

Effect of Anserine/Carnosine Supplementation on Verbal Episodic Memory in Elderly People

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Abstract. Our goal in this study was to determine whether or not anserine/carnosine supplementation (ACS) is capable of preserving cognitive function of elderly people. In a double-blind randomized controlled trial, volunteers were randomly assigned to an ACS or placebo group at a 1:1 ratio. The ACS group took 1.0 g of an anserine/carnosine (3:1) formula daily for 3 months. Participants were evaluated by psychological tests before and after the 3-month supplementation period. Thirty-nine healthy elderly volunteers (60–78 years old) completed the follow-up tests. Among the tests, delayed recall verbal memory assessed by the Wechsler Memory Scale-Logical Memory showed significant preservation in the ACS group, compared to the placebo group ($p=0.0128$). Blood analysis revealed a decreased secretion of inflammatory cytokines, including CCL-2 and IL-8, in the ACS group. MRI analysis using arterial spin labeling showed a suppression in the age-related decline in brain blood flow in the posterior cingulate cortex area in the ACS group, compared to the placebo group ($p=0.0248$). In another randomized controlled trial, delayed recall verbal memory showed significant preservation in the ACS group, compared to the placebo group ($p=0.0202$). These results collectively suggest that ACS may preserve verbal episodic memory and brain perfusion in elderly people, although further study is needed.

Keywords: Alzheimer's disease, anserine and carnosine, cognitive function, dementia, elderly people, inflammatory cytokine, perfusion MRI, randomized controlled trial, verbal memory

INTRODUCTION

The number of people worldwide living with dementia is estimated to be 47 million in 2015, and

Alzheimer's disease (AD) is the most common form of dementia [1]. Diagnosis and intervention at early stages of dementia may greatly reduce the number of individuals suffering from AD. The onset of its symptoms typically begins with a subtle decline in memory and progresses to global deterioration. Lifestyle improvements, including increased intellectual and physical activity, and nutritional improvement [2–4], that decelerate this process may delay or prevent

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the onset of AD. Among them, dietary improvements can help decelerate a decline in memory and may prevent AD [4, 5]. Here, we investigated the effects of dietary supplementation with anserine (beta-alanyl-1-methyl-L-histidine) and carnosine (beta-alanyl-L-histidine), functional dipeptides from animal meat, which also referred to as imidazole-containing dipeptides, on healthy elderly people.

Carnosine is an endogenous dipeptide consisting of beta-alanine and histidine, present in the millimolar range in skeletal muscle and in the hundred-micromolar range in the vertebrate brain [6]. Carnosine has many biochemical functions, including buffering, metal ion chelating, antioxidant, and anti-glycation activities, and a wide variety of physiological functions, mainly in excitable cells, including skeletal, cardiac, and smooth muscle cells, and nerve cells [6, 7]. We previously reported that carnosine supplementation prevents memory deficit in a transgenic AD mouse model fed a high-fat diet [8]. These mice show an increased expression of RAGE (receptor for advanced glycation end product) in blood vessels, which is inhibited by carnosine supplementation, suggesting that carnosine suppresses microvascular brain damage in this mouse model [8].

Carnosine is cleaved by carnosinase to beta-alanine and histidine. In rodents, serum carnosinase activity is low, so carnosine administration elevates the plasma carnosine level. However, in humans, high serum carnosinase activity limits the biomedical effectiveness of carnosine supplementation. Anserine is a natural carnosine derivative that is not cleaved by carnosinase. Anserine (beta-alanyl-1-methyl-L-histidine) is a methylated form of carnosine that is present at high levels in the breast skeletal muscle of chicken. Owing to their identical chemical structures, except for anserine's methylation, anserine and carnosine have equivalent reported physiological functions [6]. At present, anserine alone is not commercially available, but we could obtain an anserine/carnosine mixture prepared from chicken meat. In a clinical study, Szcześniak et al. [9] suggested that anserine and carnosine supplementation (ACS) has beneficial effects on physical capacity and cognitive functioning in elderly people.

In this study, we examined the effect of ACS on the preservation and/or improvement of a subtle memory decline in elderly people. We recruited elderly volunteers (≥ 60 years old) in the Tokyo area and evaluated the effects of ACS in a double-blind pilot randomized controlled trial (RCT). To this end, we evaluated the participants' verbal episodic memory function by

the use of the Wechsler Memory Scale-Logical Memory (WMS-R LM) [10, 11]. In addition, normal aging is associated with diminished brain perfusion measured as cerebral blood flow, and a study using a perfusion MRI method, arterial spin labeling (ASL), showed a clear age-dependent decrease in blood flow, most noticeably in the posterior cingulate cortex (PCC) region [12]. Therefore, to elucidate the effect of ACS, we also examined the participants' brain perfusion.

