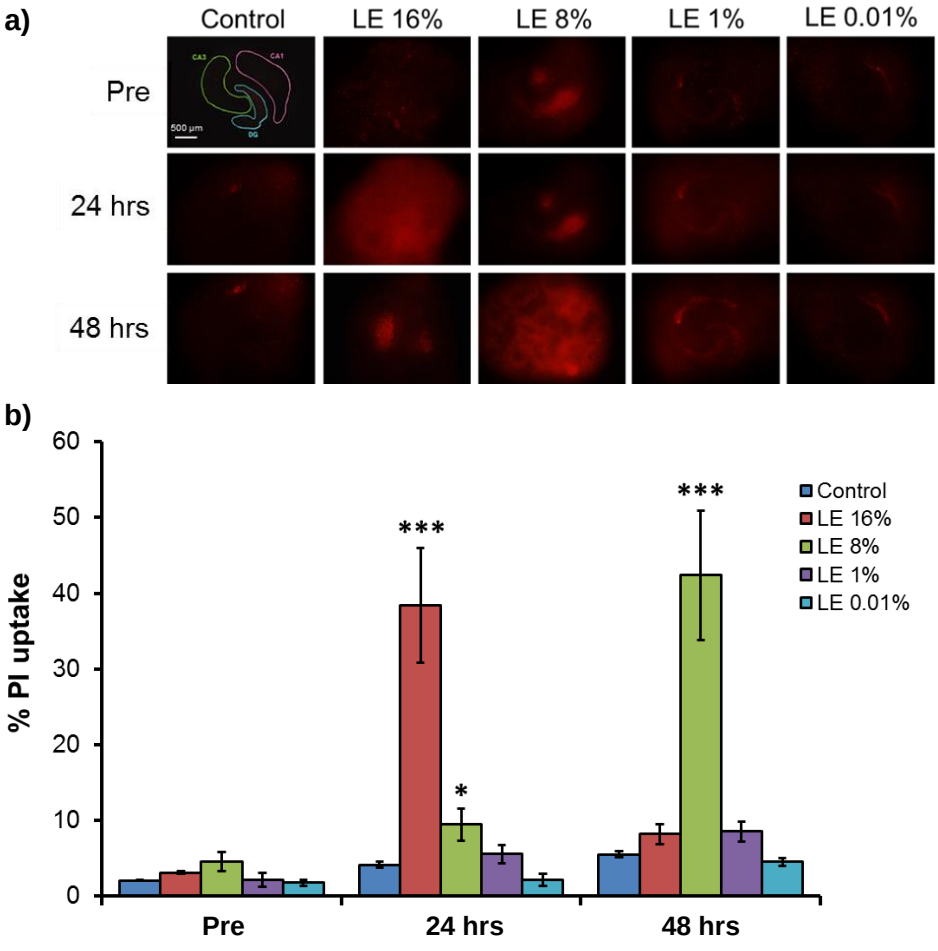

Figure 2. Propidium iodide staining of kainic acid-treated organotypic hippocampal slice cultures. (**a**) Propidium iodide (PI) staining of organotypic hippocampal slice cultures. Cultures were treated with either saline or different doses of lipid emulsion for up to 48 hrs. Positive signals indicate the occurrence of neuronal death. Fluorescence in areas of the CA1, CA3, and the dentate gyrus were summed to quantify PI uptake. Scale bar= 500 μm. (**b**) Neuronal death occurred the most in LE 16% group at 24 hrs and in LE 8% group at 48 hrs. Data are presented as mean ± SEM; n=6 for each group; *P < 0.05, ***P < 0.001 vs Control; one-way ANOVA followed by Dunnett's post-hoc analysis for comparison with the Control (saline) group at each time period. Pre= before application of saline or LE, LE= lipid emulsion.

D. Passive avoidance test

Passive avoidance test is a behavioral test that examines learning and memory. Rodents are fear conditioned via electrical foot shocks to counteract movement into a favorable environment. Unimpaired rats do not move into the darker chamber, as they have learned that a foot shock is the consequence. However, pathological rats that fail to learn the adverse consequences move into the darker chamber, regardless of conditioning.[37]

Passive avoidance test was conducted using Gemini™ (San Diego Instruments, San Diego, CA, USA) in a dark room. Three days after the administration of KA, behavioral tests commenced with a habituation period in which rats were allowed to freely explore the apparatus for 5 mins. On the next day (acquisition trial), animals were habituated to the testing room for 2 hrs before the behavioral test. The chambers were illuminated, and stepover time was recorded. Rats were then subjected to 1.5 mA electrical footshock for 3 secs (Figure 5a). Rats were returned to their cages 5 secs after the footshock. Rats that did not step over to the darker chamber after 90 secs were not considered for this behavioral test. 24 hrs after the acquisition trial, stepover time for retention was recorded for a maximum of 90 secs. Rats were subjected to identical conditions from the training session but without the delivery of the electrical footshock. The entire recording process of stepover latencies were recorded automatically using Gemini™ software (San Diego Instruments).

E. Elevated plus maze

Elevated plus maze is a behavioral test that measures anxiety in experimental animals.[38] The maze comprises four arms, two of which are bordered by walls and two of which remain open, in which rats are at risk of falling off the platform. Rodents have an innate aversion towards open and brighter environments, but they are also curious animals that actively explore novel areas to learn about their environment.[39] KA is known to be anxiogenic in rats by inducing hippocampal damage.[40] Rats deficient in CA1 neurons of the hippocampus tend to be less exploratory and remain stagnant inside the closed arms of the maze.[41]

Elevated plus maze (EPM) consisted of two open arms and two closed arms (Figure 6a). Each arm was 50 cm in length, 10 cm wide, and 50 cm elevated from the ground; while closed arms were enclosed with 40 cm opaque walls. The EPM test was conducted 4 days after KA injection for all experimental groups. Rats were left in the testing room for 2 hrs of

habituation prior to actual trials on the apparatus. The experimental animals were then placed in the center platform of the EPM facing an open arm. Rats were free to explore the apparatus for 5 mins while the duration and number of entries of either the open or closed arms were measured. Valid entries to either the closed or open arms were considered when all four paws of the rat entered the arm.

F. Histology

Morphological changes in the hippocampus at 72 hrs after KA administration has been reported in numerous studies.[20,42] In particular, CA1 and CA3 regions of the hippocampus are prone to damage,[43,44] which may lead to severe impairments in cognitive functions. Therapeutic approaches to prevent excitotoxic cell death in hippocampal regions have been extensively investigated. We examined cell viability in CA1 and CA3 using Nissl staining method (cresyl violet). Cell viability was verified based on the shape and strength of the stain (Figure 7a). Fluorojade staining images presented to the right of cresyl violet staining images show cells undergoing neurodegeneration. Neurodegenerative cells were considered positive by their fluorescence, and were quantified accordingly.

Animals were deeply anesthetized with urethane (1.25 g/kg, i.p.) at 24 hrs or 72 hrs after KA injection for each experimental group and transcardially perfused with 0.9% NaCl via the ascending aorta followed by perfusion with 4% paraformaldehyde (0.1 M phosphate buffer, pH 7.4). Extracted brain tissues were immersed in the same 4% paraformaldehyde solution for 24 hrs at 4℃. After post-fixation, tissues were stored in 10% to 30% sucrose gradient (0.1 M phosphate buffered saline, pH 7.4) for cryoprotection. Brain tissues were instantly frozen at -60℃ in cooled isopentane and sectioned onto silane-coated slides at a thickness of 20 μm.

Histological staining was conducted by washing slides in 0.1 M phosphate buffered saline (PBS) and staining in 0.25% cresyl violet acetate (C5042, Sigma Aldrich, St. Louis, MO, USA) for 2 mins. The slides were rinsed in tap water for 1 min and immersed into 70%, 95%, and 99.9% ethanol for 10 secs each. Then, the slides were placed in xylene for dehydration and mounted with coverslips using Permount (SP15, Fisher Scientific, Fair Lawn, NJ, USA).

Neurodegenerative staining was conducted by immersing slides into a basic alcohol solution consisting of 1% sodium hydroxide in 80% ethanol for 5 mins. The slides were then rinsed in 70% ethanol and distilled water

for 2 mins each. Slides were incubated in 0.1% potassium permanganate solution for 30 mins. Following a 2 mins-wash with distilled water, the slides were transferred to 0.0001% solution of Fluorojade C for 2 hrs (AG325, Merck Millipore, Temecula, CA, USA) dissolved in 0.1% acetic acid. Slides were then rinsed three times in distilled water and coverslipped using DPX mounting solution (06522, Sigma Aldrich, St. Louis, MO, USA).

G. Western blotting and qPCR

The hippocampus was the site of injection and damage; therefore, it was extracted for immunoblotting and mRNA quantification purposes. Hippocampal tissues were collected 24 hrs or 72 hrs after KA injection for each group. The experimental animals were anesthetized with isoflurane and decapitated for tissue collection. Tissue samples were immersed in liquid nitrogen immediately after extraction. For half of the experimental groups, the left hippocampus was used for qPCR analysis, and the right hippocampus was used for immunoblotting analysis. For the other half of the experimental groups, treatment was counterbalanced (left side for immunoblotting and right side for qPCR) in tissue extraction to minimize animals sacrifice. Frozen tissues were used for either qPCR or immunoblotting within 24 hrs upon tissue extraction.

(1) Western blotting

Although the mechanism of action of LE in the brain has not been clearly identified, a known protective mechanism in the heart mainly involves the phosphorylation of Akt[27] and GSK3-β,[29] which in turn promote cell survival. In particular, the phosphorylation of GSK3-β through the canonical Wnt signaling pathway is known to inhibit the degradation of β-catenin.[19] Based on the increased levels of *Wnt1* mRNA expression at 24 hrs after KA administration in LE-treated groups, we examined the protein expressions at 24 hrs and 72 hrs, and the phosphorylation of related protein markers: Wnt1, p-Akt/Akt, p-GSK3-β/GSK3-β, Porcupine (PORCN), and β-catenin.

Frozen samples were homogenized in lysis buffer (ProPrep; Intron Biotechnology, Pyeongtaek, South Korea) with phosphatase inhibitors (Phosstop; Roche, Mannheim, Germany) for protein extraction. Supernatants were collected from homogenized samples that were centrifuged at 15,000 rpm for 15 mins at 4℃. Total protein concentrations

were measured using a spectrophotometer (Nano Drop ND-1000, NanoDrop Technologies Inc., Wilmington, DE, USA), and proteins of equal amounts (30 mg per well) were inserted for denaturation at 94℃. Samples were then loaded on 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Merck Millipore, Darmstadt, Germany) for over 2 hrs. Phospho-proteins were immunodetected prior to the corresponding total protein after the membrane had been stripped. Transferred proteins on membranes were fixed using 0.05% glutaraldehyde in TBS-0.05% Tween-20 (TBST) for 15 mins at room temperature. The membrane was stained with Ponceau S (Sigma-Aldrich, St. Louis, MO, USA) for visualization of the transferred proteins and cut into strips according to the size of the target protein to minimize interactions between antibodies. Membranes were blocked with 5% bovine serum albumin (BSA) dissolved in Tris-buffered saline with Tween 20 (TBST) for 1 hr at room temperature. The membranes were incubated overnight with primary antibodies diluted in 5% BSA in TBST at 4℃. Rabbit was the host of all primary antibodies used for immunoblotting. The following antibodies were used: anti-Wnt1 (ab15251, 1:1,000, Abcam, Cambridge, UK), anti-β-Catenin (#9562, 1:3,000, Cell Signaling Technology, Beverly, MA, USA), anti-Akt (#4691, 1:3,000, Cell Signaling Technology), anti-Phospho-Akt (#9271, 1:1,000, Cell Signaling Technology), anti-GSK3-β (#9315, 1:3,000, Cell Signaling Technology), anti-Phospho-GSK3-β (#9336, 1:1,000, Cell Signaling Technology), and anti-β-Actin (#4967, 1:10,000, Cell Signaling Technology). After overnight incubation of primary antibodies, the membranes were immersed with anti-rabbit horseradish peroxidase-conjugated secondary antibody (#7074, 1:10,000, Cell Signaling Technology). Visualization of immunoreactive proteins was performed with the application of chemiluminescent detection reagent (ECL™ Prime, GE Healthcare, Little Chalfont, UK) and by ImageQuant™ LAS 4000 (GE Healthcare, Little Chalfont, UK). Protein immunoreactivity was measured using Multi-gage software (Fuji Film Inc., Tokyo, Japan).

(2) qPCR

The Wnt signaling pathway consists of 19 families, comprising *Wnt1* to *Wnt16*. Each Wnt family has different roles, which are broadly divided into two categories: the canonical and non-canonical pathways. Canonical Wnt pathway involves the GSK3-β destruction complex where β-catenin is degraded by proteasomes through ubiquitination. Non-canonical Wnt

pathway involves c-Jun N-terminal kinases (JNK) and Ca^{2+}-dependent mechanisms for cell adhesion and growth.[45,46] In this study, Wnt signals present in the rodent hippocampus were selected by screening genes of the Wnt family (Figure 9a and b). *Wnt1* and *Wnt3* of the canonical Wnt pathway, and *Wnt5a* of the non-canonical Wnt pathway, were selected as potential biomarkers related to neuroprotection.

Table 1. Primer pairs for qPCR

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
Wnt1	*GCAACCAAAGTCGCCAGAA*	*TATGTTCACGATGCCCCACCA*
Wnt2b	*GCTACCCAGACATCATGCG*	*ACACTCTCGGATCCATTCCC*
Wnt3	*AATTTGGTGGTCCCTGGC*	*GATAGAGCCGCAGAGCAGAG*
Wnt4	*GTTTCCAGTGGTCAGGATGC*	*AGGACTGTGAGAAGGCTACGC*
Wnt5a	*AAGGGAACGAATCCACGCC*	*ATACTGTCCTGCGACCTGCTTC*
Wnt7a	*CCAAGGTCTTCGTGGATGC*	*TGTAAGTTCATGAGGGTTCGG*
Wnt7b	*CGTGTTTCTCTGCTTTGGC*	*CACCACGGATGACAATGC*
Wnt9a	*GTACAGCAGCAAGTTTGTCAAGG*	*CACGAGGTTGTTGTGGAAGTCC*
Wnt10a	*CGGAACAAAGTCCCCTACG*	*AGGCGAAAGCACTCTCTCG*
Wnt16	*GCACTCTGTAACCAGGTCATGC*	*TGCAAGGTGGTGTCACAGG*
MKi67	*TTCAGTTCCGCCAATCCAAC*	*CCGTGCTGGTTCCTTTCCA*
PORCN	*CCTACCTCTTCCCCTACTTCA*	*CTTTGCGTTTCTTGTTGCGA*
β-Actin	*GTCCACCCGCGAGTACAAC*	*TATCGTCATCCATGGCGAACTGG*

Hippocampal tissue RNA extraction was executed using the Hybrid-R kit (305-010; GeneAll Biotechnology, Seoul, South Korea). RNA concentration was measured using a spectrophotometer (Nano Drop ND-1000, NanoDrop Technologies Inc.). cDNA was prepared from 1 μg of total RNA using the PrimeScript 1st strand cDNA synthesis kit (Takara Bio, Shiga, Japan). PCR amplification was executed using the SYBR-Green

reagent (Takara Bio) in the ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). PCR amplification was performed in 20-μL reaction volumes. Sequences for oligonucleotide primers were selected using the Gene Database of National Center for Biotechnology Information (NCBI) and Primer Express™ Software v3.0.1 (Thermo Fisher Scientific, Waltham, MA, USA). Primer pairs are listed in Table 1.

