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Original Research

Effects of Folic Acid and Vitamin B12 Supplementation on Cognitive Impairment and Inflammation in Patients with Alzheimer's Disease: A Randomized, Single-Blinded, Placebo-Controlled Trial

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Abstract

OBJECTIVES: To evaluate the combined action of folic acid and vitamin B12 supplementation on cognitive performance and inflammation in patients with Alzheimer's disease (AD).

DESIGN: This was a randomized, single-blind, placebo-controlled trial.

PARTICIPANTS: Patients (n=120) diagnosed clinically as probable AD and in stable condition from Tianjin Key Laboratory of Cerebrovascular and Neurodegenerative Diseases.

MEASUREMENTS: Individuals were randomly divided into the intervention group (n=60, folic acid 1.2 mg/d + vitamin B12 50 μg/d) and the placebo group (n=60). Cognitive performance, blood folate, vitamin B12, one carbon cycle metabolite, and inflammatory cytokine levels were measured at baseline and after 6 months. The data were analyzed using linear mixed models for repeated measures.

RESULTS: A total of 101 participants (51 in the intervention group and 50 in the placebo group) completed the trial. Folic acid plus vitamin B12 supplementation had a beneficial effect on the MoCA total scores (P=0.029), naming scores (P=0.013), orientation scores (P=0.004), and ADAS-Cog domain score of attention (P=0.008), as compared to those of the control subjects. Moreover, supplementation significantly increased plasma SAM (P<0.001) and SAM/SAH (P<0.001), and significantly decreased the levels of serum Hcy (P<0.001), plasma SAH (P<0.001), and serum TNFα (P<0.001) compared to in the control subjects.

CONCLUSIONS: Folic acid and vitamin B12 supplementation showed a positive therapeutic effect in AD patients who were not on a folic acid-fortified diet. The findings of this study help to delineate nutrient intervention as far as public health management for the prevention of dementia is concerned.

Key words: Alzheimer's disease, folic acid, vitamin B12, inflammation, cognitive performance.

Introduction

Alzheimer's disease (AD) is one of the most common causes of dementia, and while recent advances in symptomatic treatments targeting cognitive functions in dementia have been made, effective disease treatment remains to be achieved (1, 2).

Modifying variable risk factors has attracted special attention in the prevention of dementia, especially through nutritional strategies (3). An international consensus statement suggested that vitamin B supplementation should not be ignored for its public health significance (4). The B vitamins folic acid and vitamin B12 have many functions in the nervous system required for brain health. Folic acid and vitamin B12 can downregulate the Hcy level in AD; Hcy is an independent risk factor for AD, and folate and vitamin B12 insufficiency impels S-adenosylmethionine (SAM) to convert to S-adenosylhomocysteine (SAH), which is then converted to Hcy, and decreases methylation potential, which is associated with AD patients (5). Elevated levels of total plasma Hcy causes central nervous system damage, cognitive impairment, dementia, and AD (6). Hcy can also drive an immuno-inflammatory response by enhancing the production of pro-inflammatory cytokines related to psoriasis (7). Inflammation is reportedly involved in AD pathogenesis, and a number of important pro-inflammatory cytokines, including tumor necrosis factor alpha (TNFα), interleukin 6 (IL6), and monocyte chemotactic protein 1 (MCP1), and anti-inflammatory cytokines, including interleukin 2 (IL2) and interleukin 10 (IL10) (8), may play a role in AD progression.

However, findings on the efficacy of folic acid and vitamin B12 in the treatment of AD are inconsistent. A clinical trial of high-dose B vitamin supplementation in individuals with AD showed that it had no beneficial effect on primary cognitive function or rate of change in ADAS-cog score during 18 months in environments with folate enrichment of grains, such as in the United States (9). However, folic acid fortification might have undermined and concealed the statistical outcomes of the treatment. In the present study, we aimed to investigate the treatment outcomes of folic acid and vitamin B12 together in AD subjects who were not on a folate-fortified diet, as well as the modification of specific inflammatory

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markers.

Materials and methods

Research design and subject characteristics

The study was conducted by the Department of Nutrition and Food Science, Tianjin Medical University, and Department of Neurology, Tianjin Key Laboratory of Cerebrovascular and Neurodegenerative Diseases, Tianjin Huanhu Hospital, China (ChiCTR-IOR-16009731). The trial was a single-center, single-blind, placebo-controlled, parallel-group, randomized controlled trial. The effectiveness of the combination of 1.2 mg/d of folic acid and 50 μg/d of vitamin B12 in mitigating the progression of cognitive decline in AD subjects, which were on acetylcholinesterase inhibitor (AChEI) or memantine treatment but without vitamin B supplementation for three months before baseline, was monitored. Participants did not know if they were in the treatment or placebo groups.

