New Emulsifying Oil Powder Extracted from Pacific Krill Protects against Vascular Cognitive Impairment in a Mouse Model of Cerebral Small Vessel Disease

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Background : Marine n-3 polyunsaturated fatty acids (PUFAs) may be promising for reducing cognitive and functional decline in elderly patients with mild cognitive impairment. We examined the effects of a new emulsifying oil powder including PUFAs extracted from Pacific krill on vascular cognitive impairment in a mouse model of cerebral small vessel disease.

Methods : Small vessel disease was created using a mouse model of asymmetric common carotid artery stenosis surgery (ACAS). Mice were fed an MF diet (MFD) or MFD supplemented with the new emulsifying Pacific krill oil powder (EPKOP) from 4 weeks before ACAS to 5 weeks after ACAS. Two and/or from 4 to 5 weeks after ACAS, all experiments were performed.

Results : Cerebral blood flow (CBF) was decreased in MFD-fed ACAS compared with MFD-fed sham-operated mice. CBF was similar between MFD with EPKOP-fed ACAS and MFD-fed sham-operated mice. EPKOP reversed the ACAS-induced decrease in the latency to fall in a rotarod test of ACAS mice. Moreover, it also reversed the ACAS-induced longer escape latencies in the Morris water maze test of ACAS mice. Hematoxylin/eosin staining demonstrated that EPKOP decreased the number of shrunken neurons in the ACAS mouse hippocampus. Interleukin 1β gene expression in the hippocampus was increased in MFD-fed ACAS compared with MFD-fed sham-operated mice. In contrast, its gene expression in the hippocampus was similar between MFD with EPKOP-fed ACAS and MFD-fed sham-operated mice.

Conclusion : These results suggest that the new emulsifying oil powder extracted from Pacific krill may be effective to prevent vascular cognitive impairment. *Shinshu Med J 72 : 169—182, 2024*

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Key words : vascular cognitive impairment, Pacific krill, n-3 polyunsaturated fatty acids

Abbreviations : ACAS, asymmetric common carotid artery surgery ; CBF, cerebral blood flow ; VCI, vascular cognitive impairment ; CCA, common carotid artery ; MCP-1, monocyte chemotactic protein 1 ; IL-1β, interleukin-1β ; TNF-α, tumor necrosis factor α ; EPKOP, emulsifying Pacific krill oil powder

I Introduction

Several types of vascular dementia have been reported worldwide[1)2)] and cerebral blood vessel disease (i.e., vascular cognitive impairment, VCI)-associated cognitive impairment is one of the recent major public

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health concerns[3]. At present, vascular diseases and other risk factors for VCI have mainly been treated to prevent VCI. In fact, the management of cardiovascular risk factors, such as hypertension, diabetes, dyslipidemia, obesity, and smoking might be effective to reduce the development of VCI. The effective control of cardiovascular risk factors prevents VCI-related disease and may be more effective than current pharmacological treatment for VCI[4)5)].

It is well-known that marine n-3 polyunsaturated fatty acids (PUFAs) have health benefits[6)]. N-3 PUFAs which include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), reduce serum triacylglycerol (TAG) levels and have anti-inflammatory effects, leading to reduction of the incidence of mortality due to cardiovascular diseases[7)]. Moreover, n-3 PUFAs play a positive role in the prevention and treatment of metabolic syndrome including dyslipidemia[8)9)]. These results suggest that n-3 PUFAs can reduce the development of VCI through the reduction of cardiovascular risk factors. In fact, a clinical study demonstrated that PUFAs may be promising for reducing cognitive and functional decline in the elderly with mild cognitive impairment[10)]. Moreover, the association between cerebral white matter lesions and the plasma omega-3 to omega-6 PUFA ratio to cognitive impairment development has been demonstrated[11)].

Krill oil includes PUFAs, and the direct neuroprotective effects of *E. superba* oil against aging and Alzheimer's disease-related cognitive impairment in animals and humans were demonstrated[12)]. In fact, the protective effect of krill oil against Alzheimer's disease has been confirmed in an animal model of senescence-accelerated prone mouse strain 8 (SAMP8) mice[13)]. However, few studies showed the protective effects of krill oil on cerebrovascular dementia, especially small vessel disease-related dementia. In addition, whether krill oil directly improves hypoperfusion-induced VCI even if cardiovascular risk factors do not exist is unknown. Recently, we and ADEKA Co., Ltd. developed a new emulsifying Pacific oil powder (EPKOP) extracted from Pacific krill for human health. We studied the protective effects of the new EPKOP on vascular cognitive impairment using a mouse model of cerebral small vessel disease which was created using a mouse model of asymmetric common carotid artery surgery (ACAS)[14)].

Ⅱ Material and Methods

A Ethics

The study was performed in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health. This study was approved by the Animal Care Committee of Iwate Medical University. The protocol was approved by the Committee on the Ethics of Animal Experiments of Iwate Medical University (Permit Number : 30-18).

B Contents of a new emulsifying oil powder extracted from Pacific krill

E. pacifica is the most common krill species in the North Pacific Ocean. The common term 'krill' refers to *Euphausiids* and they are widespread in oceans worldwide. *E. pacifica* is a good source of marine n-3 PUFAs, which include EPA and DHA[15)]. Both fish oil and krill oil are sources of EPA and DHA ; however, compositions of these oils are different. Fish oil is mostly composed of TAG. Krill oil is mostly composed of TAG and phospholipids. As the phospholipid form of n-3 PUFA is incorporated into plasma faster than the TAG form, krill oil can increase the omega-3 index at a lower dose in humans[16)17)]. Krill oil also contains the antioxidant astaxanthin. The new EPKOP for human health from *E. pacifica* is mainly composed of protein including peptide (70 %), neutral lipid (10 %), diacyl phospholipid (10 %), and alkyl phospholipid (2 %) **(Fig. 1A)**. This powder contains 39.3 mg/g of EPA, 1.6 mg/g of DHA, 22.4 mg/g of alkyl phospholipid, and 285 ppm of astaxanthin **(Table 1)**.