MATERIALS AND METHODS

Participants

Sixty-nine healthy participants (41–78 years of age) were recruited from June 2012 to November 2013 from the Tokyo metropolitan area (Supplementary Table 1). The participants were required to visit the study site twice, 3 months apart. Written informed consent was obtained from all participants. Subjects with the following indications were excluded from the study: 1) individuals with a neuropsychiatric disorder or head injury; 2) individuals with a local lesion, such as a brain tumor or cerebral infarction, which could affect cognitive function; and 3) individuals with metal or electrical implants or claustrophobia that could prevent MRI scanning. The accepted participants were randomized to the ACS or placebo group. RCT-consistent assignment to the ACS or placebo group was determined by age and gender and performed by Imepro Inc. (Tokyo, Japan). All clinical and coordinating personnel and participants were blinded to the group assignments for the duration of the study. The study was approved by the Ethics Committees of the University of Tokyo and of the National Center of Neurology and Psychiatry, in 2012. The present report is of an age-restricted (≥ 60 years old) sub-analysis from this healthy volunteer study, and used the data from 39 elderly volunteers (≥ 60 years old) who completed the study (Table 1).

In another RCT, healthy participants (60–80 years of age) were recruited from April 2014 to August 2014

Table 1
Baseline characteristics of participants who completed 3 month-test

	Active (19)	Placebo (20)	<i>p</i>
Age (mean \pm SD)	67.8 \pm 5.6	70.6 \pm 5.1	0.12
Gender (M/F)	8/11	9/10	0.74
BMI	21.6 \pm 3.0	21.0 \pm 5.4	0.68
Education (y)	14.9 \pm 2.5	15.2 \pm 2.4	0.80
MMSE	28.6 \pm 1.6	29.0 \pm 1.1	0.48

Significance was determined according to Student *t*-test, and chi-square test (for gender).

from the Tokyo metropolitan area. The participants were required to visit the study site twice, 6 months apart. Written informed consent was obtained from all participants as above mentioned. The accepted participants were randomized to the ACS or placebo group.

Testing formulae

The test formula was a powder containing anserine and carnosine (3:1) derived from chicken meat, provided by NH Foods Ltd., Japan. Participants in the ACS group received twice-daily doses of the imidazole dipeptide formula (500 mg/dose). The safety of this formula was previously verified by two independent studies [13, 14]. The placebo formula contained an equivalent amount of essential amino acids as in the test formula (43 mg/day L-lysine) and 150 mg/day L-histidine, because the enzymatic digestion of carnosine (250 mg/day) generates L-histidine (150 mg/day) and beta-alanine. Both treatments were granular solids taken orally over a 3-month period.

Inventory of food intake during the 3-month test period

A dietary survey was conducted using a semi-quantitative method as reported elsewhere [15]. At the time of follow-up, the participants filled out a self-administered questionnaire on the frequency of animal meat (chicken, pork, and beef) and fish meat (red meat fish represented by tuna, white fish by salmon, blue-back fish by mackerel, and eel) intake over the previous 3 months. In Japan, this three-item fish consumption inquiry is most popular, and salmon is classified into white fish even though its meat color is pink. The representative fish in each category was based on the national consumption survey. The average anserine and carnosine concentrations in these animal and fish meats were obtained from Boldyrev et al. [6], and dietary intake was estimated from the responses to the questionnaire.

Cognitive testing

The following cognitive evaluation tools and self-reported questionnaires were used to assess the effects of ACS on cognitive function, mental status, and general health: 1) the Japanese version of the Wechsler Memory Scale-Revised Logical Memory immediate recall (WMS-LM1) and delayed recall (WMS-LM2) tests [10, 11], and 2) the Japanese version of a cognitive subscale of the Alzheimer's Disease Assessment Scale (ADAScog) [17]. Mood and subjective states

were assessed by the Japanese version of the Beck Depression Inventory (BDI) [18, 19]. In addition, mental and physical functional well-being was assessed by the Medical Outcomes Study, 36-item Short Form (SF-36) [20, 21]. The Mental Health Component Summary (MCS) score and Physical Health Component Summary (PCS) score were calculated, with higher scores indicating better functioning. The cognitive and psychological tests were performed under double-blind conditions. A Mini-Mental State Examination (MMSE) was also conducted, to assess baseline cognitive function [22].