J. Statistical analysis

Statistical evaluations were performed using one-way ANOVA or unpaired *t*-test, or otherwise specified in the figure legends. The Shapiro-Wilk test was utilized to verify the normal distribution of the numerical data. Post-hoc analyses were performed using the Tukey's multiple comparisons test or as otherwise specified in the figure legends. Data are presented as mean ± standard error of mean (SEM). A *p*-value less than 0.05 was considered statistically significant for all analyses. All statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

A. Seizure and survival

LE did not show reduction in seizures when injected prior or immediately after KA injection. The average of seizure scores for all experimental groups was 3 of the Racine scale (Figure 3a and b). For the study evaluating neuroprotective effects of LE against KA-induced excitotoxicity, seizure severity was observed in groups administered with KA excluding sham. Only rodents that experienced stage 3 seizure severity or higher were used in the latter experiments; this accounted for approximately 83% (183/220) of KA-administered rats (Table 2). 37/220 rats that have experienced seizure level 2 (facial clonus) or less have been excluded from the study due to the inconsistency in hippocampal damage severity (Table 2). Although KA-injected rats in all groups were administered with an identical dose of KA, there were phenotypic differences in individual seizure severity. The KA+Veh group exhibited a significantly lower survival rate (47/65) than that of the Veh+Veh group (65/65). The impact of LE on survival was not significant, but approached a trend for significance (P=0.0772 for KA+Veh vs. KA+LE 1%; Figure 4, Table 3) by 3 days post-KA injection.

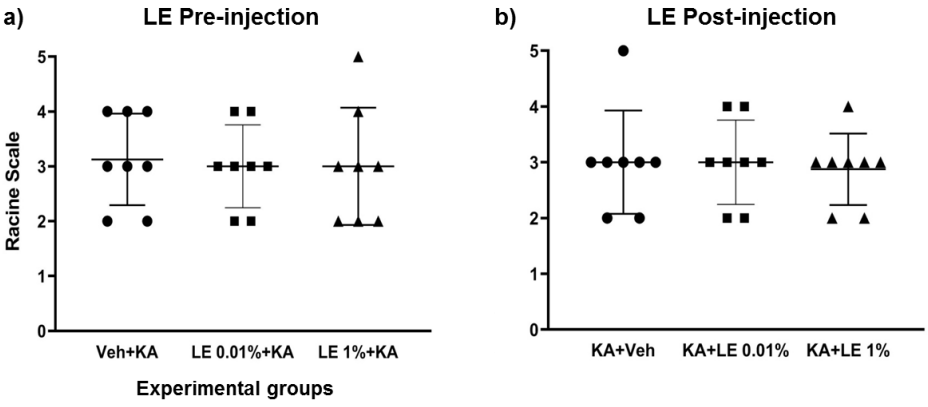

Figure 3. Effects of LE on KA-induced seizures. (**a**) Seizure severity of experimental groups when LE was injected 5 mins prior to KA injection. (**b**) Seizure severity of experimental groups when LE was injected 5 mins after KA injection. Data are presented as mean ± SEM; n=8 for each group. Statistical analysis for the Racine scale was performed using Kruskal-Wallis non-parametric test followed by Dunn's post hoc test. KA=kainic acid, Veh= vehicle, LE= lipid emulsion.

Table 2. Seizure severity of experimental animals measured using Racine's scale

Racine's scale Stage/Class	Behavior	% of total exp. animals
1	Immobility, orofacial movements	0% (0/220)
2	Head nodding, facial clonus	17% (37/220)
3	Forelimb clonus, wet dog shakes	68% (150/220)
4	Forelimb clonus with rearing	9% (20/220)
5	Clonic rearing and falling, wild jumping	6% (13/220)

A total of 220 animals were assessed for their seizure behavior and scaled accordingly to their behavior. The Veh+Veh group were not included in this table because they were not administered with kainic acid and did not experience seizures. Exp.= experimental.

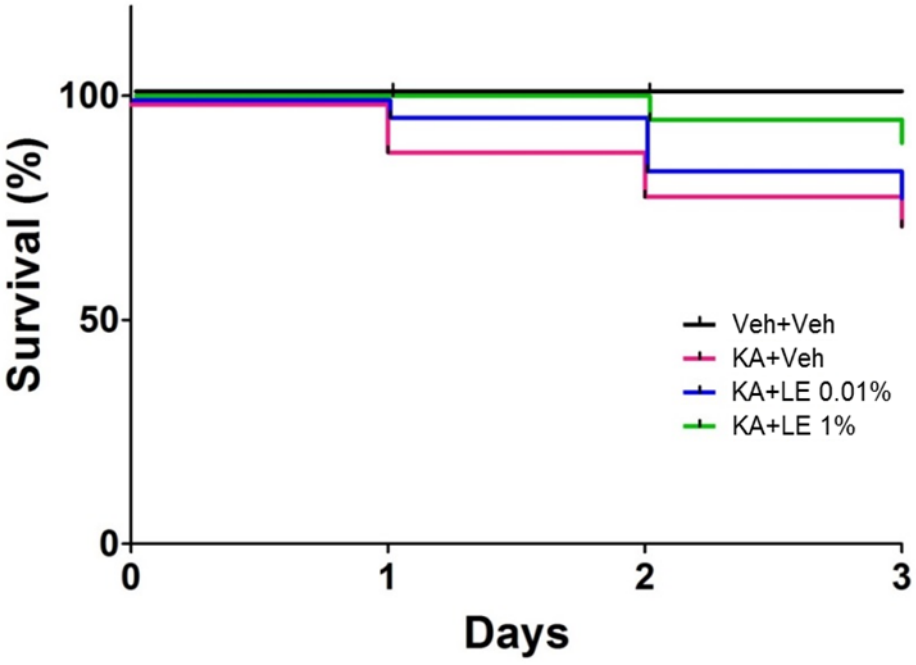

Figure 4. Survival rate for each experimental group after KA injection. Survival rate of experimental animals that experienced stage 3 or above of the Racine scale. Survival rate was recorded up to 3 days post-KA acid injection. (n=65 per group, P= 0.0024 for Veh+Veh vs. KA+Veh, P=0.6063 for KA+Veh vs. KA+LE 0.01%, P=0.0772 for KA+Veh vs. KA+LE 1%; survival analyzed by log-rank [Mantel-Cox] test). KA=kainic acid, Veh= vehicle, LE= lipid emulsion.

Table 3. Numbers at risk and survival rate by time

Numbers at risk by time	Day 0	Day 1	Day 2	Day 3
Veh+Veh	65	65	65	65
KA+Veh	65	58	52	47
KA+LE 0.01%	65	63	55	51
KA+LE 1%	65	65	62	58
Survival rates by time	Day 0	Day 1	Day 2	Day 3
Veh+Veh	1	1	1	1
KA+Veh	1	0.892	0.800	0.723
KA+LE 0.01%	1	0.969	0.846	0.785
KA+LE 1%	1	1	0.954	0.892

Total of 260 animals that experienced stage 3 or above of the Racine scale (n=65 per group) were assessed on the survival after the injection of Veh or KA. KA= kainic acid, Veh= vehicle, LE= lipid emulsion.

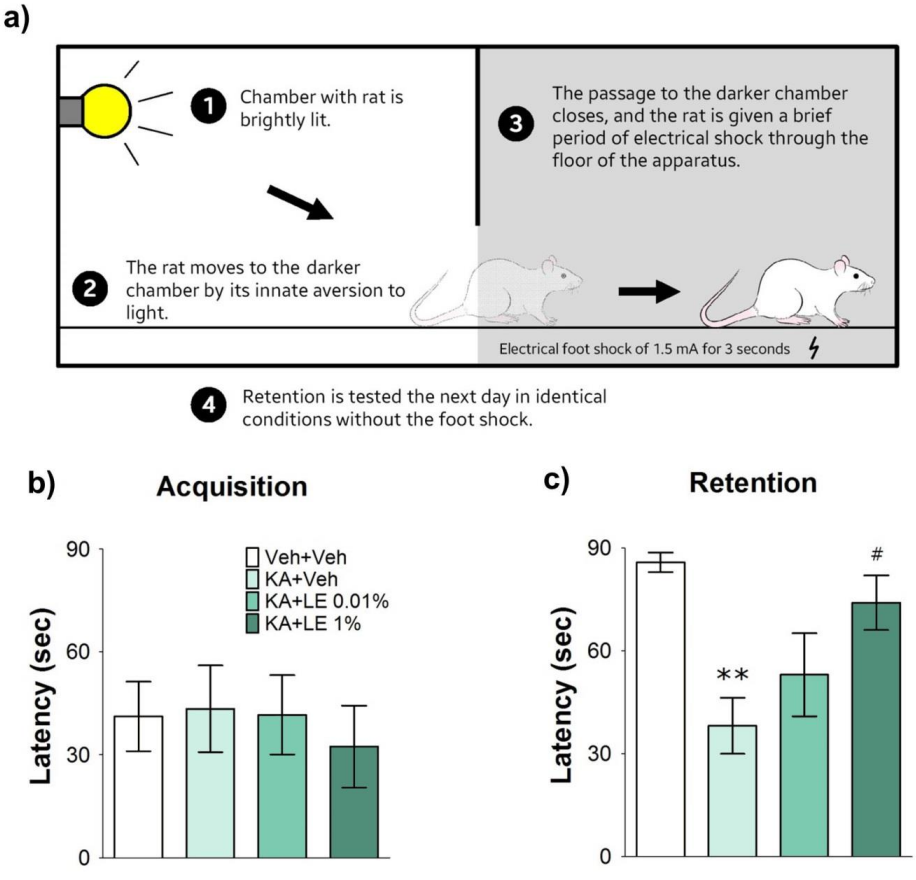

Figure 5. Illustration of passive avoidance test and results 4 days after KA and repetitive LE injection. (**a**) A schematic drawing describing the procedures of the single-trial passive avoidance test. The behavioral test consisted of habituation, acquisition, and retention trials at 2, 3, and 4 days after KA injection, respectively. (**b**) Measurements of the stepover latency during the acquisition trials (initial latency). There were no noticeable differences between all experimental groups. (**c**) The stepover latency measured during the retention trial (retention latency). (**b-c**) Data are presented as mean ± SEM; n=8 for each group; **P<0.01 vs Veh+Veh, #P<0.05 vs. KA+Veh. KA=kainic acid, Veh= vehicle, LE= lipid emulsion.

B. Memory retention in behavioral tests

Passive avoidance test is a behavioral test that examines learning and memory (Figure 5a). There were no significant differences in acquisition latency among groups (Figure 5b). The KA+Veh group experienced similar deficits in learning, whereas their retention latency was significantly shorter than that of the Veh+Veh group ($p<0.01$; Figure 5c). Failure to form new memories is associated with deficient hippocampal activity.[37,47] Rats that experienced acute hippocampal damage were more reactive to light and hyperactive when the chamber lit up. Four rats did not cross over to the darker chamber for the entire session, indicating preservation of hippocampal memory function. The KA+LE 0.01% group was hesitant to enter the darker chamber, but no significant differences in retention latency were observed between the KA+LE 0.01% and KA+Veh groups. The average difference between KA+Veh and Veh+Veh was 47 secs, KA+Veh and KA+LE 0.01% 14 secs, and KA+Veh and KA+LE 1% 35 secs (n=8).

C. Decreased anxiety via CA1 protection

Elevated plus maze is a behavioral test that measures anxiety in experimental animals (Figure 6a).[38] The KA+Veh group were less mobile and spent significantly less time in the open arms compared to the Veh+Veh group (Figure 6b). The KA+LE 1% group demonstrated significantly longer duration of exploratory behavior in the open arms and was noticeably more active during the behavioral test compared to the KA+Veh group (Figure 6c). Four animals in the KA+LE 0.01% group displayed similar results as those in the KA+LE 1% group (n=10), but the difference in exploratory activity was not statistically significant when compared to the KA+Veh group. Anxiety behavior was inconsistent between individuals. In addition, defecation and urination in the KA+Veh group were more frequent, but there were no significant differences among groups (Figure 6d).

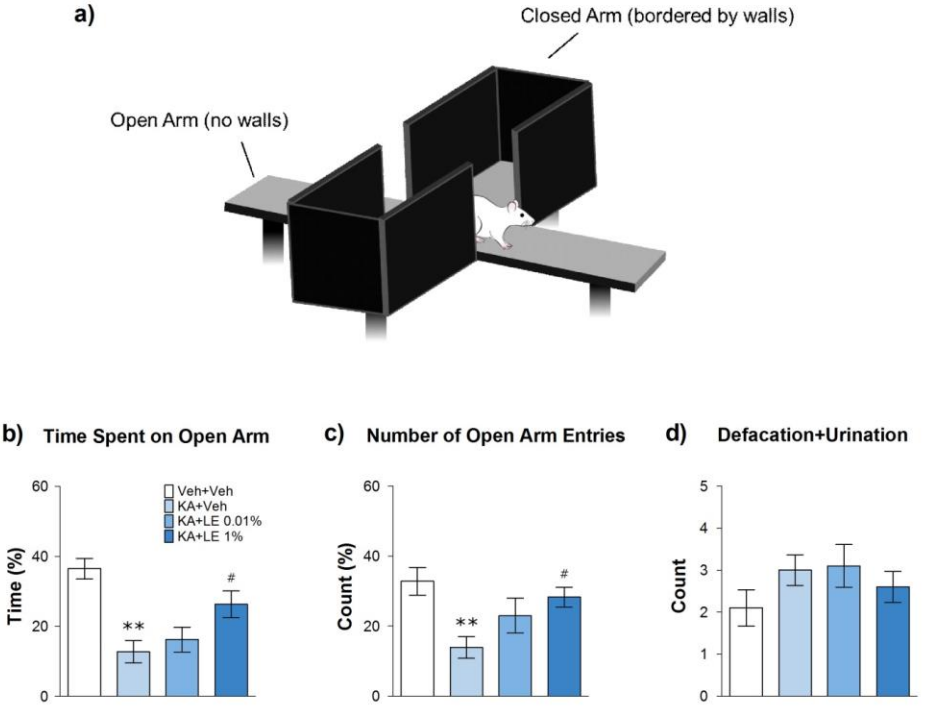

Figure 6. Illustration of the elevated plus maze and results 4 days after KA and repetitive LE injection. (**a**) A schematic drawing of the elevated plus maze. (**b**) Measurement of the time spent in the open arms compared to the total duration. (**c**) The number of entries into the open arm compared to the total entries into all arms. (**d**) Defecation and urination counts for each experimental group. Data are presented as mean ± SEM; n=10 for each group; **P<0.01 vs Veh+Veh, #P<0.05 vs. KA+Veh. KA=kainic acid, Veh= vehicle, LE= lipid emulsion.