Study design

The participants were enrolled between September 2016 and August 2018 by neurologists at Tianjin Huanhu Hospital. Patients diagnosed clinically as probable AD and in a stable condition were included in the study (10). Inclusion criteria included a Montreal Cognitive Assessment (MoCA) score of less than 22 for people over 45 years of age (11). Exclusion criteria included the presence of encephalopathy with overlapping clinical symptoms as that of AD, or the consumption of any kind of nutritional supplement within three months before baseline that interfered with nutritional status, folate, and vitamin B12 metabolism. The details of the recruitment process are shown in Figure 1.

Randomization, treatment, and compliance

All participants were on dementia medication as a basic routine therapy. After baseline diagnosis, eligible participants were randomly and equally distributed into two groups, in accordance with the random number table. The subjects did not know the random number.

The folic acid and vitamin B12 groups received three tablets of 400μg of folic acid (1.2 mg/d) and two tablets of 25 μg of vitamin B12 (50μg/d), while the placebo group received three starch tablets similar to folic acid tablets and two starch tablets similar to the vitamin B12 tablets. There were no differences in flavor, shape, color, or size of medication between the two groups. Vitamins and placebo were administered in the form of identical oral tablets. Folic acid tablets were produced by Beijing Scrien Pharmaceutical Co., Ltd., China, 400μg/tablet, national medical license no. h10970079, and vitamin B12 tablets were produced by Shanxi Lifeng Huarui Pharmaceutical Co., Ltd., 25μg/tablet, national medical license no. h14023061.

The participants were instructed to take five tablets daily immediately after breakfast for six months. The participants received vitamins per prescription by hospital doctors. The researchers encouraged and monitored the compliance of the two groups through regular telephonic follow-up calls and blood analysis. Data were captured by two different individuals and monitored electronically to ensure accuracy.

Standard protocol approvals, registrations, and patient consent

Each participant had a main caregiver, and both the participants and their caregivers signed informed consent before participating in the trial. The study was carried out in accordance with the Declaration of Helsinki and the International Conference on Harmonization Good Clinical Practice guidelines. This study was approved by the ethics committee of the Tianjin Health Service.

Outcome measures

Assessment of cognitive function

To evaluate cognitive function, the subjects underwent a professional neuropsychological assessment at the baseline and six months thereafter by a senior neuropsychologist. Cognitive function was assessed using the MoCA and the Alzheimer's Disease Assessment Scale-Cognitive subscale (ADAS-cog) (11, 12). The MoCA is a 10-minute, 30-point cognitive screening test designed to assess global cognitive function, with lower scores indicating greater cognitive impairment. The MoCA contains seven subtests, including visuospatial/executive ability, naming, attention(attention digits, attention letters, and attention subtraction), orientation, language(language repeat, and language fluency), abstraction, and delayed recall. The ADAS-Cog was used to assess the severity of AD. Total scores on the ADAS-cog range from 0 to 70, and a higher total score indicates poorer cognitive performance. The ADAS-cog subscales were grouped into three domains: language(spoken language, comprehension, word finding, naming, and remembering test instructions), memory (word recall, word recognition, and orientation), and praxis (commands, constructional praxis, and ideational praxis). Nurses and biomedical scientists experienced in cognitive testing administered the MoCA and ADAS-Cog evaluations.

Blood sampling and analysis

Venous blood was collected at baseline and after six months. Blood was collected after overnight fasting for 10-12 h into two test tubes for evaluation. The first tube containing the clotting agent was allowed to settle for 30 min and then centrifuged for 10 min at 3000 rpm to obtain the serum, which was subsequently frozen at -80°C until further use. The folate, vitamin B12, Hcy, IL2, IL6, IL10, MCP1, TNFα, Aβ40, and Aβ42 levels were evaluated. Another tube containing EDTA was centrifuged immediately for 10 min at 2500 rpm at 4°C to obtain the plasma, which was frozen at -80 °C until further use. Plasma SAH and SAM levels were assessed.

The concentrations of serum folate and vitamin B12 were evaluated using an Abbott Architect-i2000SR automated chemiluminescence immunoassay system and its supporting kit (Abbott, USA). Serum Hcy levels were analyzed using a Hitachi 7180 automatic biochemistry analyzer (Japan). The concentrations of plasma SAH and SAM were quantified relative to standards (Sigma Chemical Co., St. Louis, MO, USA) using high-performance liquid chromatography. Serum IL2, IL6, IL10, MCP1, and TNFα, were determined by the Merck Millipore liquid-chip multi-factor product detection technology. Serum Aβ40, and Aβ42 levels were quantitatively analyzed using a standard ELISA kit (Biospec, Camarillo, CA).