Blood sampling and immunoassays

The concentrations of 27 cytokines in serum samples collected from the volunteers at the baseline and during the follow-up test were measured by a Luminex-based multiplex beads array assay using the Bio-Plex Pro Human Cytokine Group I 27-plex panel (Bio-Rad Laboratories, Inc; Hercules, CA, USA), according to the manufacturer's instructions. The cytokine panel included interleukin (IL)-1 β , IL-1R α , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-12, IL-13, IL-17, CCL-2 (MCP-1), CCL-3 (MIP-1 α), CCL-4 (MIP-1 β), CCL-5 (RANTES), CCL-11 (Eotaxin), CXCL10 (IP-10), FGF-basic, G-CSF, GM-CSF, IFN- γ , PDGF-bb, TNF- α , and VEGF. Cytokine levels below the lower limit of detection were reported as the mid-point between the lowest concentration measured and zero, as reported elsewhere [23]. IL-15 was omitted from further analysis because it was beneath the detection limit in all cases. The false discovery rate method (FDR) [24] was used to correct for multiple comparisons for cytokines. *P*-values less than 0.01 were considered significant.

Microarray analysis

Peripheral blood mononuclear cells (PBMCs) from volunteers were used for total RNA extraction. The total RNA quality was assessed using the Agilent system according to the manufacturer's protocol. We eliminated one RNA sample from the Active group from further analysis. The total RNA was reverse transcribed to synthesize the first-strand cDNA, followed by second-strand synthesis. Double-stranded cDNAs were used to synthesize biotin-labeled complementary mRNA (cRNA). The cRNA samples from the volunteers were hybridized onto a Whole Human Genome oligo DNA Microarray Ver2.0 (Agilent Technologies, Inc., CA, USA), as described elsewhere [25]. The raw

signal intensities and Flags for each probe were calculated from the hybridization intensities (gProcessedSignal) and spot information (gIsSaturated, etc.), according to the procedures recommended by Agilent. The raw signal intensities of 120 samples were normalized by the quantile algorithm in the 'preprocessCore' library package [26] in the Bioconductor software [27]. The log fold-change of each selected probe in paired samples was calculated, and Student's *t*-test was applied using the MeV software [28]. The criteria for significantly different gene expressions was $p < 0.01$.

Acquisition of human MRI data and MRI data analysis

MRI analyses were performed with a 3 T scanner (Siemens, MAGNETOM Verio 3.0T), using a 32-channel phased array head coil. MRI data of the study participants were collected at two time points: prior to and after supplementation. During MRI scanning, headgear and earplugs were used to limit head motion and reduce scanner noise. For each participant, 3D T1-weighted magnetization-prepared rapid gradient echo (MPRAGE) images were collected, using the following parameters: TR = 1900 ms, TE = 2.52 ms, TI = 90 ms, flip angle = 9° , field of view (FoV) = 256×256 mm, acquisition matrix = 256×256 , slice thickness = 1.0 mm, slice gap = 0 mm, slice number = 192 [29]. The 3D pulsed ASL (pASL) perfusion images were collected by turbo gradient spin echo using the following parameters: TR = 5000 ms, TE = 38.8 ms, TI = 2350 ms, flip angle = 180° , FoV = 192×192 mm, acquisition matrix = 64×64 , slice thickness = 3.0 mm, slice gap = 1.5 mm, slice number = 40, bolus duration = 700 ms. The 3D pASL data calculated from the MRI data collected before and after supplementation were analyzed using the statistical parametric mapping 12 (SPM12) system. The data were spatially normalized to the MNI coordinates using the DARTEL method [30], and then smoothed with a full width parameter at half-maximal resolution of $12 \text{ mm} \times 12 \text{ mm} \times 12 \text{ mm}$ for 3D pASL as reported before [12]. To compare the perfusion changes that occurred over the 3-month supplementation period between the ACS and placebo groups, these spatially normalized with the 3D T1-weighted image (WI) data and smoothed pASL data were subjected to a two-way intra-subject analysis assessing the time and group interaction.

For additional MRI examinations, diffusion MRI, resting-state (rs) fMRI, and T2-FLAIR data were

obtained from the participants. The diffusion tensor images were collected by EPI-diffusion with the following parameters: TR = 14100 ms, TE = 81 ms, flip angle = 90° , FoV = 224×224 mm, acquisition matrix = 114×114 , slice thickness = 2.0 mm, slice number = 75, axes = 30, b-factor = 0 and 1000 s/mm^2 . The rsfMRI scans were acquired using a gradient-echo echo-planar sequence with repetition time TR = 3000 ms; echo time TE = 30 ms; flip angle 80° ; with 48 axial slices; 3.3-mm slice thickness with no gap; each slice consisted of 64×64 voxels, resulting in a $3.30 \times 3.31 \times 3.31$ voxel dimension. T2-fluid-attenuated inversion-recovery (FLAIR) scans were acquired with a repetition time TR = 11000 ms; echo time TE = 94 ms; inversion time TI = 2800 ms; with 20 axial slices; FoV = 198×220 mm, and were used to confirm that there were no neurological or inflammatory disorders (e.g., multiple sclerosis).