D. Histologic changes: LE alleviates damage in CA1

Cell viability was verified based on the shape and strength of the stain (Figure 7a). Cell viability in CA1 was significantly lower in the KA+Veh group than in the Veh+Veh group. Cell survival in CA1 of the KA+LE 1% group was significantly greater than that of the KA+Veh group, suggesting that LE provided neuroprotection (Figure 7b, left). In addition, neurodegeneration was significantly lower in the KA+LE 1% group compared to the KA+Veh group (Figure 7b, right). Although LE treatment showed a tendency to increase neuroprotection in a dose-dependent manner, the KA+LE 0.01% group did not demonstrate a significant degree of protection. The consequential hippocampal cell death in the CA3 induced by seizures was not reduced by LE (Figure 7c).

E. LE activates cell survival signals involved in the Wnt signaling pathway

We examined the protein expressions at 24 hrs (Figure 8a) and 72 hrs (Figure 8h), and the phosphorylation of related protein markers. In contrast to *Wnt1* mRNA expression, there were no significant changes in the levels of Wnt1 protein expression at 24 hrs (Figure 8b), but different findings were observed at 72 hrs after KA administration (Figure 8i). PORCN, an upstream marker known to activate Wnt signals,[48] decreased significantly at 72 hrs in the KA+Veh group but not at 24 hrs (Figure 8e and l). β-catenin was increased significantly in LE-treated groups compared to the KA+Veh group (Figure 8d and k). Consequently, GSK3-β was phosphorylated significantly in KA+LE 1% group compared to the KA+Veh group (Figure 8f and m). Akt (Figure 8g and n) protein expression levels in LE-treated groups also increased, which may have promoted cell survival. β-catenin protein expression levels were significantly upregulated in LE-treated groups at both time points. β-catenin protein expression was comparably lower at 72 hrs than at 24 hrs after KA administration (Figure 9b). Compared to the Veh+Veh group, Wnt3 was also downregulated in all experimental groups at both 24 hrs and 72 hrs (Figure 8c and j).

The KA+Veh group showed a significant difference in Wnt1 expression level but not in other groups (Figure 9a). The expression level of Wnt3 decreased significantly from 24 hrs to 72 hrs in the KA+Veh group but other groups did not differ significantly (Figure 9b). The expression level of β-catenin decreased significantly from 24 hrs to 72 hrs in all groups (Figure 9c) There were no differences in level of PORCN protein expression at 24 hrs and 72 hrs within groups (Figure 9d). There were no differences in level of GSK3-β and Akt protein expression at 24 hrs and 72 hrs within groups (Figure 9e and f).

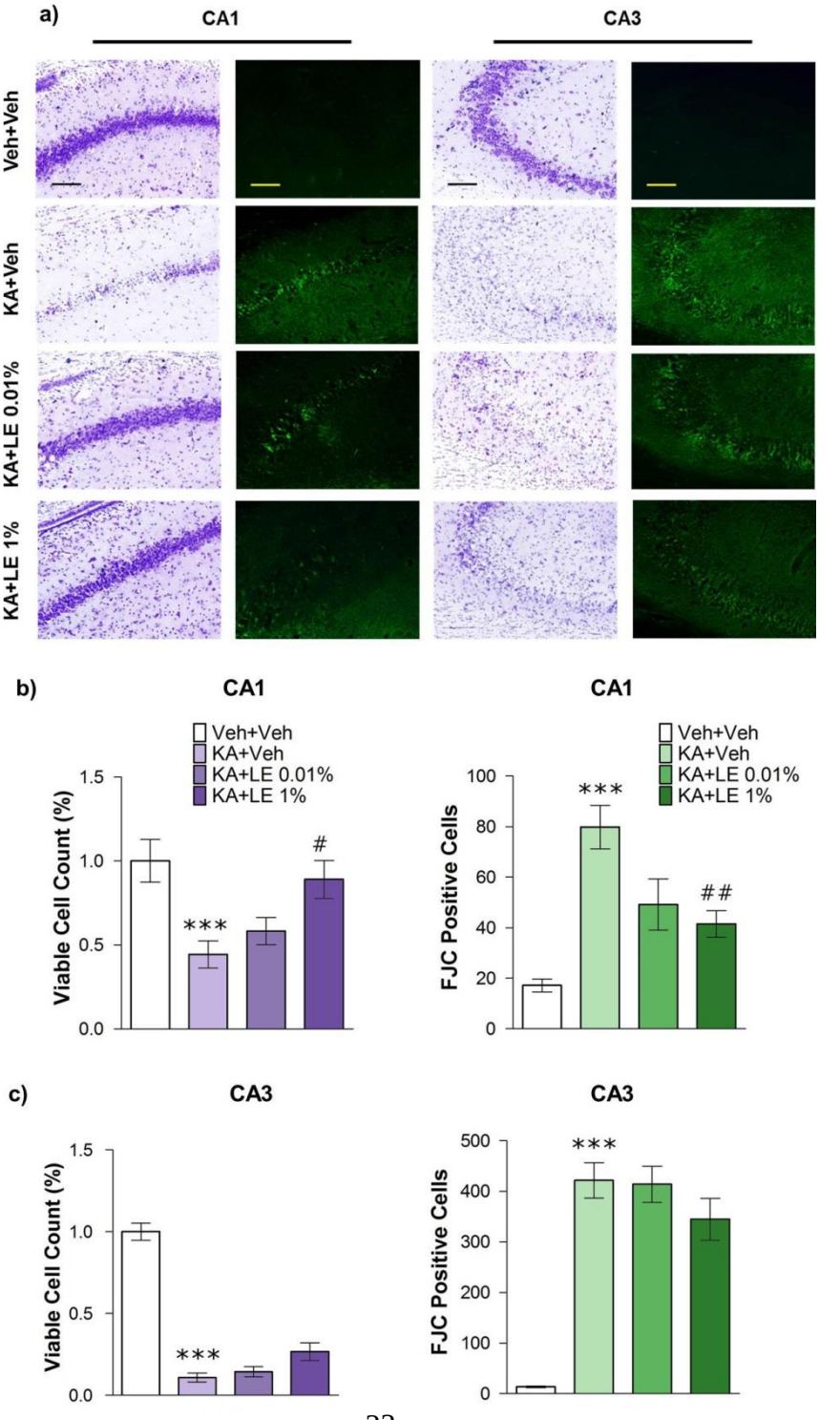

Figure 7. Effects of LE on morphology of hippocampal neurons 72 hrs after KA and repetitive LE injection (3 times). (**a**) Cresyl violet (columns 1 & 3) and Fluorojade C (FJC) (columns 2 & 4) staining for each group in the hippocampal CA1 and CA3 regions. Scale bars = 200 μm. (**b**) Quantification of viable cells compared to Veh+Veh group in the CA1 of the hippocampus. (**c**) Quantification of viable cells compared to Veh+Veh group in the CA3 region of the hippocampus. Data are presented as mean ± SEM; n=12 for each group; ***P < 0.001 vs Veh+Veh, #P < 0.05, ##P < 0.01 vs. KA+Veh. KA=kainic acid, Veh= vehicle, LE= lipid emulsion.

F. mRNA differences in canonical Wnt signaling

At 24 hrs after KA administration, a 10-fold difference in *Wnt1* expression between the KA+Veh group and LE-injected groups was observed (Figure 10a), indicating significant activation of the Wnt/β-catenin pathway. The level of *Wnt1* mRNA expression in the KA+Veh group was significantly lower than that in the Veh+Veh group, implying attenuated levels of cell survival. *Wnt1* expression substantially exceeded the baseline (represented by the Veh+Veh group), implying that overexpression of *Wnt1* underpinned neuroprotection in pathological conditions (Figure 10b). Despite peak in activities of *Wnt1* at the 24 hrs time point, it returned to baseline or lower in LE-injected groups at 72 hrs after KA injection. The attenuation in *Wnt1* at 72 hrs was notable but not significant probably due to high levels of variability in each group. The expression of the upstream signal, PORCN was significantly increased in the 24 hrs group but not in the 72 hrs group, indicating the activation of Wnt at the acute phase of the pathological state. Expression of another canonical Wnt signal, *Wnt3* was more dramatically attenuated in LE-treated groups compared to the difference in expression between the KA+Veh and Veh+Veh groups at 24 hrs after KA administration. Lower expression levels persisted until 72 hrs after KA administration, indicating a reduction in aberrant neurogenesis, which is a therapeutic goal in excitotoxic conditions.

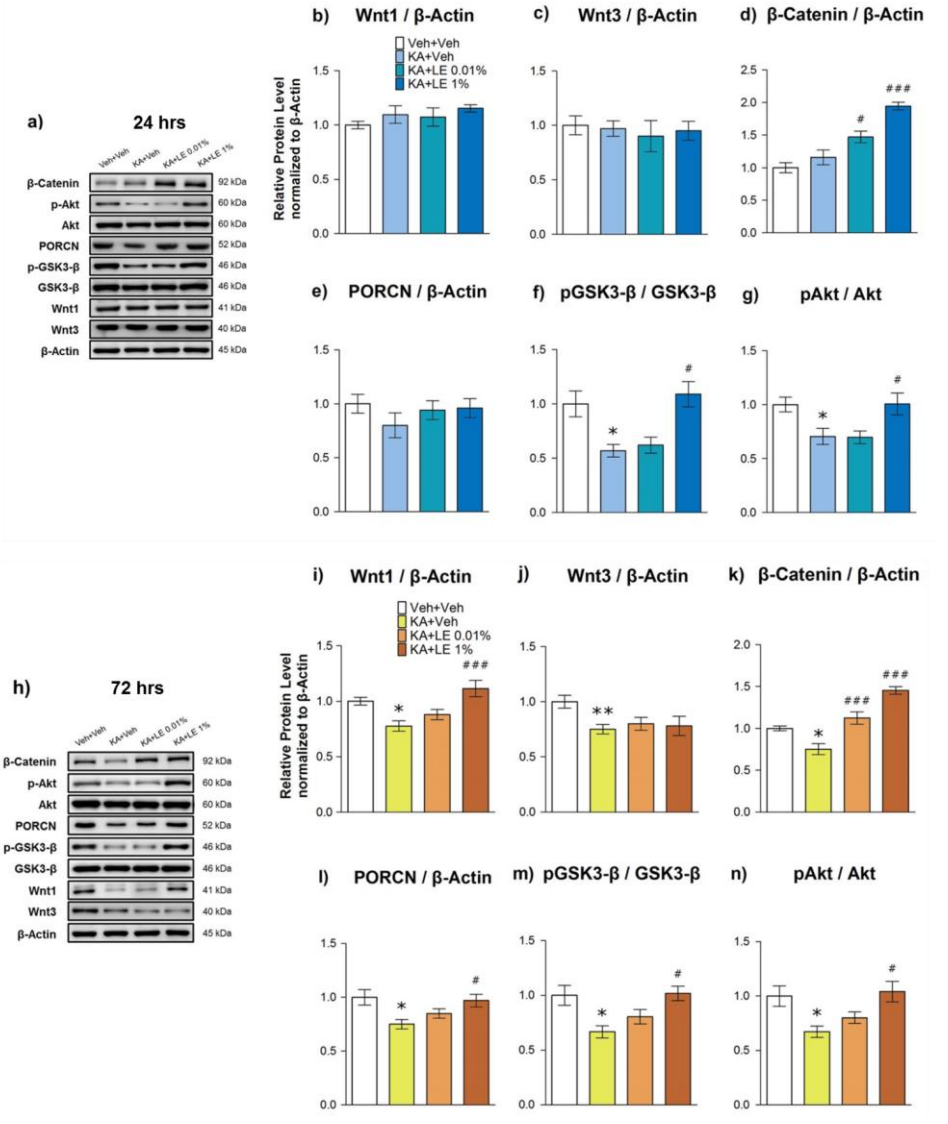

Figure 8. Effects of LE on protein expression in hippocampi extracted 24 or 72 hrs after KA and repetitive LE injection (1 and 3 times, respectively). (**a-g**) Protein expression in hippocampi extracted 24 hrs after KA injection. (**h-n**) Protein expression in hippocampi extracted 72 hrs after KA injection. Data are presented as mean ± SEM; n=10 for each group; b-n) *P < 0.05, **P < 0.01 vs Veh+Veh, #P < 0.05, ###P < 0.001 vs. KA+Veh. KA=kainic acid, Veh= vehicle, LE= lipid emulsion.

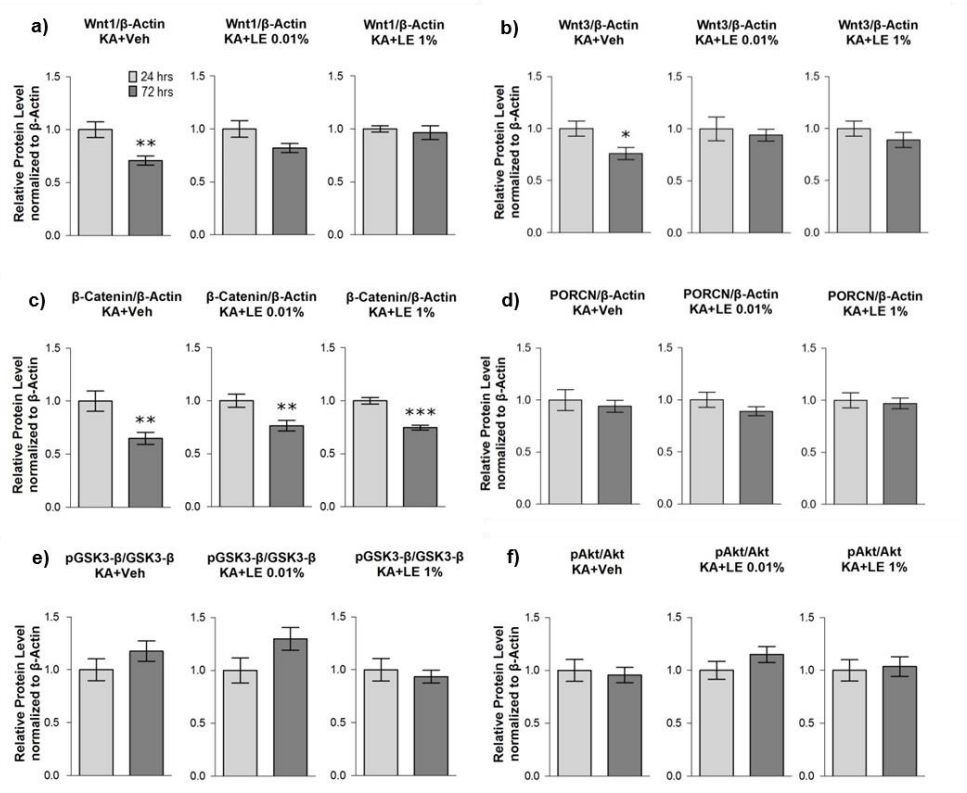

Figure 9. Comparison of LE on protein expression between 24 and 72 hrs after KA injection. (**a**) Differences in Wnt1 protein levels between 24 hrs and 72 hrs time-points. (**b**) Differences in Wnt3 protein levels between 24 hrs and 72 hrs time-points. (**c**) Differences in β-catenin protein levels between 24 hrs and 72 hrs timepoints. (**d**) Differences in PORCN protein levels between 24 hrs and 72 hrs timepoints. (**e**) Differences in p-GSK3-β/GSK3-β protein levels between 24 hrs and 72 hrs timepoints. (**f**) Differences in p-Akt/Akt protein levels between 24 hrs and 72 hrs timepoints. Data are presented as mean ± SEM; n=10 for each group. (a-f) *P<0.05, **P < 0.01, ***P < 0.001 vs 24 hrs. KA=kainic acid, Veh= vehicle, LE= lipid emulsion.