C Experimental animals and asymmetric common carotid artery stenosis surgery (ACAS)

C57BL/6J male mice (8 weeks old, 20-25-g body weight) were purchased from Japan SLC. To produce VCI, stenosis of both common carotid arteries (CCAs) was surgically created using an ameroid constrictor and a microcoil (ACAS), as previously described[18)19)]. Briefly, mice were anesthetized with isoflurane (2-4 %), then we exposed both CCAs, and these were

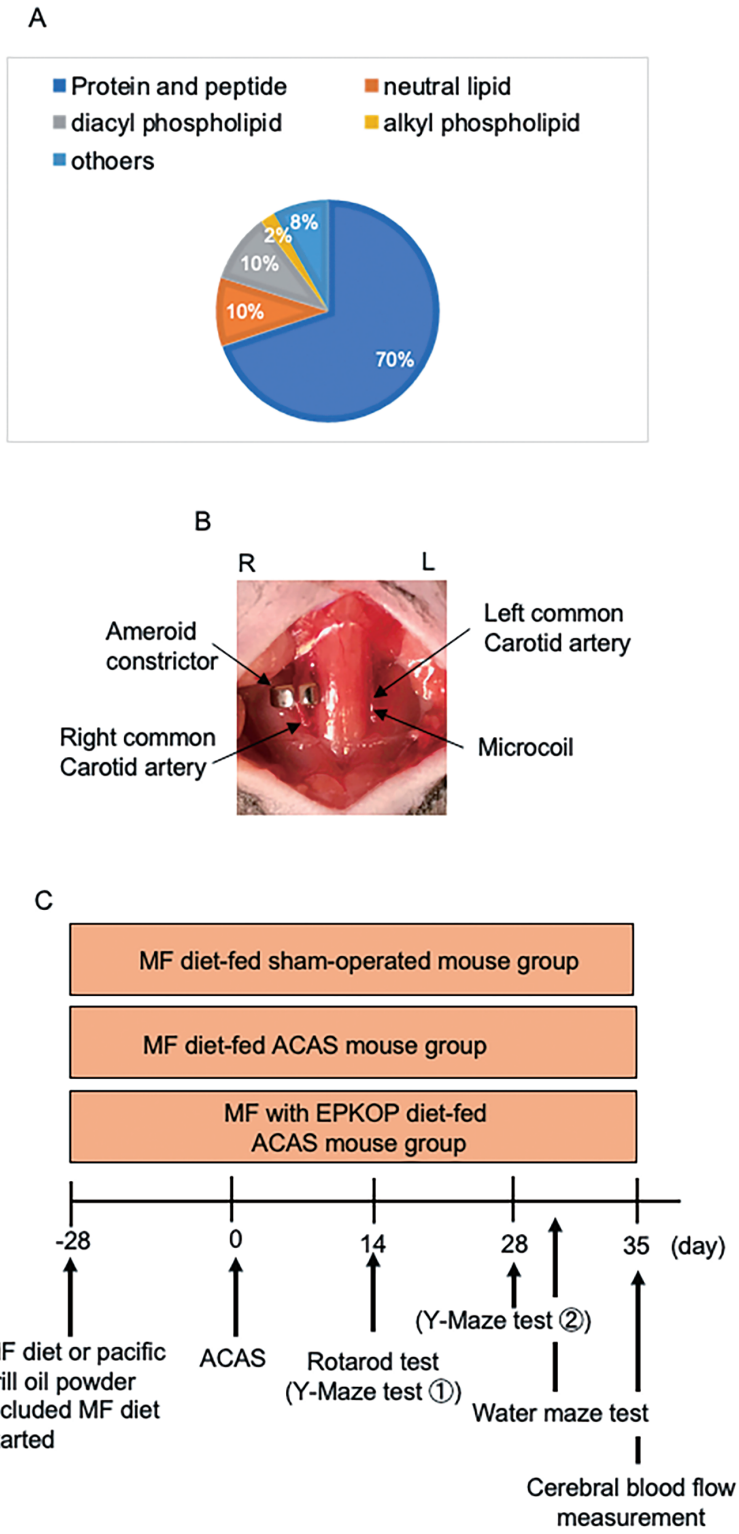

Fig. 1 Panel A, Contents of a new emulsifying oil powder extracted from Pacific krill. The new EPKOP for human health from *E. pacifica* is mainly composed of protein, including : peptide (70 %), neutral lipid (10 %), diacyl phospholipid (10 %), and alkyl phospholipid (2 %). Panel B, Small vessel disease was created using a mouse model of asymmetric common carotid artery stenosis surgery (ACAS) in two of the three groups. An image showing an ameroid constrictor (AC) around the right common carotid artery (CCA) and placement of a microcoil around the left CCA. Panel C, The schema of experimental protocols for each of the three groups (MF diet fed-sham operation mice, MF diet-fed ACAS mice, MF with EPKOP diet-fed ACAS mice). Each diet was fed from four weeks before to five weeks after sham surgery and ACAS, respectively. EPKOP, emulsifying Pacific krill oil powder.

Table 1 Components of the lipid fraction

Phospholipid	Alkyl phospholipid	DHA	EPA	Astaxanthin
mg/g				ppm
100	22.4	1.6	39.3	285

DHA, docosahexaenoic acid ; EPA, eicosapentaenoic acid

freed from their sheaths. A spiral microcoil was wrapped around the left CCA. An ameroid constrictor[14] was also fitted in the right CCA **(Fig. 1B)**. The same procedure was performed except for ACAS in sham-operated mice. Finally, the midline cervical incision was closed using 7-0 nylon suture. After the surgery, mice were maintained in an environment with day/night reversal throughout the experiments. Before the brain tissue was excised, isoflurane inhalation and/or sodium pentobarbital (30 mg/kg) application into the abdominal cavity was performed to anesthetize all animals. To confirm the adequacy of anesthesia, the degree of motion of the sternum and movement of the extremities were monitored. The toe pinch reflex test was used to confirm the appropriate depth of anesthesia.