RESULTS

Participants

This analysis used the data from elderly (≥ 60 years of age) volunteers who completed the study. The subjects were randomly assigned to the ACS and placebo groups. As shown in Fig. 1, thirty-nine elderly participants completed both the baseline and follow-up tests. The group characteristics are summarized in Table 1. The two groups did not differ significantly with respect to age, gender, body mass index, education, or MMSE score, although there was a slight age difference between the two groups due to random dropout. The baseline MMSE score of all of the participants was greater than 23.

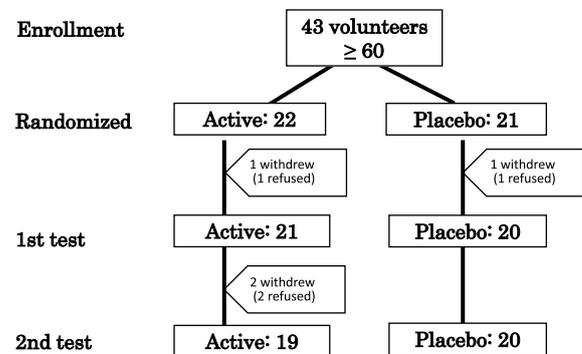


Fig. 1. Flow diagram showing the number of elderly participants (≥ 60 years old) during the study. 1st test: baseline. 2nd test: 3 months after supplementation.

The purpose of this study was to test the effect of anserine/carnosine supplementation, but these dipeptides are also obtained from the diet. To estimate daily intake from the diet, we estimated the anserine+carnosine intake from animal and fish meats using a 7-item meat intake frequency questionnaire (Fig. 2 and Table 2). There was no statistical difference in the daily anserine/carnosine intake from the diet between the two groups. Taking the dietary intake into account, the ACS group took in approximately 3 times more anserine/carnosine than the placebo group.

Cognitive tests

To evaluate cognitive function at baseline and follow-up, we performed two neuropsychological tests

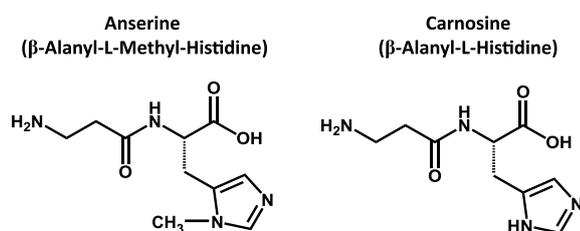


Fig. 2. Chemical structures of anserine and carnosine. Anserine (β-Alanyl-L-Methyl-Histidine) and carnosine (β-Alanyl-L-Histidine) are sometimes called as imidazole-containing dipeptides.

(Table 3). For the WMS-LM2 test, used to assess the delayed recall of verbal memory, we used two different stories (story A and story B) for the baseline and follow-up tests, respectively. Data were analyzed using a two-way repeated ANOVA (Time [baseline or follow-up] × Variant [ACS or placebo]). The interaction Time × Variant was significant before ($F[1,37]=9.067$, $p=0.0047$; Fig. 3) and after adjusting for age ($F[1]=6.8588$, $p=0.0128$). There was an effect of time ($F[1,37]=15.29$, $p=0.0001$), but not of variant ($F[1,37]=0.2621$, $p=0.61$). After a Bonferroni *post-hoc* test, we observed a significant decrease in the score for the placebo group ($DF[37]$, $t[4.958]$, $p<0.0001$), but not for the ACS group ($DF[37]$, $t[0.6281]$, $p>0.9999$). In the WMS-LM1 test, used to assess the immediate recall of verbal memory, we did not see any difference between the two groups: the interaction Time × Variant was not significant ($F[1,37]=0.7871$, $p=0.3807$).

In the ADAScog test, the interaction Time × Variant was not significant ($F[1,37]=0.5000$, $p=0.4839$). There was no effect of time ($F[1,37]=0.2280$, $p=0.6358$) or of variant ($F[1,37]=0.005572$, $p=0.9409$). In the BDI test, the interaction Time × Variant was not significant ($F[1,37]=1.641$, $p=0.2082$). There was an effect of time ($F[1,37]=9.337$, $p=0.0042$), but not variant ($F[1,37]=0.005905$, $p=0.8093$). After the Bonferroni *post-hoc* test, we