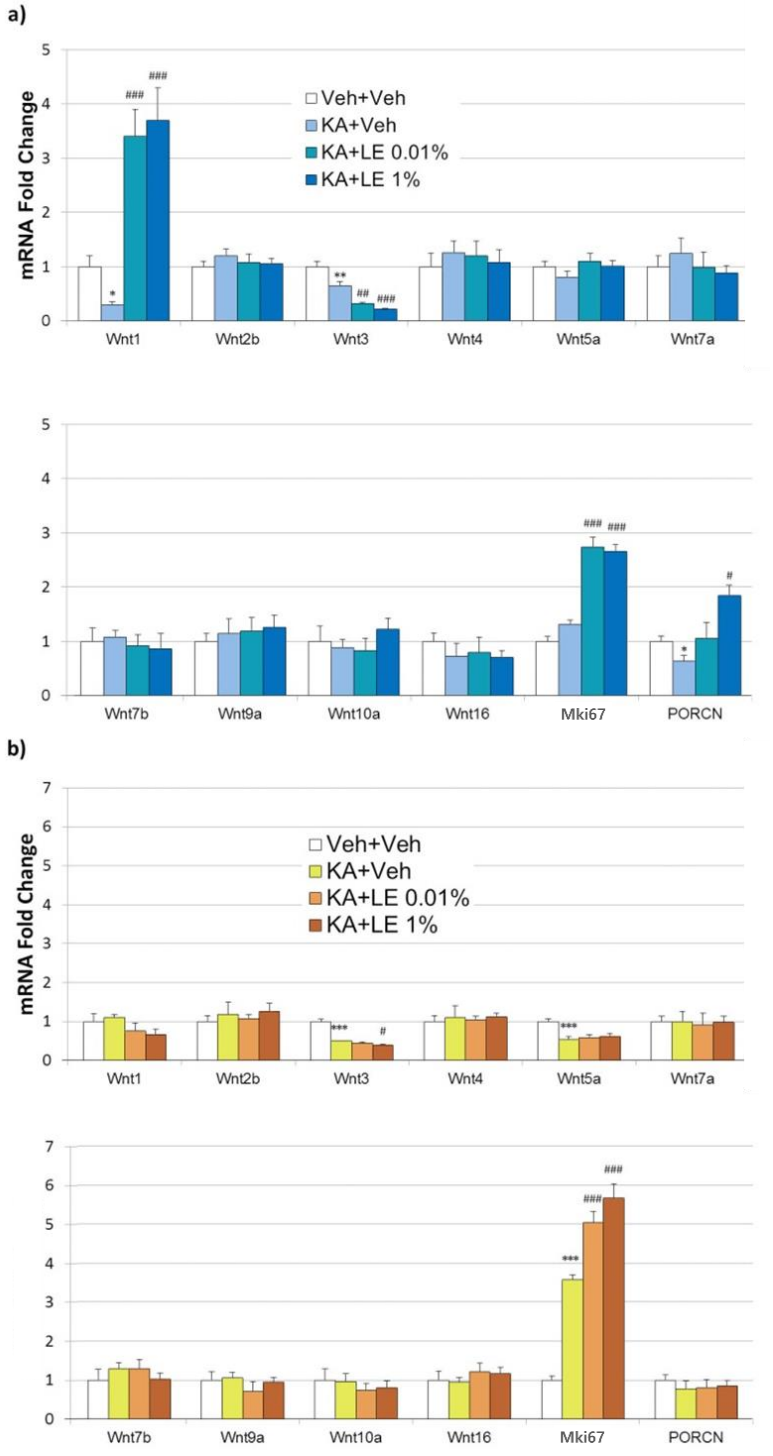

Figure 10. Effects of LE on mRNA expression levels in hippocampi extracted 24 or 72 hrs after KA and repetitive LE injection (1 or 3 times, respectively). (**a**) mRNA expression levels for each experimental group at 24 hrs after KA injection. (**b**) The mRNA expression levels for each experimental group at 72 hrs after KA injection. Data are presented as mean ± SEM; n=10 for each group; *P < 0.05, **P < 0.01, ***P < 0.001 vs Veh+Veh, #P < 0.05, ##P < 0.01, ###P < 0.001 vs. KA+Veh. KA=kainic acid, Veh= vehicle, LE= lipid emulsion.

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There were no significant differences in *Wnt5a* mRNA expression between groups at 24 hrs, but a significant decrease was observed at 72 hrs after KA administration in all KA-treated groups compared to Veh+Veh. All groups excluding the Veh+Veh group exhibited elevated expression levels of *Mki67* at 72 hrs after KA administration.

III. STUDY 2: Protective effects of LE against ischemic reperfusion injury in the brain.

1. Purpose of the study

LE has been reported to ameliorate recovery from ischemic reperfusion injury in the heart.[49] However, the protective effects of LE against such injury have not been identified in the brain. Ischemic reperfusion injury in the brain is detrimental and elicits major dysfunctions within the body, such as impairments in movement, cognition, and other vital functions.[50] Ischemic reperfusion injury in the brain encompasses abnormal production of oxygen radicals that may exacerbate initial ischemic injuries.[13] Based on neuroprotective effects of LE in Study 1, such effects of LE via *in vivo* rat model of ischemic reperfusion injury through middle cerebral artery occlusion (MCAO) were examined.

2. Materials and methods

A. Animals

Adult Sprague-Dawley rats weighing 260-300 g (Koatec, Pyeongtaek, South Korea) were used for experiments in this study. Animals were housed in groups of three per cage under 12-hour light/dark cycles, with free access to food and water. Animals were subjected to 7 days of acclimatization upon arrival at the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited Yonsei University College of Medicine Animal Care Facilities. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Yonsei University Health System (permit no.: 2019-0263) and performed according to the National Institutes of Health Guide for Care.

B. Middle cerebral artery occlusion and reperfusion

Rats were anesthetized by intraperitoneal (i.p.) injection of 50 mg/kg sodium pentobarbital (Hanlim Pharmaceutical, Seoul, South Korea) and were placed on a heated mat to maintain body temperature of 37.0 ± 1.0 ℃. The surgical and injection process is illustrated in Figure 11. The Koizumi method[51] of MCAO was used for inducing ischemic reperfusion injury. Cervical skin incision was made to expose the right common carotid artery

(CCA), external carotid artery (ECA), and internal carotid artery (ICA). The CCA was permanently ligated with a silk suture below the bifurcation to the ECA and ICA. The ECA was temporarily ligated with a silk suture while a small incision was made in the CCA to insert a silicone coated nylon filament (403723PK10Re, Doccol Corporation, Sharon, MA, USA) into the ICA until a mild resistance stopped the insertion. The length of the insertion was approximately 18-20 mm (Figure 11a). After the MCA was occluded for 90 mins, the filament was removed. The removal of the filament recovered the blood flow to the MCA through the circle of Willis as Koizumi et. al.[51] described in their study. The experimental group undergoing sham surgery did not have the filament inserted. Polyethylene tubing with an inner diameter of 0.28 mm and outer diameter of 0.61 mm (427401, Becton, Dickson and Company, Sparks, MD, USA) was inserted for injection of LE or vehicle (Figure 11b). Injections were made at 0.5 mL/min over 2 mins. The polyethylene tubing was removed after the injection and the CCA above the incision was permanently ligated. The temporary ligation of the ECA was removed. A total of 374 rats were used for this study. The MCAO surgery was conducted on 310 rats and sham surgery was conducted on 64 rats. 84 rats that deceased during MCAO, reperfusion or intra-arterial injection were excluded from the experiment. None of Sham-operated rats deceased. 34 rats that underwent MCAO and reperfusion but did not show infarction due to surgical errors were also excluded from the study.

C. Drug treatment

LE (Intralipid™ 20%, Fresenius Kabi) was dissolved in sterile 0.9% NaCl to a final concentration of 10% for the injection of the LE 10% group. Vehicle, 10% LE, and 20% LE were intra-arterially administered during reperfusion after 90 mins. of MCAO. Intra-peritoneal injection XAV939 (S1180, Selleck Chemicals, Houston, TX, USA) 40mg/kg or DMSO were injected for 2 days prior to the MCAO surgery and on the day of the surgery before MCAO.

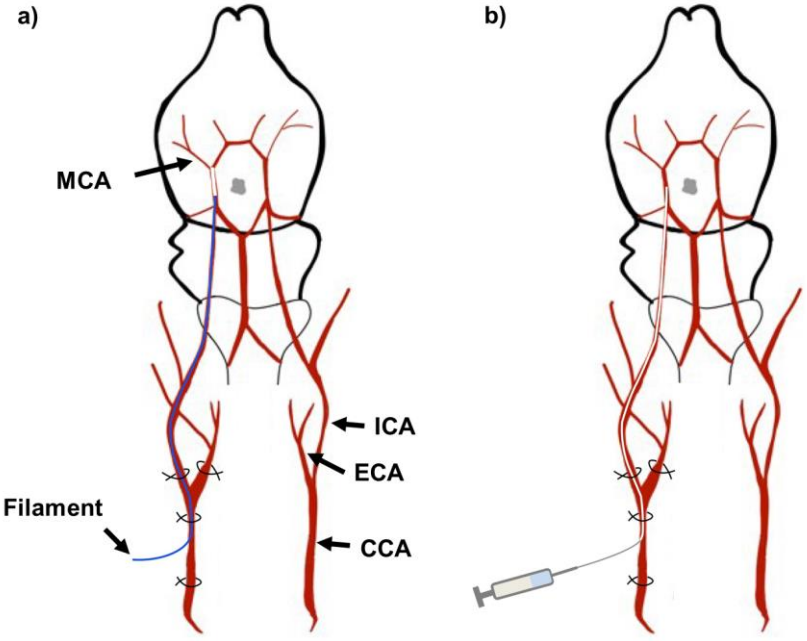


Figure 11. Illustration of middle cerebral artery occlusion and injection. (**a**) Insertion of nylon filament for MCAO. (**b**) Insertion of polyethylene tubing for injection of lipid emulsion or vehicle. CCA= common carotid artery, ECA= external carotid artery, ICA= internal carotid artery, MCA= middle cerebral artery.

D. Neurological deficit assessment

A neurological examination of experimental animals was performed in accordance with the modified Bederson method[52] 24 hrs after MCAO or sham surgery by a blinded researcher. The scoring criteria are as follows: score 0: no deficit; score 1: lost forelimb flexion; score 2: as for 1, plus decreased resistance to lateral push; score 3: unidirectional circling; score 4: longitudinal spinning or seizure activity; score 5: no movement.

E. Infarction volume assessment

The infarction volume was assessed by triphenyltetrazolium chloride (TTC) staining (T8877, Sigma Aldrich) of consecutive 2.0 mm coronal sections bregma +4-6.0 mm. The sections were immersed into 2% TTC solution for 30 mins at 37.0 ℃. The sections were transferred into 4% paraformaldehyde solution for 24 hrs. The sections were then

photographed and analyzed using the ImageJ software (ImageJ, National Institute of Health, Bethesda, MD, USA). The infarction volume was calculated using the following formula[51]: (contralateral volume – non-infarct ipsilateral volume)/contralateral volume x 100%. The contralateral volume refers to the opposite brain hemisphere of the infarcted side.

F. Western blotting

Frozen samples were homogenized for protein extraction in lysis buffer (ProPrep; Intron Biotechnology) with phosphatase inhibitors (Phosstop; Roche). Supernatants were collected from homogenized samples that were centrifuged at 15,000 rpm for 15 mins at 4℃. Total protein concentrations were measured using a spectrophotometer (Nano Drop ND-1000, NanoDrop Technologies Inc.), and equal amount of protein (30 mg per well) were resolved on 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Merck Millipore, Darmstadt, Germany) for 2 hrs. Phospho-proteins were detected by immunoblotting prior to their corresponding total protein levels, which were detected after the membrane had been stripped. Transferred proteins on membranes were fixed using 0.05% glutaraldehyde in Tris-buffered saline containing 0.05% Tween-20 (TBST) for 15 mins at room temperature. The membrane was stained with Ponceau S (Sigma-Aldrich) for visualization of the transferred proteins and cut into strips according to the size of the target protein to minimize interactions between antibodies. Membranes were blocked with 5% bovine serum albumin (BSA) dissolved in TBST for 1 hr at room temperature. The membranes were incubated overnight with primary antibodies diluted in 5% BSA in TBST at 4℃. Rabbit was the host of all primary antibodies used for immunoblotting. The following antibodies were used: anti-Wnt1 (ab15251, 1:1,000, Abcam), anti-Wnt3 (ab32249, Abcam), anti-PORCN (ab105543, Abcam), anti-β-catenin (#9562, 1:3,000, Cell Signaling Technology, Beverly, MA, USA), anti-Akt (#4691, 1:3,000, Cell Signaling Technology), anti-Phospho-Akt (#9271, 1:1,000, Cell Signaling Technology), anti-GSK3-β (#9315, 1:3,000, Cell Signaling Technology), anti-Phospho-GSK3-β (#9336, 1:1,000, Cell Signaling Technology), anti-pβ-catenin (#9561, 1:1,000, Cell Signaling Technology), anti-tankyrase 1 (MBS8531631, 1:1,000, MyBioSource, San Diego, CA, USA), anti-IL-1β (AB1832P, 1:1,000, Sigma Aldrich), anti-IL-6 (ARC0062, 1:500, Invitrogen, Carlsbad, CA, USA), anti-IL-8 (MBS9385550, 1:500, MyBioSource), anti-TNF-α (AAR33, 1:1,000,

Bio-Rad, Hercules, CA, USA) and anti-β-actin (#4967, 1:10,000, Cell Signaling Technology). After overnight incubation of primary antibodies, the membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (#7074, 1:10,000, Cell Signaling Technology). Visualization of immunoreactive proteins was performed with the application of chemiluminescent detection reagent (ECL™ Prime, GE Healthcare) and images were taken by ImageQuant™ LAS 4000 (GE Healthcare). Protein immunoreactivity was measured using Multi-gauge software (Fuji Film Inc., Tokyo, Japan).