D Experimental groups and diet

Male mice were divided into three groups, as follows : MF diet (Certified diet, Oriental Yeast Co., Ltd., Tokyo, Japan) fed sham-operated mice, MF diet-fed ACAS mice, MF diet with new EPKOP-fed ACAS mice **(Fig. 1C)**. EPKOP intake continued from 4 weeks before ACAS to 5 weeks after ACAS. All mice were allowed to access water *ad libitum* and housed under a 12-h light/dark cycle. Feed was replaced every three days. Body weight was measured weekly for 10 weeks during experiments and did not differ among the three different groups of mice **(Fig. 2A)**.

E Rotarod test

Motor coordination and balance were tested with the rotarod test. The rotarod test was performed 14 days after ACAS or sham surgery (n = 10 for each) **(Fig. 1C)** by placing the mouse on a rotating drum (Muromachi Kikai CO., Japan) and measuring the time the mouse maintained its balance on the rod, as previously reported[20]. The speed of the rotarod was accelerated from 4 to 40 rpm over a 5-min period. The test was repeated five times with an interval of 5 min between attempts.

F Y-maze test

To assess spatial working memory and spontaneous activity, the Y-maze test was performed as previously described[19)21]. The Y-maze test was conducted at 2 and 4 weeks after ACAS or sham surgery (n = 10 for each) **(Fig. 1C)**. Mice were allowed to freely explore the three arms during an 8-min session after introduction to the end of the start arm. A video camera (Logicool HD Webcam C615, Logicool, Tokyo, Japan) was used to record the numbers of arm entries and trials. A mouse was considered to have entered an arm when all four limbs were within the arm. Spatial working memory was elucidated by the percentage of alternation behavior. Actual alternation behavior was defined as entry into all three arms on consecutive occasions. The maximum alternation behavior was calculated as the total number of arm entries minus two, and the percentage of alternation was calculated as the number of actual alternations divided by that of maximum alternations multiplied by 100. Spontaneous activity was elucidated by the total number of arms entered during a session.

G Morris water maze test

The Morris water maze test was performed consecutive days from 29th to 34th after ACAS or sham surgery (n = 10 for each) **(Fig. 1C)**, as previously described[19)22]. Briefly, to learn the platform position, a visible platform task was performed using a transparent circular platform with a diameter of 8 cm three times per day from the first to sixth days. After the visible platform task, the circular platform was immersed 1 cm below the water surface in the center of a quadrant of the pool to perform a hidden platform task. The platform was kept in the same position throughout the experiment. We performed the hidden