Table 2
Estimated intake of anserine and carnosine in each group

	Food	Active Group Ave.±SD	Placebo Group Ave.±SD	<i>p</i> value
Anserine (mg/day)	Poultry	217 ± 179	198 ± 167	0.73
	Pork	9.3 ± 8.0	4.4 ± 3.1	0.02
	Beef	7.9 ± 7.9	7.2 ± 9.7	0.79
	Eel	0 ± 0	0 ± 0	N. D.
	White fish	120 ± 138	98 ± 86	0.56
	Blue back Fish	1.2 ± 1.2	1.5 ± 1.6	0.48
	Red meat Fish	54.9 ± 53.3	67.0 ± 63.5	0.53
	Anserine from daily food	411 ± 278	377 ± 271	0.70
	Anserine from supplementation	750	0	N. D.
	Anserine (Total)	1,161 ± 278	377 ± 271	9.5E-11
	Carnosine (mg/day)	Poultry	60.9 ± 50.3	55.6 ± 46.9
Pork		124 ± 106	58.8 ± 41.4	0.02
Beef		38.6 ± 38.3	34.9 ± 46.9	0.79
Eel		7.4 ± 5.3	6.1 ± 1.7	0.34
White fish		0 ± 0	0 ± 0	N. D.
Blue back Fish		32.5 ± 33.4	41.4 ± 43.7	0.48
Red meat Fish		4.3 ± 4.2	5.3 ± 5.0	0.53
Carnosine from daily food		268 ± 135	202 ± 110	0.10
Carnosine from supplementation		250	0	N. D.
Carnosine (Total)		518 ± 135	202 ± 110	1.3E-09
Anserine+Carnosine (mg/day)		1,679 ± 384	579 ± 360	4.0E-11

Estimated intake of anserine and carnosine was calculated from the results of a 7-item food frequency questionnaires filled out by each volunteer (Materials and Methods section 2.3. Inventory of food intake during 3-month test period) and the average amount of anserine and carnosine in various types of meat described by Boldyrev et al. [6].

Table 3
Scores of neuropsychological tests of the ACS and placebo groups

	Baseline (ave.±SD)		Follow-up (ave.±SD)		Change (ave.±SD)		Interaction (df = 1)	
	Active	Placebo	Active	Placebo	Active	Placebo	F	p value
WMS-LM1	13.4 ± 4.2	13.2 ± 3.9	11.4 ± 3.6	10.2 ± 3.7	-2.0 ± 4.2	-3.0 ± 2.8	0.787	0.3807
WMS-LM2	11.9 ± 4.1	13.1 ± 3.6	11.4 ± 3.6	9.1 ± 4.1	0.5 ± 3.8	4.1 ± 3.5	9.067	0.0047**
ADAScog	9.6 ± 3.7	9.1 ± 9.2	8.8 ± 4.6	9.2 ± 4.7	-0.80 ± 3.5	0.16 ± 4.9	0.500	0.4839
BDI	10.2 ± 6.4	8.8 ± 6.9	6.8 ± 4.8	7.4 ± 4.4	-3.4 ± 4.2	-1.4 ± 5.6	1.641	0.2082
SF-36 MCS	54.0 ± 8.6	50.6 ± 7.0	54.0 ± 7.5	52.8 ± 5.9	-0.02 ± 6.5	2.12 ± 6.8	1.024	0.3182
SF-36 PCS	47.4 ± 8.1	48.0 ± 7.3	48.5 ± 5.9	47.3 ± 9.3	1.04 ± 8.0	-0.67 ± 8.0	0.443	0.5100

Scores of the baseline and follow-up tests are shown (mean ± SD). Active: ACS group. Change: score at follow-up – score at baseline. Significance was determined by two-way repeated ANOVA (Time [baseline or follow-up] × Variant [ACS or placebo]), by utilizing GraphPad Prism (GraphPad Software, Inc. La Jolla, CA. www.graphpad.com). The F(df1) and P value obtained after the interaction analysis (Time × Variant) are shown. ** $p < 0.01$ between the two groups without any adjustment. After age-adjustment, $F(1) = 6.8588$ and $p = 0.0128$ between the two groups.

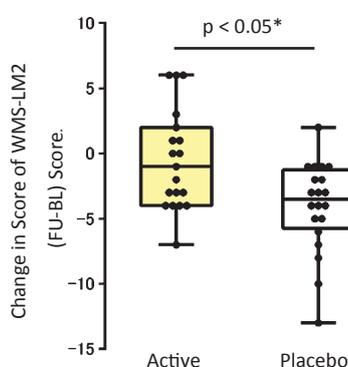


Fig. 3. Longitudinal change in score of WMS-LM2 between the two groups. Box plot of WMS-LM2 data described in Table 3. Each black dot represents the change of score in each volunteer. Active ($n = 19$), Placebo ($n = 20$). Solid bar shows median, and box shows 25–75 percentile. BL, Baseline; FU, Follow-up. We observed a significant improvement of WMS-LM2 score in the ACS group before ($F[1,37] = 9.067$, $p = 0.0047$), after adjusting for age ($F[1] = 6.8588$, $p = 0.0128$).

observed a significant decrease in the score in the ACS group ($DF[37]$, $t[3.028]$, $p = 0.0089$), but not in the placebo group ($DF[37]$, $t[1.271]$, $p = 0.4231$). In the MCS score from SF-36, the interaction Time × Variant was not significant ($F[1,37] = 1.024$, $p = 0.3182$). There was no effect of time ($F[1,37] = 0.9854$, $p = 0.3273$) or variant ($F[1,37] = 1.234$, $p = 0.2737$). In the PCS score from SF-36, the interaction Time × Variant was not significant ($F[1,37] = 0.4426$, $p = 0.5100$). There was no effect of time ($F[1,37] = 0.02027$, $p = 0.8876$) or variant ($F[1,37] = 0.01733$, $p = 0.8960$).