G. qPCR

RNA was extracted by using the Hybrid-R kit (305-010; GeneAll Biotechnology, Seoul, South Korea). The concentration of RNA was measured using a spectrophotometer (Nano Drop ND-1000, NanoDrop Technologies Inc). cDNA was prepared from 1 μg of total RNA using the PrimeScript 1st strand cDNA synthesis kit (Takara Bio, Shiga, Japan). PCR amplification was executed using the SYBR-Green reagent (Takara Bio) in the ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). PCR amplification was performed in 20 μL reaction volumes. Sequences for oligonucleotide primers were selected using the Gene Database of National Center for Biotechnology Information (NCBI) and Primer Express™ Software v3.0.1 (Thermo Fisher Scientific, Waltham, MA, USA). Primer pairs are listed in Table 4.

H. Statistical analysis

Statistical evaluations were performed using one-way ANOVA or unpaired *t*-test, as indicated in figure legends. The Shapiro-Wilk test was utilized to verify the normal distribution of the numerical data. Post hoc analyses were performed using the Tukey's multiple comparisons test or as otherwise specified in the figure legends. All statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). A *p*-value less than 0.05 was considered statistically significant for all analyses.

Table 4. Primer pairs for qPCR

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
Wnt1	*GCAACCAAAGTCGCCAGAA*	*TATGTTCACGATGCCCCACCA*
Wnt2b	*GCTACCCAGACATCATGCG*	*ACACTCTCGGATCCATTCCC*
Wnt3	*AATTTGGTGGTCCCTGGC*	*GATAGAGCCGCAGAGCAGAG*
Wnt4	*GTTTCCAGTGGTCAGGATGC*	*AGGACTGTGAGAAGGCTACGC*
Wnt5a	*AAGGGAACGAATCCACGCC*	*ATACTGTCCTGCGACCTGCTTC*
Wnt7a	*CCAAGGTCTTCGTGGATGC*	*TGTAAGTTCATGAGGGTTCGG*
Wnt7b	*CGTGTTTCTCTGCTTTGGC*	*CACCACGGATGACAATGC*
Wnt9a	*GTACAGCAGCAAGTTTGTCAAGG*	*CACGAGGTTGTTGTGGAAGTCC*
Wnt10a	*CGGAACAAAGTCCCCTACG*	*AGGCGAAAGCACTCTCTCG*
Wnt16	*GCACTCTGTAACCAGGTCATGC*	*TGCAAGGTGGTGTCACAGG*
Mki67	*TTCAGTTCCGCCAATCCAAC*	*CCGTGCTGGTTCCTTTCCA*
PORCN	*CCTACCTCTTCCCCTACTTCA*	*CTTTGCGTTTCTTGTTGCGA*
IL-1β	*AATGCCTCGTGCTGTCTG*	*TCCATTGAGGTGGAGAGC*
IL-6	*ATGAAGTTTCTCTCCGCAAG*	*CAACAACATCAGTCCCAAG*
IL-8	*AGCCTTCCTGATTTCTGC*	*AGCACTCCTTGGCAAAAC*
TNFα	*CAGCCGATTTGCCATTTC*	*TCTTGATGGCAGAGAGGAG*
β-Actin	*GTCCACCCGCGAGTACAAC*	*TATCGTCATCCATGGCGAACTGG*

3. Results

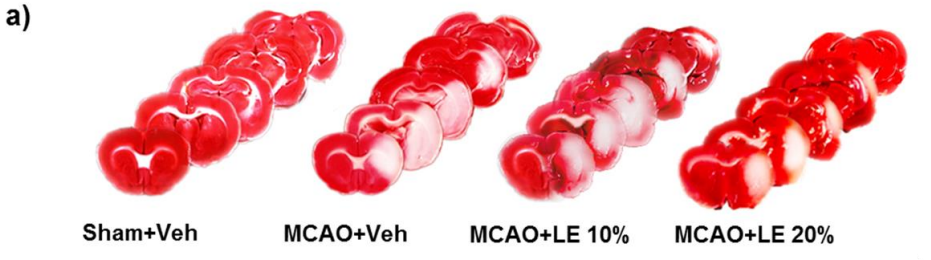
A. Dosage-dependent reduction of infarction volume and behavior by LE

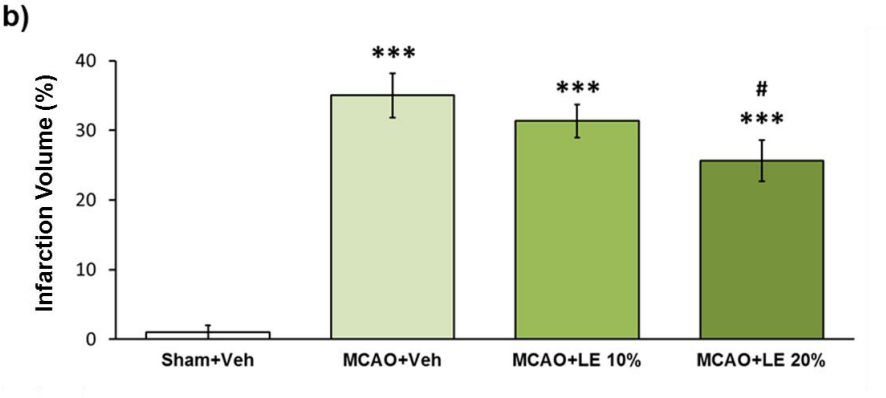
The severity of infarction was visible through the unstained areas of the brain in Figure 12a. The Sham+Vehicle (Veh) group did not show notable infarction. The MCAO+Veh group suffered an injury of

approximately 35% of the left hemisphere. The MCAO+LE 10% group exhibited decrease in infarction volume to about 31%, which was not significant compared to MCAO+Veh group ($p > 0.05$). However, there was a significant reduction in infarction to about 26% in the MCAO+LE 20% group compared to the MCAO+Veh group ($p < 0.05$) (Figure 12b). In behavioral test, all experimental groups suffered a certain degree of behavioral deficit except for the Sham+Veh group. The MCAO+Veh group had an average Bederson score of 3. The MCAO+LE 10% group scored lower in the behavior test and did not differ significantly compared to the MCAO+Veh group ($p > 0.05$). The administration of LE 20% significantly decreased the average Bederson score to approximately 2 ($p < 0.05$). The majority of the MCAO+LE 20% group achieved a Bederson score of 2 or under, while the majority MCAO+Veh group recorded between 2 and 4 (Figure 12c).

B. Wnt/β-catenin-dependent reduction in infarction volume and behavior by LE

The control group was administered with DMSO instead of XAV939. The severity of infarction was visible through the unstained areas of the brain as depicted in Figure 13a. The DMSO+Sham+Veh group did not experience notable infarction. Approximately 33% of the left hemisphere of the DMSO+MCAO+Veh group suffered an injury, while a significant decrease in infarction to about 24% was observed in the DMSO+MCAO+LE 20% group ($p < 0.05$). The XAV939+MCAO+LE 20% failed to protect the brain with approximately 29% infarction volume, which was not significantly different from the DMSO+MCAO+Veh group ($p > 0.05$) (Figure 13b). All experimental groups suffered a certain degree of infarction except for the DMSO+Sham+Veh group. The DMSO+Sham+Veh group did not experience notable neurological deficits. The DMSO+MCAO+Veh group recorded an average Bederson score of 3. The administration of LE 20% decreased the Bederson score to approximately 2 ($p < 0.05$). The XAV939+MCAO+Veh group achieved an average Bederson score of 3, which was similar to the DMSO+MCAO+Veh group ($p > 0.05$) (Figure 13c).





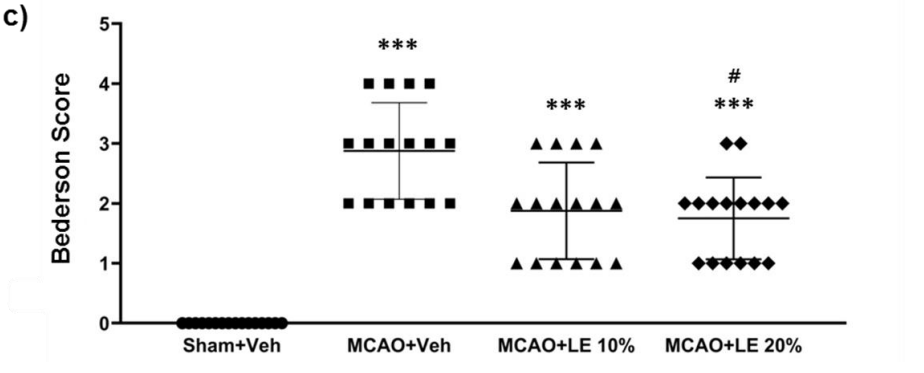


Figure 12. Neuroprotective effects of LE or Veh after the MCAO and reperfusion injury. (**a**) Triphenyltetrazolium chloride (TTC)-stained brain slices for infarction measurement. (**b**) Measurement of infarction volume from TTC staining. (**c**) Bederson scores of the experimental groups. Data are presented as mean ± standard error of the mean (SEM); $n = 16$ for each group; *** $p < 0.001$ vs. Sham+Veh, $^{\#}$ $p < 0.05$ vs. MCAO+Veh. Statistical analysis for the measurement of infarction volume was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Statistical analysis for Bederson scores was performed using Kruskal-Wallis non-parametric test followed by Dunn's post hoc test. MCAO= middle cerebral artery occlusion, Veh= vehicle, LE= lipid emulsion.

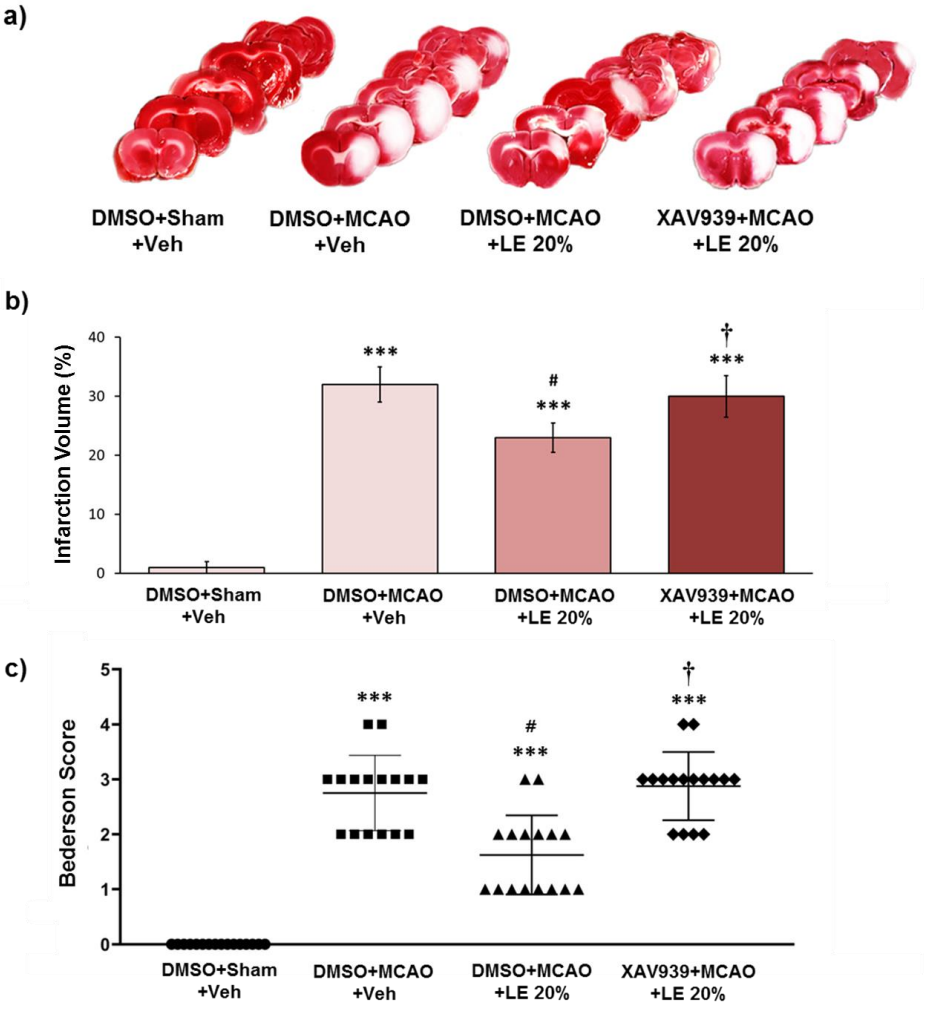


Figure 13. Neuroprotective effects of LE or Veh on the MCAO and reperfusion injury after the administration of DMSO or XAV939. (**a**) TTC-stained brain slices for infarction measurement. (**b**) Measurement of infarction volume by TTC staining. (**c**) Bederson scores of experimental groups. Data are presented as mean ± standard error of the mean (SEM); $n = 16$ for each group; *** $p < 0.001$ vs. DMSO+Sham+Veh, $^{\#}$ $p < 0.05$ vs. DMSO+MCAO+Veh, † $p < 0.05$ vs. DMSO+MCAO+LE 20%. Statistical analysis for the measurement of infarction volume was performed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical analysis for Bederson scores was performed using Kruskal-Wallis non-parametric test followed by Dunn's post hoc test. MCAO= middle cerebral artery occlusion, Veh= vehicle, LE= lipid emulsion, DMSO= dimethyl sulfoxide.

C. Dosage-dependent alleviation of ischemic reperfusion injury through the Wnt/β-catenin signaling pathway and reduction of inflammatory protein markers by LE

The administration of LE affected the protein expression of survival and inflammation-related signals (Figure 14a). The expression of total Akt did not differ among the experimental groups (Figure 14b). The MCAO+Veh group showed significant decrease in phosphorylation of Akt levels (pAkt) compared to the Sham+Veh group ($p < 0.05$). Although the MCAO+LE 10% group exhibited an increase in pAkt levels, such an increase was not statistically significant compared to the MCAO+Veh group ($p > 0.05$). The significantly increased level of pAkt in MCAO+LE 20% group compared to the MCAO+Veh group indicated the increased survival of neurons after ischemic reperfusion injury ($p < 0.05$) (Figure 14c). The ratio of pAkt and Akt was markedly decreased in the MCAO+Veh group compared to the Sham+Veh group ($p < 0.05$). pAkt/Akt appeared to increase expression in a dosage dependent manner in MCAO+LE 10% and MCAO+LE 20% groups; however the MCAO+LE 20% group was significantly different from the MCAO+Veh group ($p < 0.05$) (Figure 14d).