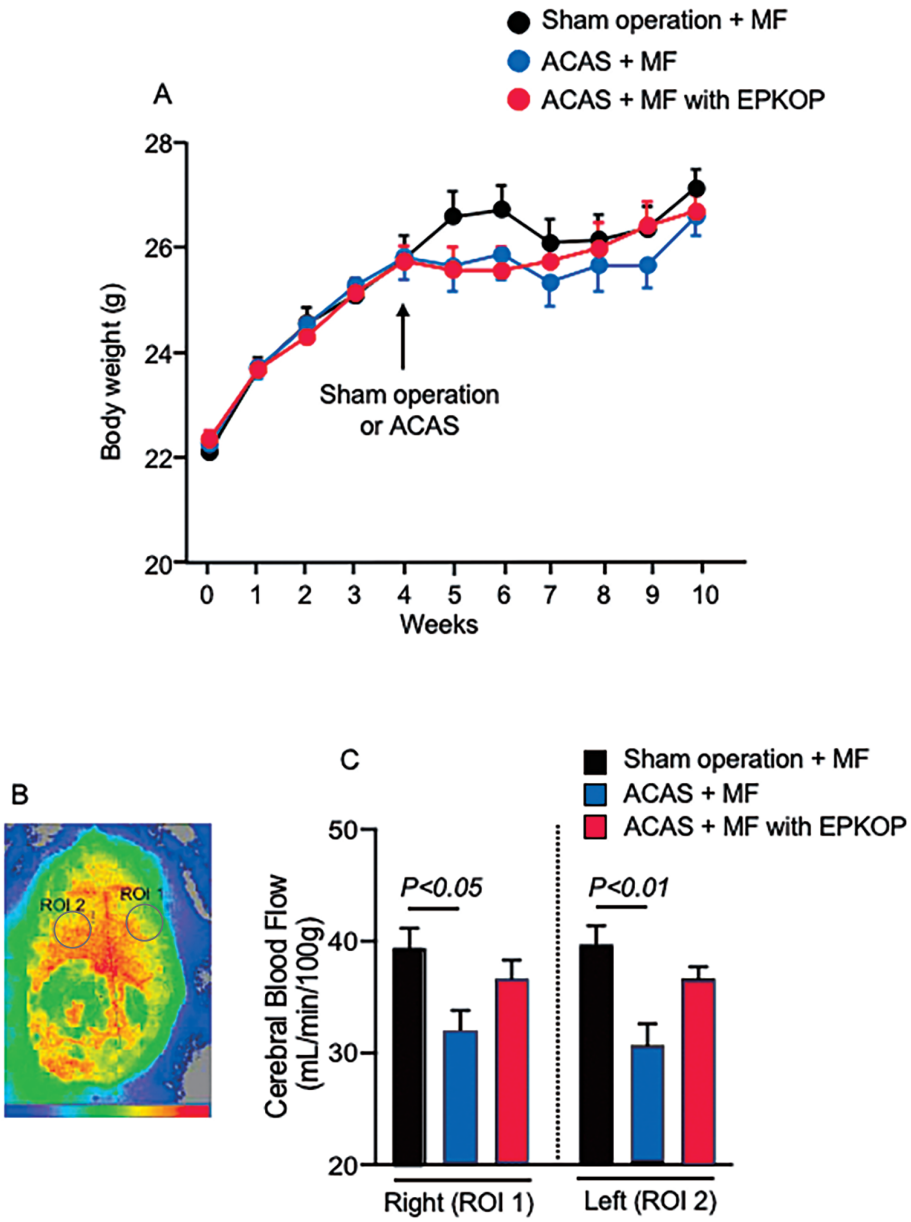

Fig. 2 Body weight and cervical blood flow (CBF) in the three different groups of mice. Panel A, Body weight was measured for 10 weeks during experiments and did not differ among the three different groups of mice. Sham operation or ACAS was performed 4 weeks after the experiment started. Panel B, Representative CBF images of laser speckle flowmetry. Panel C, Mean local CBFs in circular regions of ROI1 and ROI2 in the three different groups of mice at five weeks after ACAS or the sham operation. Data are the mean ± SE obtained from 10 mice for each group. Sham operation + MF, MF diet-fed sham operation mice ; ACAS + MF, MF diet-fed ACAS mice ; ACAS + MF with EPKOP, MF diet with emulsifying Pacific krill oil powder-fed ACAS mice.

platform task five times with a 5-min interval between attempts each day. Mice were released into the water at the starting position in the three other quadrants. We monitored each mouse tested and recorded the time required until it arrived at the platform. When a mouse arrived at the platform in 90 sec we allowed it to stay there for 15 sec. When a mouse did not arrive at the platform in 90 sec we guided it to the platform and allowed it to remain there for 15 sec. In each mouse, we calculated the latency to reach the platform (escape latency) based on the average of the 5 times of performing the hidden platform task each day.

H Cerebral blood flow (CBF) measurement

We measured CBF at 35 days after ACAS or sham

surgery using a 2D laser blood flow imager (OMEGA-ZONE, Omegawave Inc., Tokyo, Japan), (n＝10 for each), as previously described[18)19)]. Briefly, laser speckle flowmetry with the average of highspeed 2D imaging was used to measure the relative CBF. After anesthesia (1–2 % isoflurane), we surgically removed the mouse scalp to expose the skull. The periosteum, which adheres to the skull, was widely removed with fine-tip forceps. The skull surface was wiped with saline-soaked gauze to prevent drying of the surface. CBF recordings were performed through the skull. Circular regions in identically sized ROIs (900 pixels), located 1 mm posterior and 2 mm lateral to the bregma were defined on the image for quantitative measurement **(Fig. 2B)**. These data reflect the CBF of the subsurface microvessels in the brain with a depth of 0.5 mm[23)].

I Histology

Hematoxylin/eosin staining for histopathological analysis was performed as previously described[19)]. Briefly, after anesthesia with isoflurane (2 %), and sodium pentobarbital (15 mg/kg i.p.) the mouse brain was transcardially perfused and fixed with a 4 % solution of paraformaldehyde in 0.01 mol/L PBS for more than 5–8 min in the three different groups of mice at five weeks after ACAS or the sham operation (n＝4 for each). After brain fixation, each brain was quickly removed and divided coronally at the bregma, the bregma at 1 mm, and bregma at 2 mm through the hippocampus. The brain samples were embedded in paraffin, and then cut into 3-μm-thick coronal sections. For histopathological analysis, five sections stained with hematoxylin/eosin were prepared. After HE staining, the shrunken neurons with pyknotic nuclei located within the square area (100×100 μm) in hippocampus CA1 region were counted to assess neuropathy as previously reported[24)].

J Quantification of mRNA by real-time PCR

The amount of mRNA by real-time reverse transcription-polymerase chain reaction (RT-PCR) was quantified as previously described[25)]. Briefly, after the brain tissue was resected, total RNA was prepared from the frontal lobe (F) and hippocampus area (HS) of the three different groups of mouse brains (n＝7 for each) using ReliaPrep™ RNA Tissue Miniprep System (Promega, Madison, WI, USA), according to the manufacturer's instructions. We used 500 ng of total RNA as a template for reverse transcription with the SuperScript® III First-Strand synthesis system (Invitrogen, Carlsbad, CA, USA). We performed real-time RT-PCR analysis with ABI Step One Real-Time PCR System using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) to detect monocyte chemotactic protein 1 (MCP-1), interleukin (IL)-1β, tumor necrosis factor α (TNF-α), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **Table 2** shows the sequences of oligonucleotide primers. As the expression of GAPDH mRNA was constant between groups, the expression of each gene was normalized to that of GAPDH mRNA.

Table 2 Sequences of oligonucleotide primers

Primer		Array
GAPDH	S	TGTGTCCGTCGTGGATCTGA
	A	TTGCTGTTGAAGTCGCAGGAG
IL-1β	S	TCCAGGATGAGGACATGAGCAC
	A	GAACGTCACACACCAGCAGGTTA
TNF-α	S	AAGCCTGTAGCCCACGTCGTA
	A	GGCACCACTAGTTGGTTGTCTT
MCP-1	S	GGCTCAGCCAGATGCAGTTAAC
	A	GCCTACTCATTGGGATCATCTTG

K Statistical analysis

All data are shown as the mean±SE. We used analysis of variance (ANOVA) with Dunnett's test for statistical analysis of multiple comparisons of data. Two-way and one-way ANOVA with Dunnett's test were used for statistical analysis of the Morris water maze test and other experiments, respectively. $P<0.05$ was considered significant.

Ⅲ Results

A CBF was not decreased on feeding emulsifying Pacific krill oil powder (EPKOP) to ACAS mice

To examine the effects of EPKOP on ACAS-induced decreases in CBF, CBF was measured and local CBFs were calculated in circular regions of ROI1 and ROI2

(Fig. 2B). ACAS decreased local CBF in both circular regions of MFD-fed ACAS compared with MFD-fed sham-operated mice **(Fig. 2C)**. In contrast, the local CBFs in both circular regions were similar between MFD with EPKOP-fed ACAS and MFD-fed sham-operated mice.