Cytokine and microarray analysis

Among 26 cytokines tested, three (CCL-2 (MCP-1), IL-8, and IL-5) showed significant decrease (FDR ; $p < 0.01$) in the sera from ACS volunteers at the follow-up (Fig. 4), and no cytokine was increased at the

follow-up. In the placebo group, no significant changes were detected in any cytokines between the baseline and follow-up time points. All data (mean ± SD) for the baseline and follow-up time points for the two groups are shown in Supplementary Table 2.

We performed a microarray analysis of the blood samples obtained from volunteers at the baseline and follow-up time points. For the analysis, we focused on the genes categorized as encoding ‘inflammatory related genes including soluble factors such as chemokines and cytokines’, which would be expected to affect nerve cells or cerebrovascular cells. Supplementary Figure 1 shows the changes in expression of ‘inflammatory related’ genes by microarray analysis, even if the significance level was below the multiple correction level. We also examined the gene expression levels for the 26 cytokines analyzed above, to evaluate changes between the baseline and follow-up time points in each patient, but we did not detect any significant differences in their gene expression levels in PBMCs (data not shown).

MRI analysis

Participants were evaluated by a perfusion MRI method, pulsed arterial spin labeling (pASL). To assess longitudinal brain perfusion changes induced by the supplementation, we compared 3D pASL data between the ACS and placebo groups, using the baseline and follow-up test data. Whole brain pASL data analysis revealed a tendency for brain perfusion preservation at the PCC area in the ACS group (Fig. 5), whereas the inverse calculation did not reveal any findings. By using a PCC ROI offered by the toolbox wfu_pickatlas in the SPM software, we observed a significant preservation of brain perfusion in the ACS group ($p = 0.0228$; Fig. 6), after adjusting for age ($F[1] = 5.5051$, $p = 0.0248$).

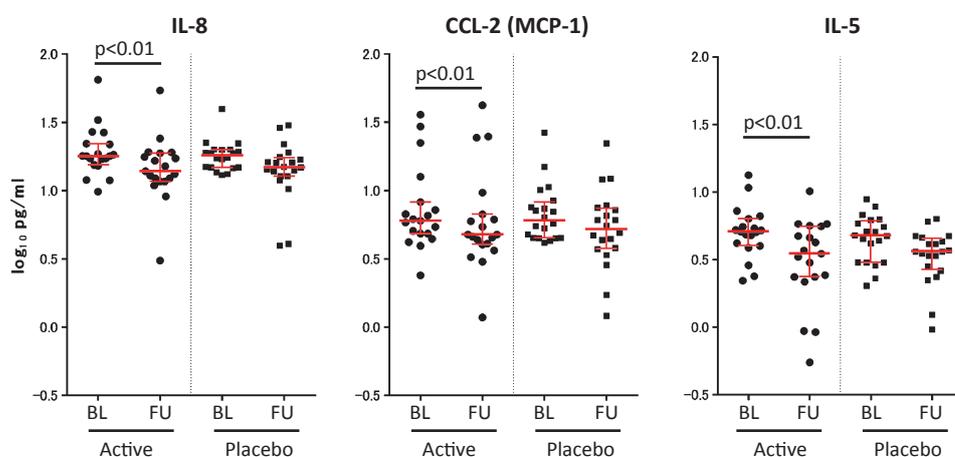


Fig. 4. Significant decreases in inflammatory cytokines in the ACS Group. Of 26 cytokines tested, three (IL-8, CCL-2 (MCP-1), and IL-5) were significantly decreased ($p < 0.01$, after FDR) in the sera of the ACS group. No cytokines significantly decreased in the placebo group. Each black dot represents the concentration of cytokine in each volunteer at the indicated test. Active ($n = 19$), Placebo ($n = 20$). Red bar shows median, and red error bar shows 25–75 percentile. BL, Baseline; FU, Follow-up.