Significant decrease in total GSK-3β expression was observed in the MCAO+Veh group compared to the Sham+Veh group ($p < 0.05$). The MCAO+LE 10% group showed substantial increase in GSK-3β levels but this increment was not statistically significant compared to the MCAO+Veh group ($p > 0.05$). The total GSK-3β expression of the MCAO+LE 20% was also significantly increased compared to the MCAO+Veh group ($p < 0.05$) (Figure 14e). The phosphorylation of GSK-3β (pGSK-3β) was significantly decreased in the MCAO+Veh

group compared to the Sham+Veh group ($p < 0.01$), which might be indicating a diminished Wnt activity. The phosphorylation of GSK-3β was significantly increased in the MCAO+LE 10% ($p < 0.05$) and MCAO+LE 20% ($p < 0.001$) group when compared to the MCAO+Veh group. There was also a significant increase in pGSK-3β in the MCAO+LE 20% group compared to the Sham+Veh group ($p < 0.05$), thereby indicating an increased activity of the Wnt/β-catenin signaling pathway (Figure 14f). The pGSK-3β/GSK-3β activity exhibited marked decrease in the MCAO+Veh group compared to the Sham+Veh group ($p < 0.05$). The pGSK-3β/GSK-3β expression for MCAO+LE 10% did not differ significantly compared to the MCAO+Veh group ($p > 0.05$). pGSK-3β/GSK-3β activity significantly increased in the MCAO+LE 20% group compared to the MCAO+Veh group ($p < 0.05$) (Figure 14g).

Wnt1, a canonical Wnt signal and upstream marker of GSK-3β, was significantly decreased in the MCAO+Veh group compared to the Sham+Veh group ($p < 0.05$) and significantly increased in the MCAO+LE 20% group compared to the MCAO+Veh group ($p < 0.05$) (Figure 14h). All experimental groups ($p < 0.05$), excluding the Sham+Veh group, displayed robust decrease in the neurogenesis marker, Wnt3. Although Wnt3 is one of canonical Wnt signals, it does not seem to affect the Wnt/β-catenin signaling pathway induced by LE (Figure 14i). Porcupine (PORCN), a key regulator of Wnt proteins,[53] decreased significantly in the MCAO+Veh group compared to the Sham+Veh group ($p < 0.05$). PORCN expression increased in the MCAO+LE 20% group compared to the MCAO+Veh group, implying elevated activity in the modulation of Wnt proteins (Figure 14j). The downstream survival marker of GSK-3β, β-catenin, was decreased in all MCAO injury groups compared to the Sham+Veh group due to infarction. However, β-catenin was significantly preserved in the MCAO+LE 20% group compared to the MCAO+Veh group ($p < 0.05$) (Figure 14k). Phosphorylation of β-catenin (pβ-catenin) was increased significantly in the MCAO+Veh group compared to the Sham+Veh group ($p < 0.05$), indicating an elevated level of β-catenin degradation. The MCAO+LE 20% group had a significantly lower expression level of pβ-catenin compared to the MCAO+Veh group ($p < 0.05$), which supported the survival of cells (Figure 14l). The expression level of tankyrase 1 was significantly increased in the MCAO+Veh group compared to the Sham+Veh group ($p < 0.05$), indicating the accumulation of tankyrase 1 through increased axis inhibition protein (AXIN) stabilization for β-catenin degradation.

The MCAO+LE 20% group had a significantly lower expression level of tankyrase 1 compared to the MCAO+Veh group ($p < 0.05$) (Figure 14m).

Inflammatory protein markers of ischemic reperfusion damage, IL-1β, IL-6, IL-8 and TNF-α,[54,55] increased significantly for all MCAO-injured groups when compared to the Sham+Veh group. However, attenuated levels of inflammatory markers were observed in the MCAO+LE 20% group compared to the MCAO+Veh group ($p < 0.05$), indicating the reduction of ischemic reperfusion injury (Figure 14n–q).

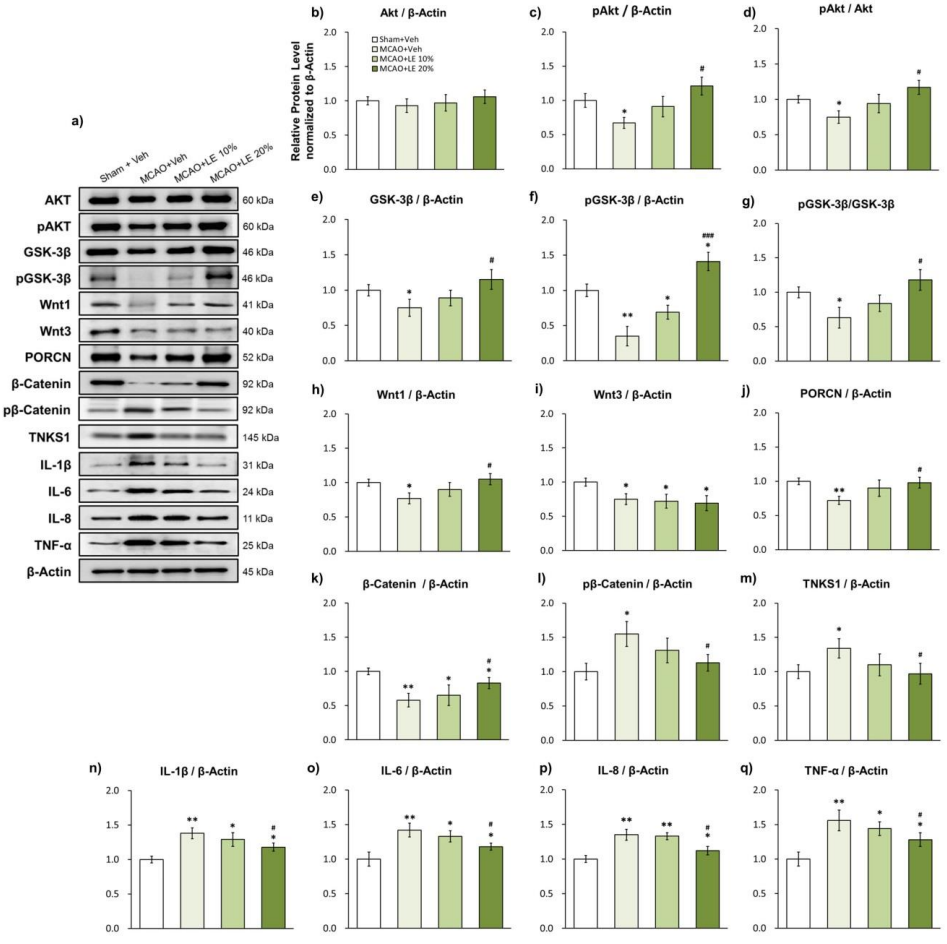


Figure 14. Effects of LE or Veh on protein expressions after MCAO and reperfusion injury. (**a**) Representative Western blots indicating the expression of specific proteins in the peri-infarct region of the left hemisphere. (**b**–**d**) Expression and phosphorylation of Akt in the experimental groups. (**e**–**g**) Expression and phosphorylation levels of GSK-3β (pGSK-3β) in the experimental groups. (**h**–**m**) Wnt signal-related protein expressions of experimental groups. (**n**–**q**) Inflammatory protein expressions of experimental groups. Data are presented as mean ± standard error of the mean (SEM); $n = 8$ for each group; * $p < 0.05$, ** $p < 0.01$ vs. Sham+Veh, $^{\#}$ $p < 0.05$, $^{\#\#\#}$ $p < 0.001$ vs. MCAO+Veh. MCAO= middle cerebral artery occlusion, Veh= vehicle, LE= lipid emulsion.

D. Wnt/β-catenin-dependent alleviation of ischemic reperfusion injury and reversal of protection-related proteins by LE

The administration of XAV939 inhibited the activity of the Wnt/β-catenin signaling pathway, which was reflected in the protein expression (Figure 15a) of distinct genes. The expression of total Akt did not differ among the experimental groups (Figure 15b). In the DMSO+MCAO+Veh and XAV939+MCAO+LE 20% groups, substantial decreases in pAkt levels were shown when compared to the DMSO+Sham+Veh group ($p < 0.05$). However, there was significant increase in the level of pAkt in DMSO+MCAO+LE 20% group compared to the DMSO+MCAO+Veh group ($p < 0.05$) and XAV939+MCAO+LE 20% group ($p < 0.01$), respectively (Figure 15c). The pAkt/Akt expression decreased significantly in DMSO+MCAO+Veh and XAV939+MCAO+LE 20% groups, compared to the DMSO+Sham+Veh group ($p < 0.05$). The expression of pAkt/Akt in the DMSO+MCAO+LE 20% group was significantly increased compared to DMSO+MCAO+Veh and XAV939+MCAO+LE 20% groups ($p < 0.05$) (Figure 15d).

Significant decrease in total GSK-3β expression was observed in the DMSO+MCAO+Veh and XAV939+MCAO+LE 20% groups when compared to the DMSO+Sham+Veh group ($p < 0.05$). Total GSK-3β expression of the DMSO+MCAO+LE 20% group was also significantly increased compared to the DMSO+MCAO+Veh and XAV939+MCAO+LE 20% groups ($p < 0.05$), respectively (Figure 15e). The level of pGSK-3β was significantly decreased in the DMSO+MCAO+Veh and XAV939+MCAO+LE 20% groups compared to the DMSO+Sham+Veh group ($p < 0.01$), which might be indicative of a compromised Wnt activity. The phosphorylation of GSK-3β was significantly increased in the DMSO+MCAO+LE 20% group compared

to the DMSO+Sham+Veh group ($p < 0.05$). There was also a significant increase in the pGSK-3β levels in the DMSO+MCAO+LE 20% group compared to the DMSO+MCAO+Veh and XAV939+MCAO+LE 20% groups ($p<0.001$) (Figure 15f). The pGSK-3β/GSK-3β activity was significantly decreased in the DMSO+MCAO+Veh and XAV939+MCAO+LE 20% groups when compared to the DMSO+Sham+Veh group ($p < 0.05$) and significantly increased in the DMSO+MCAO+LE 20% group compared to the DMSO+MCAO+Veh and XAV939+MCAO+LE 20% groups ($p < 0.05$). The administration of XAV939 effectively decreased the phosphorylation GSK-3β induced by LE (Figure 15g).

Wnt1 was significantly decreased in the DMSO+MCAO+Veh and XAV939+MCAO+LE 20% groups compared to the DMSO+Sham+Veh group ($p < 0.05$) and significantly increased in the DMSO+MCAO+LE 20% group compared to the DMSO+MCAO+Veh and XAV939+MCAO+LE 20% groups ($p < 0.05$) (Figure 15h). All experimental groups, excluding the DMSO+Sham+Veh group, showed steep decrease ($p < 0.05$) in Wnt3 (Figure 15i). PORCN decreased significantly in the DMSO+MCAO+Veh group ($p < 0.01$) and XAV939+MCAO+LE 20% group ($p < 0.05$) compared to the DMSO+Sham+Veh group. PORCN expression increased in expression in the DMSO+MCAO+LE 20% group compared to the DMSO+MCAO+Veh and XAV939+MCAO+LE 20% groups ($p < 0.05$) (Figure 15j). β-catenin was decreased in all MCAO injury groups compared to the DMSO+Sham+Veh group due to infarction. Significantly attenuated level of β-catenin was also observed in the XAV939+MCAO+LE 20% group, while DMSO+MCAO+LE 20% group were preserved significantly compared to DMSO+MCAO+Veh group ($p < 0.05$). Average β-catenin expression decreased in the XAV939+MCAO+LE 20% group compared to the DMSO+MCAO+LE 20% group but not to a significant level ($p > 0.05$) (Figure 15k). pβ-Catenin was increased in the DMSO+MCAO+Veh group compared to the DMSO+Sham+Veh group ($p < 0.05$), indicating lowered cellular survival. The level of pβ-catenin was significantly decreased in the DMSO+MCAO+LE 20% group compared to the DMSO+MCAO+Veh group ($p < 0.05$). The pβ-catenin expression level was significantly increased in the XAV939+MCAO+LE 20% group compared to the DMSO+MCAO+LE 20% group ($p < 0.05$) (Figure 15l). The accumulation of tankyrase 1 was significant in the DMSO+MCAO+Veh compared to DMSO+Sham+Veh ($p < 0.05$). The tankyrase 1 expression

of DMSO+MCAO+LE 20% group significantly decreased compared to the DMSO+MCAO+Veh group ($p < 0.05$). The XAV939+MCAO+LE 20% group had significantly higher levels of tankyrase 1 expression levels compared to the DMSO+MCAO+LE 20% group ($p < 0.05$). The administration of XAV939 successfully inhibited tankyrase 1 activity which increased degradation of β-catenin (Figure 15m).

Inflammatory protein markers of ischemic reperfusion damage, IL-1β, IL-6, IL-8 and TNF-α, also significantly increased for all MCAO-injured groups compared to the DMSO+Sham+Veh group. Attenuated levels of inflammatory markers were observed in the DMSO+MCAO+LE 20% group compared to the DMSO+MCAO+Veh group ($p < 0.05$). The expression levels of the XAV939+MCAO+LE 20% group were not significantly different from the DMSO+MCAO+Veh group ($p > 0.05$) The TNF-α expression level of the XAV939+MCAO+LE 20% group increased significantly compared to the DMSO+MCAO+LE 20% group ($p < 0.05$) (Figure 15n–q).

E. Dosage-dependent mRNA expression against ischemic reperfusion injury by LE

According to the results of qPCR, *Wnt1* can be implicated as one of the main regulators for neuroprotection. In the MCAO+Veh group, *Wnt1* expression level was approximately 0.4 folds compared to the Sham+Veh group ($p < 0.05$). The mRNA expression of *Wnt1* signals of MCAO+LE 20% was upregulated by approximately 2.4 folds compared to the Sham+Veh group ($p < 0.01$) and 4 folds compared to the MCAO+Veh group ($p < 0.001$) (Figure 16a). *Wnt3* expression was attenuated in all MCAO-injured groups compared to the Sham+Veh group. The MCAO+LE 10% ($p < 0.05$) and MCAO+LE 20% groups' ($p < 0.001$) *Wnt3* expression levels were significantly decreased compared to the MCAO+Veh group ($p < 0.05$) (Figure 16b). *Mki67*, a cell proliferation marker, increased in all MCAO-injured groups compared to the Sham+Veh group. The *Mki67* expression level increased significantly in the MCAO+LE 20% group compared to the MCAO+Veh group ($p < 0.05$), which may have been affected by the elevated expression level of *Wnt1* (Figure 16c). The *Wnt* regulator, *PORCN*, decreased significantly in the MCAO+Veh group compared to the Sham+Veh group ($p < 0.05$). Significant increase of *PORCN* was observed in the MCAO+LE 20% group compared to the MCAO+Veh group ($p < 0.05$), which may be induced by the increase in *Wnt1* activity (Figure 16d).