B EPKOP reversed ACAS-induced shortening of latency to fall measured by rotarod test

The rotarod test showed that the latency to fall was shorter in MFD-fed ACAS compared with sham-operated MFD-fed mice. Moreover, ACAS significantly shortened the latency to fall in MFD-fed compared with MFD with EPKOP-fed mice **(Fig. 3A)**. In contrast, the latency to fall was similar between MFD with EPKOP-fed ACAS and MFD-fed sham-operated mice.

C EPKOP did not affect spatial working memory or spontaneous activity measured by Y-maze test

The Y-maze test showed that both the percentage of alternation behaviors and rates of arm entries were similar among the three different groups of mice at two and four weeks after ACAS **(Fig. 3B)**.

D EPKOP reversed ACAS-induced prolongation of escape latency measured by Morris water maze test

The Morris water maze test showed that the escape latency was significantly longer in MFD-fed ACAS mice than in sham-operated MFD-fed mice during the acquisition phase of 6 days. MFD with EPKOP significantly shortened the prolonged escape latency in MFD-fed ACAS mice during the acquisition phase of 6 days **(Fig. 3C)**. Mean swimming speeds in the acquisition phase did not differ among the three different groups of mice (data not shown).

E EPKOP improved increases in number of shrunken neurons in ACAS mice

To examine the effects of EPKOP on ischemia-induced neuronal damage, shrunken neurons with pyknotic nuclei in the hippocampal area were observed. Representative images of the hippocampal area revealed shrunken neurons with pyknotic nuclei (white arrows) in MFD-fed ACAS mice but not in the two other groups of mice **(Fig. 4A)**. Quantitative analysis of shrunken neurons with pyknotic nuclei in the C1 area of the hippocampus showed an increased number of shrunken neurons in MFD-fed ACAS mice compared with the two other groups of mice **(Fig. 4B)**.

F EPKOP inhibited the increases in IL-1β mRNA expressions in ACAS mice

mRNA expression of the IL-1β gene was significantly increased in MFD-fed ACAS compared with MFD-fed sham-operated mice in the hippocampus **(Fig. 5A)**. In contrast, it was similar between MFD with EPKOP-fed ACAS and MFD-fed sham-operated mouse brains. Expressions of MCP-1 and TNF-α mRNA were similar among the three different groups of mice in the frontal lobe **(Fig. 5B-C)**. In contrast, those gene expressions in the hippocampus tended to be increased in MFD-fed ACAS and decreased in MFD with EPKOP-fed ACAS mice **(Fig. 5B-C)**.

Ⅳ Discussion

We demonstrated that EPKOP : 1) improved the decreased cerebral blood flow in ACAS mice, 2) reversed the shortened latency to fall based on rotarod testing in ACAS mice, 3) reversed the longer escape latencies in ASCS mice based on the Morris water maze test, and 4) decreased the number of shrunken neurons with pyknotic nuclei in the hippocampal area of ACAS mice. EPKOP ameliorated the increased IL-1β gene expressions in the ACAS mouse hippocampus. These results suggest that EPKOP improves VCI and neural injury through the improvement of cerebral blood flow and IL-1β gene expressions in the mouse model of ACAS-induced vascular dementia.

Cerebral small vessel disease causes blood-brain barrier (BBB) functional damage, leukocyte infiltration, and increases in IL-1β and TNF-α expressions, leading to the injury of oligodendrocytes and neurons[26]. A rat cerebral small vessel disease model demonstrated that IL-1β and TNF-α expressions were increased in the hippocampus[27]. Moreover, the Morris water maze test demonstrated that cerebral hypoperfusion induced a longer escape latency in rats[28]. Our previous study demonstrated that ACAS-induced decreases in cerebral blood flow (i.e., cerebral hypoperfusion) caused a longer escape latency based on the Morris water maze test, hippocampal neuronal injury shown