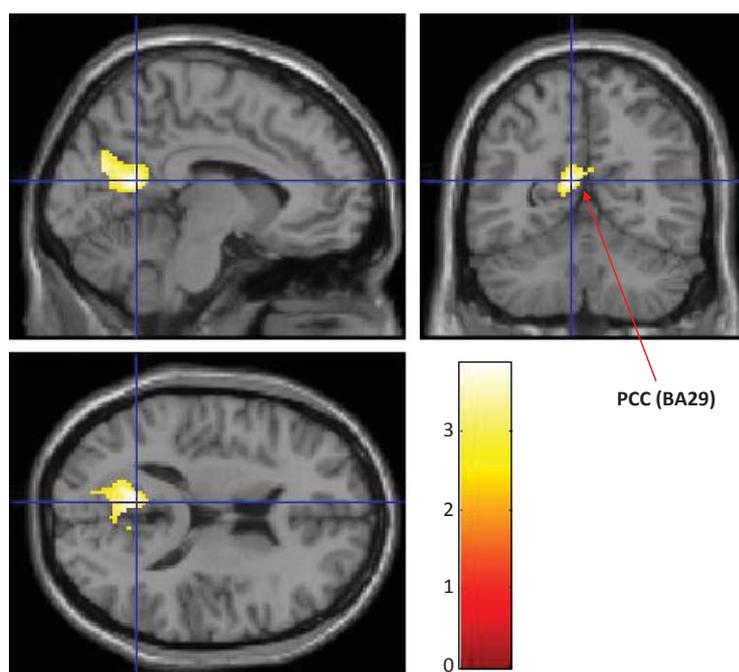


Fig. 5. Analysis and location of longitudinal brain perfusion changes. Color indicates the brain region that showed a difference in brain perfusion changes between the two groups, after repeated two-way ANOVA, interaction Time \times Variant (active $>$ placebo, $p < 0.01$, Voxel threshold > 500). Due to low image quality, we eliminated one individual from the ACS group; therefore, we compared 38 participants in the ACS ($n = 18$) and placebo ($n = 20$) groups. Inverse calculation showed no plot. The locations are plotted on a template human brain. Red arrows with PCC (BA29) shows a cluster of perfusion preservation after supplementation (Cluster Size: 797; Peak $(x, y, z) = (-8, -58, 16)$ at Z-score of 3.51).

Another set of RCT

To confirm whether ACS would preserve verbal memory in elderly people, we recruited healthy participants (60–80 years of age) and tested the WMS-LM2

test at the baseline and the 6-month follow-up. The group characteristics are summarized in Table 4. The two groups did not differ significantly with respect to age, gender, body mass index, education, or MMSE score. For the WMS-LM2 test, we used story A for the

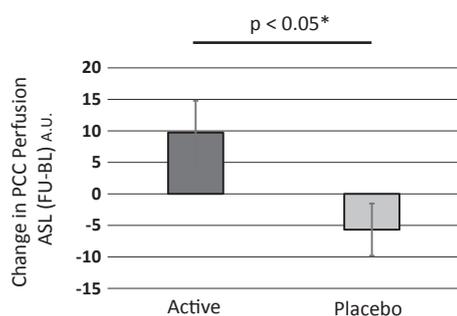


Fig. 6. Longitudinal change in PCC perfusion change between the two groups. We used a PCC ROI defined by a public domain program for the SPM software: toolbox, wfu_pickatlas, and performed a ROI analysis. We observed a significant preservation of the brain perfusion of the PCC in the ACS group ($p=0.0228$). After adjusting for age, we detected a significant difference between the two groups ($F(1)=5.5051$, $p=0.0248$).

Table 4

Baseline characteristics of another RCT participants who completed 6 month-test

	Active (42)	Placebo (42)	<i>p</i>
Age (mean \pm SD)	69.4 \pm 5.9	70.4 \pm 5.7	0.43
Gender (M/F)	19/23	18/24	0.83
BMI	22.9 \pm 2.7	22.3 \pm 2.7	0.29
Education (y)	14.9 \pm 1.9	14.6 \pm 3.0	0.52
MMSE	27.7 \pm 1.7	27.7 \pm 1.9	0.85

Significance was determined according to Student *t*-test, and chi-square test (for gender).

baseline and the follow-up tests. Data were analyzed using a two-way repeated ANOVA (Time [baseline or 6-month follow-up] \times Variant [ACS or placebo]). The interaction Time \times Variant was significant before ($F[1,82]=5.509$, $p=0.0213$; Fig. 7), and after adjusting for age ($F[1]=5.6125$, $p=0.0202$).

DISCUSSION

In this study, we detected a significant preservation of verbal episodic memory function in healthy elderly people treated with an anserine/carnosine supplement in the two separate tests. Using a WMS-LM test to evaluate cognitive function [11, 31], we obtained significant results in the delayed recall (WMS-LM2) test, but not in the immediate recall (WMS-LM1) test, suggesting that ACS may have a beneficial effect on verbal memory registration, but not on short-term working verbal memory. We also applied a set of psychological tests and health questionnaires, including the ADAScog, BDI, and SF-36 (both mental and physiological domains), to evaluate the effect of ACS on the cognitive and mental functions of the elderly