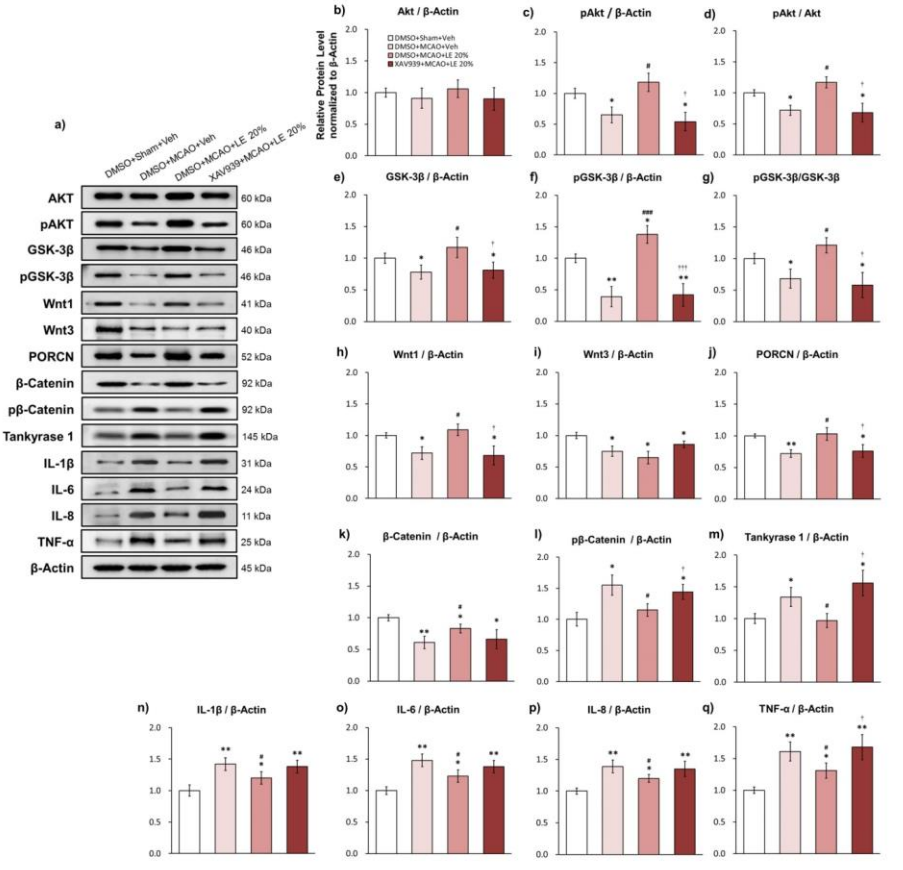


Figure 15. Effects of LE or Veh on protein expression on the MCAO and reperfusion injury after the administration of DMSO or XAV939. (**a**) Representative Western blots of proteins in the peri-infarct region of the left hemisphere. (**b**–**d**) Levels of Akt and pAkt in the experimental groups. (**e**–**g**) Levels of GSK-3β and pGSK-3β in the experimental groups. (**h**–**m**) Wnt signal-related protein expressions of experimental groups. (**n**–**q**) Inflammatory protein expressions in the experimental groups. Data are presented as mean ± standard error of the mean (SEM); $n = 8$ for each group; * $p < 0.05$, ** $p < 0.01$ vs. DMSO+Sham+Veh, $^{\#}$ $p < 0.05$, $^{\#\#\#}$ $p < 0.001$ vs. DMSO+MCAO+Veh, † $p < 0.05$, ††† $p < 0.001$ vs. DMSO+MCAO+LE 20%. MCAO= middle cerebral artery occlusion, Veh= vehicle, LE= lipid emulsion, DMSO= dimethyl sulfoxide.

Inflammatory markers, *IL-1β*, *IL-6*, *IL-8 and TNF-α*, significantly increased in MCAO-injured groups compared to the Sham+Veh group. Significantly lower inflammatory mRNA markers were observed in the MCAO+LE 20% group compared to the MCAO+Veh group ($p < 0.05$) (Figure 16e–h).

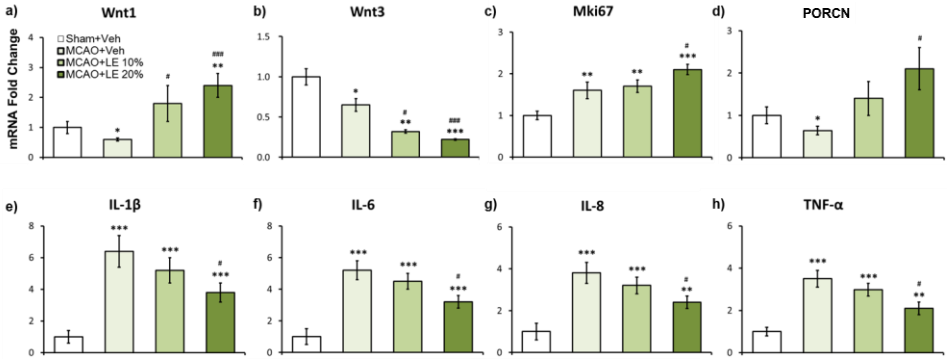


Figure 16. Effects of LE or Veh on mRNA expression after the MCAO and reperfusion injury. (**a**–**b**) *Wnt* expressions in experimental groups. (**c**) *Mki67* expression was increased in all MCAO-injury groups compared to the Sham+Veh group. (**d**) *PORCN* expression. (**e**–**h**) mRNA expression of inflammatory markers. Data are presented as mean ± standard error of the mean (SEM); $n = 8$ for each group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Sham+Veh, $^{\#}$ $p < 0.05$, $^{\#\#\#}$ $p < 0.001$ vs. MCAO+Veh. MCAO= middle cerebral artery occlusion, Veh= vehicle, LE= lipid emulsion.

F Wnt-dependent mRNA expression of LE against ischemic reperfusion injury

XAV939 injection inhibited *Wnt* activity in mRNA levels to reverse neuroprotection. In the DMSO+MCAO+Veh group ($p < 0.05$) and XAV939+MCAO+LE 20% group, *Wnt1* mRNA expression levels were approximately 0.5 folds and 0.6 folds compared to the DMSO+Sham+Veh group, respectively. Expression of *Wnt1* signals in the DMSO+MCAO+LE 20% was upregulated by approximately 2.2 folds compared to the DMSO+Sham+Veh group ($p < 0.01$) and 4.2 folds compared to the DMSO+MCAO+Veh group ($p < 0.001$). There was a significant decrease in *Wnt1* expression in the XAV939+MCAO+LE 20% group compared to the DMSO+MCAO+LE 20% group ($p < 0.01$) (Figure 17a). *Wnt3* expression was attenuated in all MCAO-injured groups compared to the DMSO+Sham+Veh group. The DMSO+MCAO+LE 20% group's *Wnt3* expression was significantly decreased compared to the DMSO+MCAO+Veh group ($p < 0.001$). The

XAV939+MCAO+LE 20% group also showed significant decrease in *Wnt3* expression level compared to the DMSO+Sham+Veh group ($p < 0.01$) but displayed marked increase when compared to the DMSO+MCAO+LE 20% group ($p < 0.001$) (Figure 17b). *Mki67* levels increased in all MCAO-injured groups compared to the DMSO+Sham+Veh group. In the DMSO+MCAO+LE 20% group, *Mki67* expression level increased significantly compared to the DMSO+MCAO+Veh group ($p < 0.05$), while, in the XAV939+MCAO+LE 20% group, it was significantly downregulated compared to the DMSO+MCAO+LE 20% group ($p < 0.05$) (Figure 17c). *PORCN* decreased significantly in the DMSO+MCAO+Veh group ($p < 0.05$) and XAV939+MCAO+LE 20% group compared to the DMSO+Sham+Veh group. Significant increase of *PORCN* was observed in the DMSO+MCAO+LE 20% group when compared to the DMSO+MCAO+Veh group ($p < 0.05$). The XAV939+MCAO+LE 20% group decreased in *PORCN* compared to the DMSO+Sham+Veh group but did not differ significantly. The XAV939+MCAO+LE 20% group expression levels of *PORCN* were significantly decreased compared to the DMSO+MCAO+LE 20% group ($p < 0.05$) (Figure 17d). Inflammatory markers, *IL-1β, IL-6, IL-8 and TNF-α,* significantly increased in MCAO-injured groups compared to the DMSO+Sham+Veh group. Significantly lower inflammatory mRNA markers were observed in the DMSO+MCAO+LE 20% group compared to the DMSO+MCAO+Veh group ($p < 0.05$). XAV939 injection significantly reversed attenuated levels of inflammation by LE 20% (Figure 17e–h).

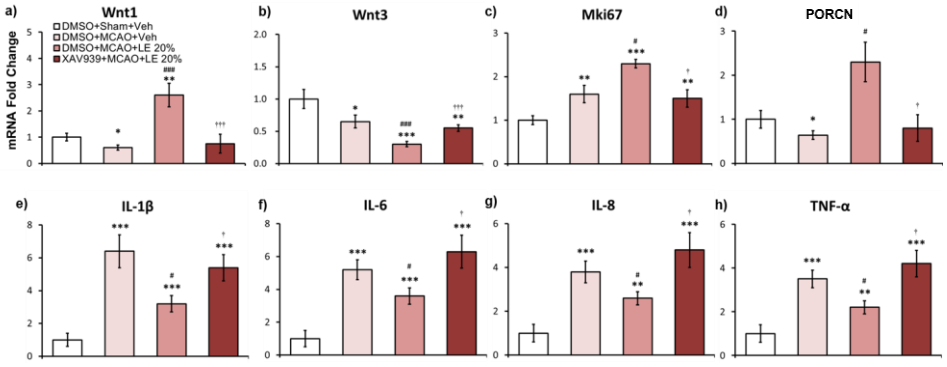


Figure 17. Effects of LE or Veh on mRNA expression on the MCAO and reperfusion injury after the administration of DMSO or XAV939. (**a**–**b**) *Wnt* expressions in experimental groups. (**c**) *Mki67* expression. (**d**) *PORCN* expression. (**e**–**h**) The mRNA expression of inflammatory markers. Data are presented as mean ± standard error of mean (SEM); $n = 8$ for each group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. DMSO+Sham+Veh, $^{\#}$ $p < 0.05$, $^{\#\#\#}$ $p < 0.001$ vs. DMSO+MCAO+Veh, † $p < 0.05$, ††† $p < 0.001$ vs. DMSO+MCAO+LE 20%. MCAO= middle cerebral artery occlusion, Veh= vehicle, LE= lipid emulsion, DMSO= dimethyl sulfoxide.

IV. DISCUSSION

The results of studies 1 and 2 suggest that LE provides neuroprotection against neural damage in a dose-dependent manner. In study 1, the concentration of LE 1% was more effective at providing neuroprotection in the hippocampus than LE 0.01%. The administration of LE also lowered mortality induced by KA nearly to a significant level (Figure 4). Many animals of the KA+Veh group showed difficulty in maintaining a healthy status. On the other hand, stabilized behavior was observed in most animals of the KA+LE 1% group, which may be due to the partial alleviation of neurotoxicity. Lower concentrations were less effective than the higher dose when administered locally. A previous study reported that intraperitoneal injections of higher doses did not seem to alter brain state,[56] but differed in intrahippocampal injections from the present study. The level of *Wnt1* mRNA expression was up-regulated at 24 hrs after KA+LE injection, but this returned to baseline or lower at 72 hrs after KA+LE injection. These results suggest that LE triggers neuroprotection at the acute phase of excitotoxicity.

Memory and anxiety levels were protected in the LE 1%-treated group when compared to the LE 0.01%-treated group. Despite the loss of neurons in the CA3 region, protection in CA1 was sufficient to secure hippocampal function. However, it is important to note that the behavioral tests were not heavily influenced by CA3 impairment. The CA3 region is involved in the processing of spatial memory,[57] and is affected by convulsive seizures accompanied by KA administration.[43,47] A previous study reported that synaptic silencing of the CA3 region significantly reduced seizures and cell death in that particular region.[58] Although LE does not possess known tranquilizing components for relieving seizures, accumulating evidences suggest that LE aids the survival of neurons in excitotoxic conditions. In a previous study, the protective effects of LE and propofol were measured by intracerebroventricular microinjections into the brains of rats with ischemia. LE significantly reduced the level of extracellular glutamate in the CA1 region during ischemia,[59] suggesting that LE may be an important factor against excitotoxicity. These results indicate that hippocampal cells in the CA1 region were significantly protected by LE. The attenuation of anxiety-like behavior, known to involve CA1,[60,61] was also consistent with the dose-dependent protective effects of LE in the behavioral tests. These findings indicate that LE provides neuroprotection in the hippocampus.

The Wnt signaling pathway has received attention as a therapeutic target for neurodegenerative diseases across numerous studies based on upregulated antagonistic activity in pathological conditions.[62,63] Promoting Wnt activation through the inhibition of antagonists resulted in significant recovery from pathological conditions.[20,64] Wnt1 is an upstream signal of GSK3-β that was up-regulated in LE-treated groups, supporting cell survival through the regulation of β-catenin. The preservation of β-catenin through the phosphorylation of GSK3-β has been reported as a consequence of canonical Wnt signaling.[19] Although the effects of LE on GSK3-β and Akt have not been clearly elucidated, the increased levels of these signals indicate an interaction induced by LE. One possible explanation for the initiation of Wnt may involve the lipid modification of Wnt1. Wnts are glycoproteins that are modified by glycosylation and lipids through palmitoylation partly by PORCN. Such modifications can trigger cell survival mechanisms in the pathological state of excitotoxicity. Protective effects of LE in pathological conditions increased *Wnt1* mRNA expression levels according to findings of this study. The downstream protection related protein markers, Akt and GSK3-β, was consistent with previous studies in myocardial cells.[29,65] Although metabolic factors differ between neurons and cardiomyocytes, it is notable that survival signals were activated regardless. Also, Wnt3 was attenuated in LE-treated groups, implying that not all canonical Wnts displayed uniform trends in the levels of expression for neuroprotection against excitotoxicity. Canonical Wnt3 signal has been reported to be associated with neurogenesis in the hippocampus.[66] The expression of Wnt3 may have persisted at a low level in the studies 1 and 2, as KA has been reported to encompass aberrant neurogenesis in the hippocampus at chronic but not acute phases of its pathology.[67] A possible explanation for the significantly lower expression levels of Wnt3 in LE-treated groups may be related to the survival of CA1 neurons. Protection provided to CA1 neuronal populations may have affected inflammatory responses that triggered gliosis surrounding the damaged area. On the other hand, the non-canonical *Wnt5a* has been associated with configuration of postsynaptic compartments and cellular structures.[68,69] The difference in *Wnt5a* expression was insignificant between KA+Veh and LE-treated groups, indicating minimal differences in structures affected by non-canonical signaling.