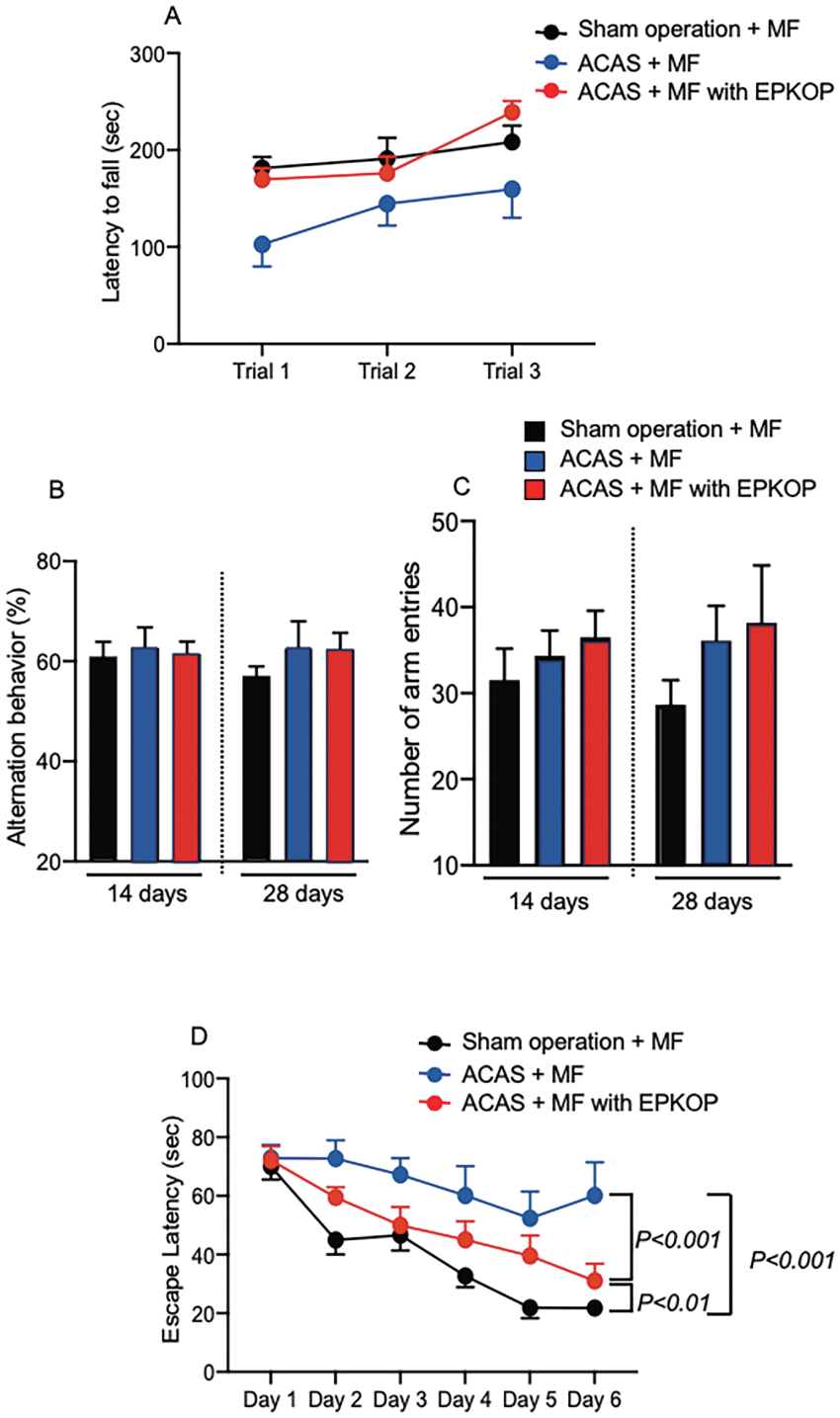

Fig. 3 Behavioral performance of ACAS mice in Rotarod, Y-maze, and Morris water maze tests. Each diet was fed from four weeks before to five weeks after sham operation and ACAS, respectively. Panel A, Motor coordination and balance were tested with the rotarod test. The rotarod test was performed at two weeks after ACAS or sham operation in the three different groups of mice. Panels B-C, Spatial working memory elucidated by alternation behavior and spontaneous activity elucidated by the number of arm entries in the Y-maze test at two and four weeks after ACAS or the sham operation in the three different groups of mice. Panel D, Time-course of escape latency assessed with the Morris water maze test from days 1 to 6 (consecutive days from 29th to 34th) after ACAS or sham operation in the three different groups of mice. Data are the mean ± SE obtained from 10 mice for each group. EPKOP, emulsifying Pacific krill oil powder.