Statistical analysis

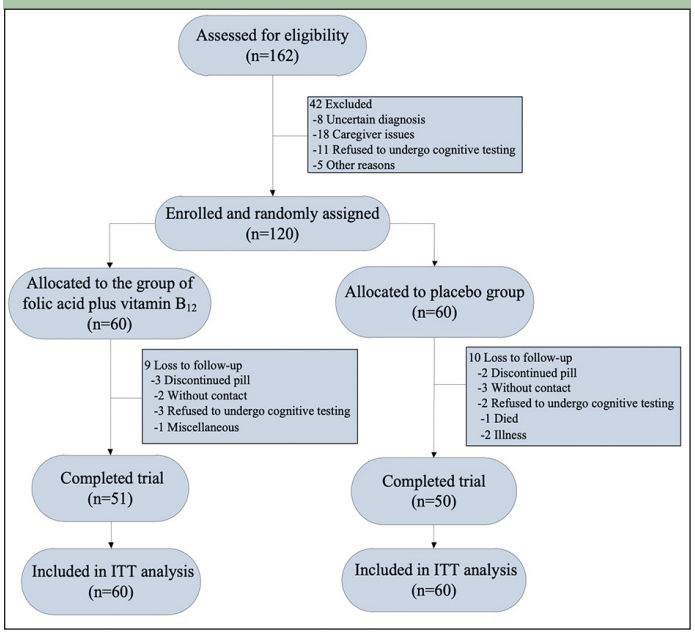
Statistical analysis was performed using SPSS version 18.0 (SPSS Inc., Released 2009: Chicago, IL, USA). Data are represented as medians (quartiles), mean±standard deviations (SD), or proportions. The chi-square test for categorical variables and two-tailed Student's t-tests for quantitative variables at baseline were used to compare the groups. Linear mixed models for repeated measures were used to evaluate the difference between groups in each outcome variable at different time points as compared to the baseline. Linear mixed models for repeated measures were used to estimate β(95% CI) to evaluate the differences among treatments. In the linear mixed models, the β coefficient indicates the change in neuropsychological index after six months of treatment. A positive β coefficient for longitudinal analysis indicated that an increase in the neuropsychological index was associated with treatment over time. The group effect indicates the difference between the two groups, the time effect indicates the difference between time points, and the interaction effect indicates the difference between the two groups over time. Statistical significance was set at $P<0.05$. The enrolled patients were randomly assigned to the intention-to-treat analysis at baseline.

Results

Participant characteristics

The process of participant recruitment and enrollment during the trial are shown in Figure 1. Out of a total of 162 AD patients, 42 patients with AD were excluded because 8 had an uncertain diagnosis, 18 had caregiver issues, 11 refused to receive a cognitive evaluation, and 5 were excluded for unknown reasons. A total of 120 patients were included in the trial and randomly divided into two groups. Sixty patients were placed in the vitamin B12 and folic acid supplementation groups, and 60 patients were placed in the placebo group.

Figure 1. Flow diagram for recruitment, randomization, and follow-up in the trial



The baseline characteristics of the participants are presented in Table 1. Family history refers to patients with dementia in their parents or siblings. No significant differences were observed between individuals in the two groups. Furthermore, no significant differences were observed in the folate and vitamin B12 levels at baseline between individuals in the treatment and placebo groups ($P>0.05$).

Cognitive status

The effects of treatment on cognitive performance are shown in Table 2. Linear mixed models for repeated measures indicated that after 6 months, the significance of the interaction effect in MoCA total scores (β [95% CI]:1.033 [0.105, 1.961], P=0.029) indicates that an increase in MoCA total scores was associated with treatment over time. The significance of the interaction effect in MoCA

Table 1. Baseline characteristics of the study population

Item	Folate and vitB12 group (n = 60)	Control group (n = 60)	P-value
Age at diagnosis, years (mean ±SD)	68.58±7.29	68.02±8.34	0.692
Males, n (%)	30 (50.00)	26 (43.33)	0.464
Education, years (mean ±SD)	10.32±3.89	10.23±4.37	0.912
Right-handedness, n (%)	54 (90.00)	56 (91.80)	0.509
Marital status-married, n (%)	50 (83.33)	49 (81.67)	0.810
Live with others, n (%)	55 (91.67)	58 (95.08)	0.243
Smoke, n (%)	18 (30.00)	25 (41.67)	0.183
Alcohol, n (%)	12 (20.00)	9 (14.75)	0.471
BMI (mean ±SD, kg/m^2	22.88±3.13	23.61±2.99	0.196
Profession- Intellectual work, n (%)	27 (45.00)	24 (40.00)	0.580
Family history, n (%)	20 (33.33)	14 (22.95)	0.224
Comorbid disorders, n (%)	32 (53.33)	40 (66.67)	0.136
AChEI	55(91.67)	54 (90.00)	0.752
Memantine	7 (11.67)	3 (5.00)	0.322
Folate (nmol/L)	14.37 (9.52, 19.32)	16.08 (11.35, 24.58)	0.436
Vitamin B12 (pmol/L)	280.73 (182.97, 422.02)	267.45 (208.06, 427.00)	0.878
Hcy (μmol/L)	13.75 (11.70, 15.96)	13.21 (11.40, 16.79)	0.637
MoCA	11.92±6.51	12.72±6.56	0.434