In study 2, LE provided neuroprotection against ischemic reperfusion injury in a dosage-dependent manner. Intra-arterial injection of LE 20% during reperfusion after MCAO was able to reduce infarction

volume significantly. The decreases in infarction volumes have been reflected with improved performance in the neurological deficit assessment. Rats injected with LE 20% demonstrated better control over their paralyzed limb. Increased levels of Wnt1 signaling in both protein and mRNA levels in LE 20%-treated rats were observed, which in turn enhanced the resistance to reperfusion injury following ischemia. Especially, a notable increase in *Wnt1* mRNA expression was observed, which may have led to increased Wnt1 protein expression. The elevated levels of Wnt1 are consistent with previous studies that have indirectly stimulated Wnt/β-catenin signaling pathway for cell survival after cerebral ischemic injury.[25,70] In addition, the phosphorylation of GSK-3β increased by LE 20% injection, which inactivated the β-catenin destruction complex. β-catenin was preserved to promote cell survival, which resulted in enhanced resistance to the ischemic reperfusion damage. Elevation of PORCN expression may have been due to the increase in demand for Wnt1 to resist damage; however, a clear link between PORCN and LE cannot be formulated at this stage. A previous study on the ischemic reperfusion injury in the heart has reported that palmitic acid of LE affects the lipid-modification of Wnt ligands for protection.[71] Clear distinctions between cardiac cells and neurons exist, but Wnt modifications seem to occur regardless of their metabolic factors. Inflammatory markers, IL-1β, IL-6, IL-8, and TNF-α, are known to increase after ischemic reperfusion injury.[54,55] Inflammation by oxidative stress was significantly decreased in the LE 20%-treated group, indicating an anti-inflammatory action of LE in the central nervous system.

In order to verify the protective mechanism of LE, which increased Wnt1, the protective process needed to be interrupted. XAV939, a Wnt/β-catenin signaling pathway inhibitor, has been commonly utilized to inhibit Wnt activity in many studies.[72,73] In study 2, the i.p. injection of XAV939 successfully inhibited Wnt activity and reversed the protective effects induced by LE. The XAV939+MCAO+LE 20% group exhibited infarction volumes nearly as large as the control, the DMSO+MCAO+Veh group. Akin to studies 1 and 2, the results show that the Wnt/β-catenin signaling pathway might be a potential therapeutic target for neuroprotection. The administration of XAV939 inhibited the mRNA expression level of *Wnt1*, indicating that *Wnt1* may be dependent on the activity of GSK-3β. XAV939 is known to inhibit tankyrase 1, which prevents the degradation of axis inhibition protein 2 (AXIN2), allowing the accumulation of the GSK-3β destruction complex.[74]

Therefore, pGSK-3β was attenuated in the XAV939+MCAO+LE 20% group, which reversed the protective effect of LE by the degradation of the downstream survival marker, β-catenin. Implications can be made that LE has effects on the upstream signals of GSK-3β and not on the downstream signals of GSK-3β (Figure 18). If LE affected the GSK-3β downstream marker, β-catenin, XAV939 might not have affected the protection effect. In addition, if LE affected GSK-3β directly, a less effective or partial reversal of protection may have occurred. Considering that the infarction damage was not significantly different between the DMSO+MCAO+Veh group and XAV939+MCAO+LE 20% group, implying an effective reversal from the effects of LE 20% injection.

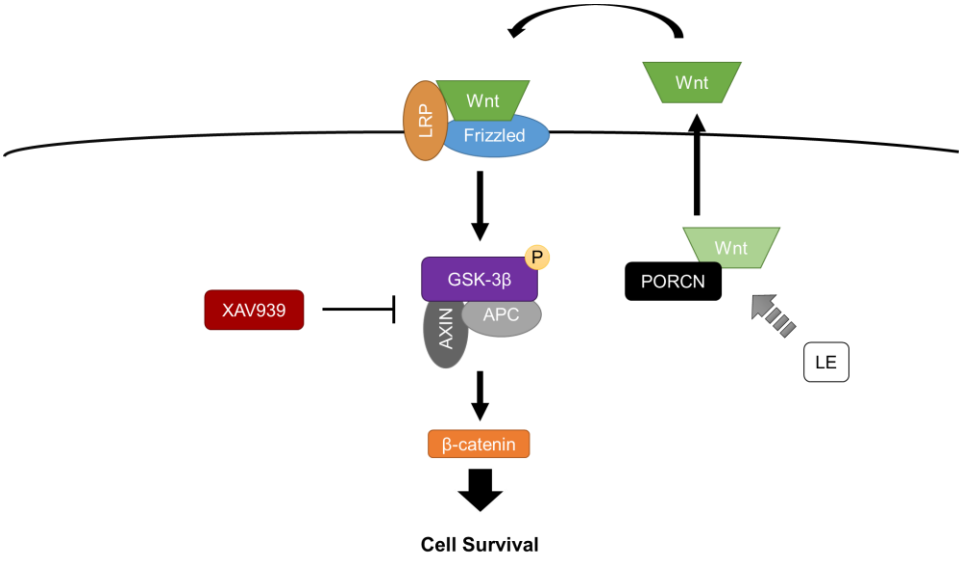


Figure 18. Schematic diagram of the protective mechanism of LE in ischemic reperfusion injury. LE increases the lipid modification of Wnt proteins by PORCN. Lipid-modified/active Wnt proteins are released to the extra-cellular space for the binding to Frizzled/LRP receptors. The activated receptors induce the phosphorylation of GSK-3β, which prevents the destruction complex from the degradation of β-catenin by proteasomes. Preserved levels of β-catenin lead to cell survival. The injection of XAV939 prevents the phosphorylation of GSK-3β, which allows β-catenin degradation for cell death. LE=lipid emulsion, PORCN= porcupine, Wnt= wingless integration, LRP= low-density lipoprotein receptor-related protein, GSK-3β= glycogen synthase kinase 3β, APC= adenomatous polyposis coli, AXIN= axis inhibition protein.

Although significant neuroprotection was observed through the outcomes of these studies, there are some limitations. Studies 1 and 2 focused on the local effects of LE in the brain and observed neuroprotective properties by direct injection or intra-arterial injection. Moreover, systemic effects of LE in the brain are at question. The local injection or intra-arterial administration of LE in clinical settings may lack practicality before it has been compared with other routes of administration. Dosage screening would need to be accompanied for different routes of administration for the search of effective concentrations. In a previous study, the treatment of LE 16% to the heart in a recirculation condition mildly depressed contractile function by approximately 20% implying that LE consists of toxicity in higher concetrations.[75] In study 1, cell death was observed starting from LE 8% in the dosage screen using organotypic hippocampal slice cultures (Figure 2) indicating that the brain may be relatively more sensitive to the concentration of LE compared to the heart. The exposure to excess amounts of LE may have adverse effects in the brain. Therefore, further studies are required to search for appropriate doses for different routes of administration.

Intravenous injection is a possible route that can examine the systemic effects of LE. In clinical settings, a few studies have studied intravenous injections of LE against neurological disorders. Intravenous injections of LE initiated the ketogenic diet prior to utilizing the enteral route to reduce severe refractory status epilepticus in an 8 year old child.[76] In another study, acute ischemic stroke patients were treated with intravenous injections of LE which served as a placebo for the experiment. LE was effective in reducing the National Institutes of Health Stroke Scale score of these patients along with their tested drug, dodecafluoropentane emulsion.[77] LE was also reported effective in a failed clinical trial regarding the treatment of progesterone on patients suffering from traumatic brain injury. The authors have mentioned that the clinical trial faced difficulties in finding significant differences between their experimental groups and control group due to the effectiveness of the vehicle chemical, LE.[78]

Lipids are known to be metabolized by the glial population in the brain which may bring change to glial activity. Studies regarding the activity of glial cells such as astrocytes and microglia may be required to better elucidate the neuroprotective process. Increased levels of *Mki67*, a cell proliferation marker which is also influenced directly by the

canonical Wnt signaling pathway,[79] was measured in LE-treated groups at 24 hrs after KA administration. Furthermore, *Mki67* was increased by neuroprotection against ischemic reperfusion injury while inflammatory markers were decreased in study 2. Such results indicate that roles of astrocytes may be important since microglial activity is assumed to be reduced due to decreased inflammatory action.

In addition, further studies regarding other signals that are involved in the Wnt/β-catenin signaling pathway, such as frizzled-1, low-density lipoprotein receptor-related protein 5/6, or PORCN, that can be subjected for inhibition are required. Study 1 have investigated the upregulation of Wnt-related signals however have not verified the mechanism through inhibition of related factors. Elucidation of specific signals may lead to more effective therapeutic targets in the brain. Also for study 2, behavior tests regarding motor functions may be additionally required to fully validate the degree of protection that LE provides. Study 2 utilized the Bederson scale to measure the degree of neurological deficits, but additional tests such as the rota-rod test may further verify behavioral dysfunctions. In regards to the assessment of cognitive functions, behavior tests that do not demand much locomotive activity such as the passive avoidance test may be appropriate for evaluating cognitive abilities of rats that have gone through ischemic reperfusion injury. Concerns may arise on behavior tests regarding cognitive functions that demand high levels of motor activity such as the Morris water maze test. Rats that have gone through the MCAO surgery may not have the adequacy to complete the given tasks such as swimming or moving across long platforms in a short amount of time.

V. CONCLUSION

In conclusion, LE administration resulted in alleviation of hippocampal damage in the acute phase of excitotoxicity induced by KA and ischemic reperfusion injury in the model of MCAO. The results for study 1 were consistent with previous studies regarding the phosphorylation of Akt[27] and GSK3-β[29] through LE administration in the heart. Furthermore, the aforementioned survival markers as part of the canonical Wnt signaling pathway validated. In particular, *Wnt1* mRNA expression levels were significantly increased, and β-catenin was preserved from degradation, thereby promoting the survival of neurons under excitotoxic conditions. Neuroprotection provided by LE significantly reduced the exacerbation of cognitive function and anxiety induced by KA through protection of the hippocampal CA1 region. For study 2, the intra-arterial administration of LE 20% alleviated ischemic reperfusion injury induced by MCAO and reperfusion. Infarction volumes and Bederson scores were attenuated in a dosage-dependent manner. Protein and mRNA expression levels of the Wnt/β-catenin signaling pathway were elevated and inflammatory markers decreased significantly in the LE 20%-treated group. Especially, GSK-3β was phosphorylated significantly, which in turn preserved β-catenin to promote cell survival. The reversal of protective actions by XAV939 indicates that the protection mechanism of LE has been induced through the Wnt/β-catenin signaling pathway. These findings regarding the anti-inflammatory and neuroprotective properties of LE may provide a foundation for further investigation of therapies for brain protection.

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ABSTRACT(IN KOREAN)

뇌손상 동물 모델에서 지질 유상액의 뇌 보호 효과

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최근 연구에 의하면, 지질 유상액 (lipid emulsion)은 심장에서 허혈성 재관류 손상에 의한 심근세포 사멸을 감소시킨다. 신경세포와 심근세포 사이에는 흥분성과 전도성 등의 유사성이 있는데, 특히 신경세포는 흥분성 상태에 취약한 것으로 알려져 있다. 그러나 중추신경계에서 지질 유상액의 보호 효과에 대한 연구는 부족하다. 따라서 본 연구에서는 지질 유상액의 신경 보호 효과를 규명하고자 하였다.

본 연구에서는 두 가지 실험을 시행하였다. 첫 번째 실험은 카인산 (kainic acid)에 의한 흥분 독성이 지질 유상액에 의해 완화되는지 규명하는 것을 목표로 하였다. 이를 검증하기 위해, 실험쥐 (Sprague Dawley)의 해마에 카인산을 주입한 후 Racine scale을 사용하여 발작 정도를 측정하였다. 3단계 이상에 해당하는 발작을 일으키지 않은 쥐는 실험에서 제외하였다. 벤조디아제핀을 주입하여 발작을 진정시킨 후, 지질 유상액 또는 vehicle을 쥐의 해마에 투여했다. 각 실험군의 생존율은 log-rank 검사를 사용하여 분석하였다. 그리고 행동 평가를 위해 수동회피 검사 (passive avoidance test) 및 십자형 상승 미로

(elevated plus maze) 실험을 수행하였다. 이후, 조직학적 분석을 위해 cresyl violet과 Fluorojade-C 염색을 하였다. 또한, 분자적인 기전을 규명하기 위하여 western blotting과 quantitative Polymerase Chain Reaction (qPCR) 실험을 실시하였다. 이 실험에서는 쥐에게 카인산을 주입한 후 24시간 혹은 72시간이 경과한 다음에 뇌 조직을 추출하였다. 실험 결과, 1% 지질 유상액 처리 집단에서 생존율이 증가하는 경향을 보였으며 불안 증상이 개선되었고, 학습과 기억 능력이 향상되었다. 해마의 CA1영역에서 신경퇴행이 현저하게 감소하였고 세포 생존은 증가하였다. 지질 유상액은 주입 농도에 따라 β-카테닌의 단백질 발현과 GSK3-β와 Akt의 인산화 발현량의 차이를 보였다. 또한 Vehicle을 주입한 집단보다 지질 유상액을 주입한 집단에서 Wnt1 mRNA 발현량이 높아진 것을 확인하였다. 위의 결과들을 종합해 보았을 때, 지질 유상액은 canonical Wnt 신호 전달 체계를 변화시킴으로써 카인산에 의해 유도된 신경세포의 흥분독성을 완화시키고 신경세포를 보호하는 기능을 수행함을 알 수 있었다.

두 번째 실험은 중대뇌동맥폐색 (middle cerebral artery occlusion)을 통한 허혈성 재관류 손상 (ischemia reperfusion injury) 동물 모델에서 지질 유상액의 신경 보호 효과를 규명하는 것을 목표로 했다. 이를 위해, 실험쥐에게 중대뇌동맥폐색 시술을 한 후 재관류 과정에서 동맥내 주사를 통해 지질 유상액을 투여하였다. 신경퇴행 정도를 측정하기 위해, 염화 삼페닐테트라졸륨 (triphenyltetrazolium chloride) 염색, western blotting, 그리고 qPCR 실험을 실시하였다. 실험 결과, 신경퇴행 정도는 지질 유상액의 농도에 영향을 받는 것으로 나타났다. 특히 20% 지질 유상액을 주입한 쥐는 경색량이 현저히 감소하였으며 또한 Bederson 점수도 낮았다. 20% 지질 유상액 처리 집단에서 Akt와 GSK3-β의 인산화가 증가하였다. 한편, 20% 지질 유상액이 주입된 집단에서 Wnt 관련 인자들이

현저히 증가하였으며, 염증성 표지자들은 감소하였다. 지질 유상액의 보호 효과와 Akt, GSK3-β, Wnt1, 그리고 β-catenin과 같은 생존 관련 인자들의 발현은 복강 내 XAV939의 투여에 의해 상쇄되었다.

종합해 보았을 때, 지질 유상액은 Wnt 신호 경로를 통해 신경 보호 효과를 가지고 있으며, 더 나아가 임상에 적용 가능한 치료 표적의 규명에 기여할 가능성을 지니고 있다고 할 수 있다.

핵심 되는 말: 지질 유상액, 카인산, 뇌전증, 뇌졸중, Wnt, 뇌

PUBLICATION LIST

Tanioka M, Park WK, Shim I, Kim K, Choi S, Kim UJ, et al. Neuroprotection from excitotoxic injury by local administration of lipid emulsion into the brain of rats. Int J Mol Sci. 2020; 21: 2706.

Tanioka M, Park WK, Park J, Lee JE, Lee BH, Lipid emulsion improves functional recovery in an animal model of stroke. Int J Mol Sci. 2020; 21: 7373.