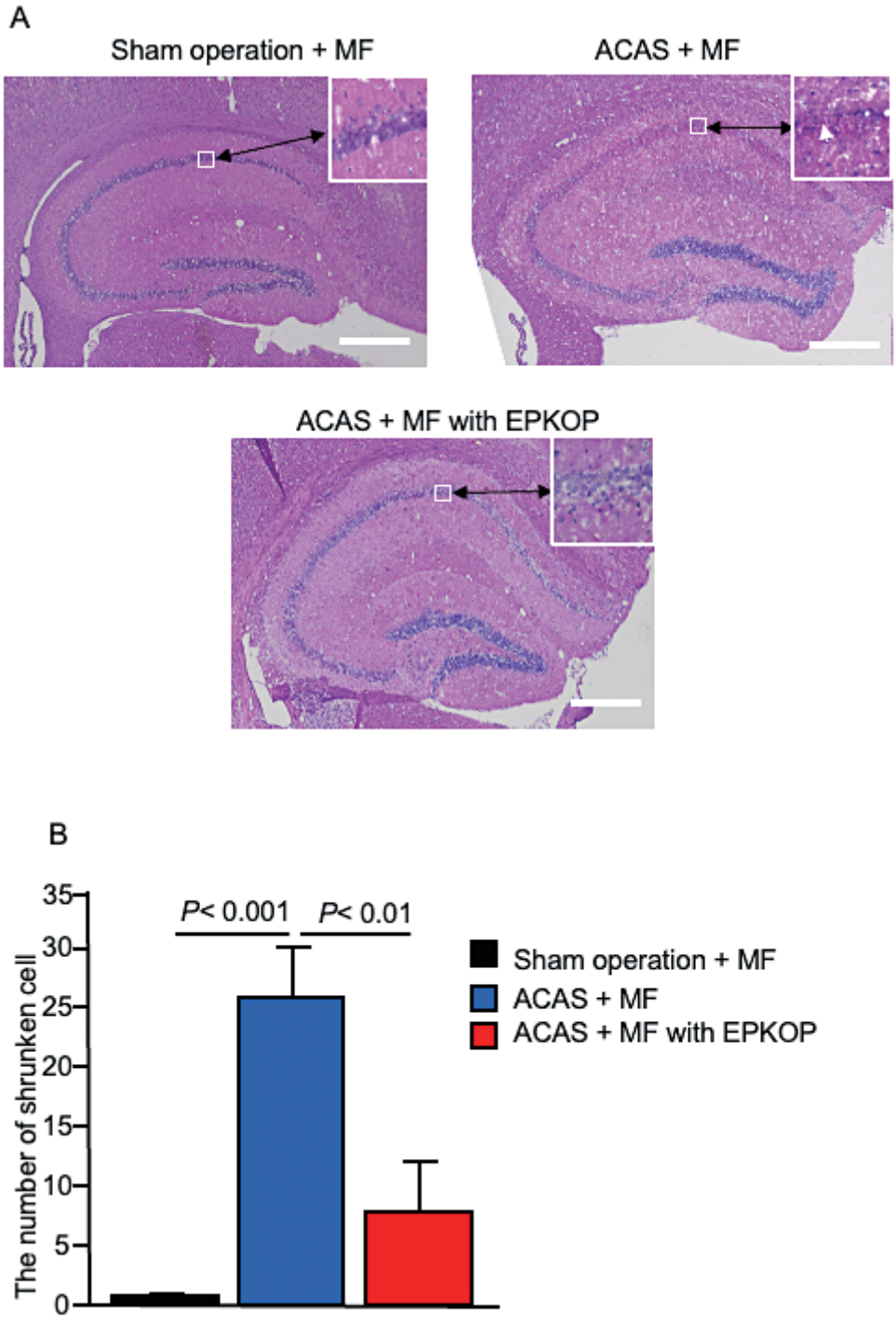

Fig. 4 Historical analysis of ischemia-induced neuronal injury in the mouse hippocampus in the three different groups of mice at five weeks after ACAS or the sham operation. Each diet was fed from four weeks before to five weeks after sham operation and ACAS, respectively. Panel A, Typical image of HE staining (Original magnification : 2× Scale bar = 500 μm). White arrow shows shrunken neurons with pyknotic nuclei. Panel B, Quantitative analysis of shrunken neurons with pyknotic nuclei calculated from neurons located within the white square area (100 × 100 μm, CA1 region) shown in panel A. Data are the mean ± SE obtained from 4 mice for each group. EPKOP, emulsifying Pacific krill oil powder.

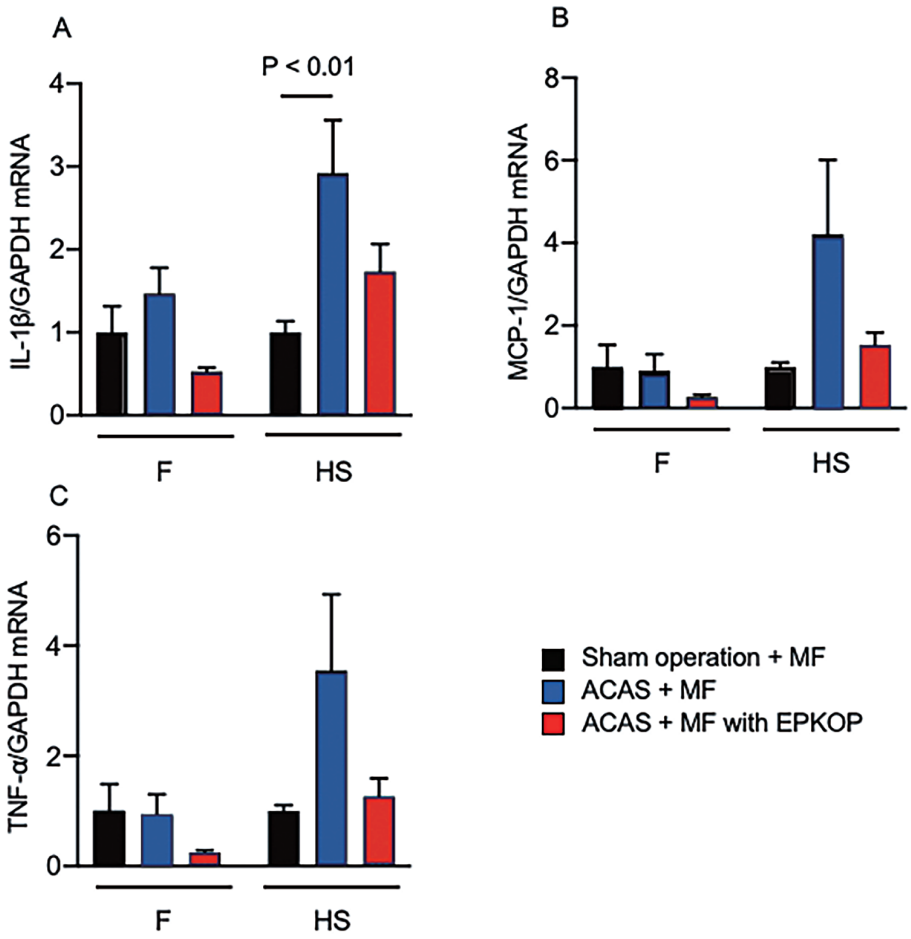

Fig. 5 Quantitative analyses of pro-inflammatory cytokines and chemokine gene expressions by real-time RT-PCR in the three different groups of mouse brain tissues at five weeks after ACAS or the sham operation. Each diet was fed from four weeks before to five weeks after sham operation and ACAS, respectively. mRNA levels of interleukin 1β (IL-1β), monocyte chemotactic protein 1 (MCP-1), and tumor necrosis factor α (TNF-α) are shown. Data for IL-1β, MCP-1, and TNF-α were normalized to those for GAPDH. Data are the mean ± SE obtained from 7 mice for each group. EPKOP, emulsifying Pacific krill oil powder ; F, frontal lobe ; HS, hippocampus area.

by shrunken neurons with pyknotic nuclei, and increased IL-1β and TNF-α gene expressions in mice, suggesting that cerebral hypoperfusion-induced increases in IL-1β and TNF-α gene expressions play important roles in the development of neural injury and VCI. The present study demonstrated that EPKOP reduced the increased gene expression of IL-1β in ACAS mice, and TNF-α gene expression was similar between EPKOP-fed ACAS mice and sham-operated mice. Moreover, EPKOP reversed the longer escape latency based on the Morris water maze test and decreased the number of shrunken neurons in the hippocampal area in ACAS mice. A previous study demonstrated that lipopolysaccharide-induced BV2 microglia activation released IL-1β, leading to PC12 cell apoptosis[29]. Thus, EPKOP might improve hippocampal neuronal injury and VCI through reduction of the increased IL-1β gene expression.