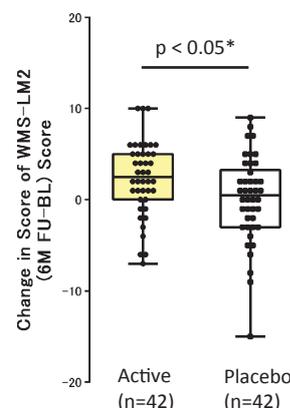


Fig. 7. Longitudinal change in score of WMS-LM2 between the two groups in another RCT. Each black dot represents the change of score in each volunteer between the baseline score and the 6 month follow-up score. Active ($n=42$), Placebo ($n=42$). Solid bar shows median, and box shows 25–75 percentile. BL, Baseline; FU, 6 Month Follow-up. We observed a significant improvement of WMS-LM2 score in the ACS group before ($F[1,82]=5.509$, $p=0.0213$), and after adjusting for age ($F[1]=5.6125$, $p=0.0202$).

volunteers, as we expected we did not detect any significant differences between the ACS and placebo groups. We assessed the effect of the supplementation on cognitive function using the data from all of the volunteers (age 41–78 years), but we did not detect any significant differences between the ACS and placebo group (data not shown).

Given that the delayed-recall tests of verbal memory, such as the WMS-LM delayed recall (WMS-LM2) test or the free and cue selective reminding test (FCSRT) [32–34], are considered a sensitive battery for detecting a cognitive decline of elderly people, our findings indicate that ACS may inhibit a cognitive decline, as detected by the verbal memory test. In line with our data, Szcześniak et al. [9] also suggested that ACS inhibits the decline in delayed recall, but not immediate recall, of memory in elderly people, as assessed using the STMS test [32].

To evaluate brain perfusion, especially in the PCC region, where an age-dependent decline in perfusion is reported to occur [12, 35, 36], we used a perfusion MRI method, 3D-pulsed ASL, with a FAIR labeling scheme [37] and a post-label delay time of 2350 ms. With this long delay time, capillary components rather than artery components contribute to the signal, according to the model proposed by Li et al. [38].

Given this background, we examined the level of inflammatory cytokines in serum, which may reflect atherosclerotic changes in the body's cardiovascular system. We detected significant decreases in the

concentrations of IL8 and CCL2 in the ACS group after supplementation, in agreement with our previous finding that carnosine treatment decreases the production of IL8 in TNF-treated cells [39]. CCL2 is upregulated during inflammation and can disrupt the integrity of the brain-blood barrier [40]. Our microarray analysis of the blood cells from the volunteers did not reveal any decline in the gene expression levels of inflammatory cytokines, suggesting that the decrease in the blood concentration of IL8 and CCL2 may have resulted from regulation at the cardiovascular cells, such as endothelial cells or smooth muscle cells, rather than at the blood cells.

To explore alternative scenarios for the effect of ACS on verbal episodic memory learning as assessed by the WMS-LM2 delayed recall, we performed a number of MRI scans, including VBM and DTI. Although previous studies showed a correlation of the verbal episodic memory score with a decline in hippocampal volume [41, 42] or with white matter microstructural change [43], we did not observe any significant difference in these brain structures between the two groups. Taken together, it is reasonable to speculate that ACS has a preservative effect against a cognitive decline through its ability to suppress neurovascular damage.

How ACS suppresses neurovascular damage and maintains brain blood flow is still unknown. In humans, the concentration of carnosine and its derivatives, such as anserine, is high in muscle tissues, in the millimolar range [6, 44, 45]. In our immunoassay, we detected a decrease in the serum concentration of inflammatory cytokines, but we did not detect a change in their gene expression level in PBMCs, suggesting that the difference emerged, not from blood cells, but from cardiovascular cells, such as pericytes. Recently, Zlokovic and his colleagues elegantly demonstrated the role of brain blood vessel pericytes in enabling brain microcapillaries to maintain brain blood flow and suggested that pericyte degeneration contributes to the etiology of AD [46, 47]. It can be speculated that ingested anserine and carnosine are taken up by the pericytes of brain microcapillaries, where they inhibit degenerative cellular changes; however, further studies are required to determine the precise mechanisms by which ACS affects brain function.

Our study has various limitations. The sample size was limited, and represented an age-based subgroup analysis from a larger original group that included data from younger subjects. To confirm main effect of ACS on verbal memory, we added an interim data from another RCT in this communication. In this study, we used a mixture of anserine and carnosine from

chicken meat extract, which also contained a small amount of creatinine that induced a transient increase in the blood creatinine level. Anserine and carnosine were also ingested through the subjects' normal diet. In this study, we tried to estimate the participants' dietary intake of anserine and carnosine by using a questionnaire. Although the survey results indicated that the anserine and carnosine consumption from the diet was equivalent between the ACS and placebo groups in our study, a better method is needed to estimate their levels more accurately.

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SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: <http://dx.doi.org/10.3233/JAD-150767>.

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