Our present study demonstrated that the latency to fall tested with the rotarod test was significantly shorter in MFD-fed ACAS than in MFD-fed sham-operated mice, indicating that muscle strength, motivation not to fall, and/or CNS motor function were significantly impaired in ACAS mice. Interestingly, EPKOP improved the shortened the latency to fall to the normal level in ACAS mice, suggesting that EPKOP can maintain muscle strength, motivation not to fall, and/or CNS motor function in mice with small vessel disease.

CBF was similar between MFD with EPKOP-fed ACAS mice and MFD-fed sham-operated mice. This indicates that EPKOP improves the decreases in cerebral blood flow in ACAS mice. Moreover, almost all of the factors tested were similar between MFD with EPKOP-fed ACAS mice and MFD-fed sham-operated mice. Our previous study[30] demonstrated that phlorizin ameliorated the decreased cerebral blood flow in ACAS mice, and that cerebral blood flow was similar between phlorizin-treated ACAS mice and sham-operated mice. Moreover, phlorizin also reversed the increases in IL-1β and the number of shrunken neurons in the ACAS mouse brain. Therefore, EPKOP might ameliorate neural injury and VCI through the improvement of cerebral blood flow as phlorizin did. However, the mechanism of EPKOP-induced improvement of cerebral blood flow in ACAS mice is still unknown. Several studies showed that EPA induced cerebral arteriolar dilatation in rabbits[31]. Moreover, N-3 PUFAs are likely to improve vascular endothelial function and arterial stiffness[32][33], which are both important mechanistic determinants of CBF[34]. In fact, transgenic fat-1 mice that overproduce n-3 PUFAs showed functional and histological protection against focal cerebral ischemia[35]. Interestingly, these mice exhibited improvements in revascularization and angiogenesis. These results suggest that n-3 PUFAs induce cerebral vasodilation and take part in cerebrovascular remodeling. Thus, EPKOP-induced improvement of cerebral blood flow in ACAS mice may be caused through n-3 PUFA-induced cerebral vasodilation and cerebrovascular remodeling.

It is well-known that the increase in MCP-1 expression in brain and endothelial cells plays important roles in cerebral ischemia-induced cognitive deficits[36]-[38]. Moreover, MCP-1 can increase IL-1β and TNF-α expressions in microglia[39]. This indicates that the cerebral ischemia increases MCP-1 expression in the brain, leading to inflammatory responses such as increases in IL-1β and TNF-α expression. In this study, cerebral gene expression of MCP-1 tended to be increased in ACAS mice and was similar between MFD with EPKOP-fed ACAS and MFD-fed sham-operated mice. Our previous study demonstrated that ACAS increased MCP-1 gene expression in brain tissues. Although whether EPKOP inhibits ACAS-induced increases in MCP-1 gene expression is still unclear, it may participate in the suppression of MCP-1 gene expression in small vessel disease.

We demonstrated in this study that EPKOP decreased the number of shrunken neurons with pyknotic nuclei in the hippocampal area of ACAS mice similar to sham-operated mice. Hattori et al.[18] demonstrated that in the hippocampus stained with H&E staining, neural marker NeuN antibody, and glial marker Iba1 antibody, shrunken neurons and neuronal loss in the hippocampus were observed only on the right ameroid constrictor side but not the left microcoil side in ACAS mice. Moreover, activated microglia was also observed surround the area of neuronal loss in the right side of hippocampus[18]. We demonstrated that shrunken neurons were observed in only the right side of hippocampus stained with H&E staining in ACAS mice. Based on these results, it is considered that the dementia model using the ACAS procedure has been completed in this experiment. Thus, EPKOP might improve neuronal injury shown by shrunken neurons with pyknotic nuclei in the hippocampal area of ACAS mice.

This study had limitations. In this experiment, cerebral blood flow was measured only at 35 weeks after ACAS. Hattori et al.[18] demonstrated that the decrease in cerebral blood flow due to ACAS gradually progressed until the 7th day after ACAS, with blood flow decreasing by 65-70 % on the 7th day. Subsequent cerebral blood flow remained at the same level after 14 and 28 days. EPKOP intake continued from 4 weeks before ACAS to 5 weeks after ACAS, so it is thought that the blood concentration of EPKOP-containing components might reach a sufficient amount to maintain blood flow through n-3 PUFA-induced cerebral vasodilation and cerebrovascular remodeling. These suggest that EPKOP fed mice do not cause ACAS-induced decrease in cerebral blood flow since the beginning of ACAS. It revealed that EPKOP might ameliorate neural injury and VCI through the improvement of cerebral blood flow as phlorizin did. However, a direct protective effect of

EPKOP on ischemic brain tissues is still unknown. Our previous study demonstrated that phlorizin directly improved brain tissue injury in addition to improving cerebral blood flow[30]. N-3 PUFAs directly protect against hypoxia and ischemia-induced brain damage in neonates by activating the Akt pathway in compromised neurons[40]. Moreover, DHA and EPA directly ameliorated oxygen and glucose deprivation-induced neuronal death in primary neuronal cultures[41], suggesting the possibility of a direct protective effect of EPKOP on ischemic brain tissues. Therefore, the effects of EPKOP on brain tissue would need to be examined in future project. As we did not measure effects of EPKOP on hemodynamics the effects are still unknown. Hattori et al.[18] previously measured mean blood pressure and heart rate in the ACAS model and showed that there were no differences in these values in the ACAS group compared to the sham group. Therefore, it is unlikely that hemodynamic changes are involved in hippocampal tissue damage or cognitive decline. Although it is not clear whether EPKOP has an effect on hemodynamics, protective effects of EPKOP on hippocampal tissue damage and cognitive decline might not be effects on hemodynamics.

In conclusion, we are the first to demonstrate the possibility of using EPKOP as a functional food for the prevention of VCI in a mouse model of small vessel disease.

Disclosure

No author has an actual or perceived conflict of interest regarding the contents of this article.

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