The range of complications included hypertension, diabetes, cerebral infarction, cerebral trauma, coronary heart disease, CO poisoning, cerebral hemorrhage, exposure to heavy metals, Parkinson's disease, tumor, anxiety, and depression. BMI, body mass index; AChEI, acetylcholinesterase inhibitor; MoCA, Montreal Cognitive Assessment

naming scores (β [95% CI]:0.250 [0.053, 0.447], P=0.013) indicates that an increase in MoCA naming scores was associated with treatment over time. The significance of the group effect in MoCA orientation scores (P=0.015) indicates that there was a difference between individuals in the intervention and placebo groups.The significance of the time effect in MoCA orientation scores (P=0.024) indicates that there was a difference between time points.The significance of the interaction effect in MoCA orientation scores (β [95% CI]: 0.550 [0.183, 0.917], P=0.004) indicates that an increase in MoCA orientation scores was associated with treatment over time. The significance of the time effect in MoCA abstraction scores (P=0.036) indicates that there was a difference between the time points. The significance of the group effect in ADAS-Cog language scores (P=0.027) indicates that there was a difference between individuals in the intervention and placebo groups, and the significance of the time effect in ADAS-Cog language scores (P=0.047) indicates that there was a difference between time points. The significance of the group effect in ADAS-Cog attention scores (P=0.017) indicates that there was a difference between individuals in the intervention and placebo groups, and the significance of the interaction effect in ADAS-Cog attention scores (β [95% CI]: -0.675[-1.162, -0.188], P=0.008) indicates that a decrease in ADAS-Cog attention scores was associated with treatment over time.

Level of vitamin cofactors in Alzheimer's dementia

The levels of vitamin cofactors were evaluated at baseline and six-months in this trial (Table 3). According to the linear mixed models for repeated measures, individuals in the folic acid and vitamin B12 groups showed a significant group effect in serum folate level(P <0.001), indicating that there was a difference between individuals in the intervention and placebo groups. Significance of interaction effect in serum folate level (β [95% CI]:29.690 [21.315, 38.066], P<0.001) indicates that an increase in folate was associated with treatment over time. The significance of the interaction effect in serum vitamin B12 level (β [95% CI]:110.632 [56.070, 165.193], P<0.001) indicates that an increase in vitamin B12 was associated with treatment over time. The significance of the interaction effect in serum Hcy level (β [95% CI]:-3.101[-4.351, -1.852], P<0.001) indicates that a decrease in serum Hcy level was associated with treatment over time. The significance of group effect in plasma SAM level (P <0.001) indicates that there was a difference between individuals in the intervention and placebo groups. The significance of the interaction effect in plasma SAM level (β [95% CI]:52.707 [37.555, 67.858], P<0.001) indicates that an increase in plasma SAM level was associated with treatment over time. The significance of group effect in

Table 2. MoCA and ADAS-Cog test outcomes at baseline and at 6 months

Item	Group	n	Baseline	6 months	Interaction effect		Time effect		Group effect	
					β (95%CI)	P	β (95%CI)	P	β (95%CI)	P
MoCA	Treatment	60	11.92±6.51	12.72±6.56	1.033 (0.105, 1.961)	0.029*	-0.167 (-0.823, 0.489)	0.616	-1.850 (-4.171, 0.471)	0.117
	Placebo	60	12.73±4.75	12.42±5.65						
Visuospatial/Executive abilities	Treatment	60	1.97±1.41	2.08±1.50	0.183 (-0.103, 0.469)	0.207	-0.067 (-0.269, 0.136)	0.515	-0.267 (-0.891, 0.358)	0.399
	Placebo	60	2.05±1.31	1.98±1.31						
Naming	Treatment	60	2.05±1.05	2.22±0.92	0.250 (0.053, 0.447)	0.013*	-0.083 (-0.222, 0.056)	0.238	-0.383 (-0.847, 0.080)	0.104
	Placebo	60	2.18±0.87	2.10±0.88						
Attention	Treatment	60	3.43±2.10	3.63±2.09	0.150 (-0.239, 0.539)	0.446	0.050 (-0.225, 0.325)	0.719	-0.483 (-1.352, 0.385)	0.273
	Placebo	60	3.77±1.78	3.82±1.97						
Orientation	Treatment	60	3.02±1.97	3.27±1.96	0.550 (0.183, 0.917)	0.004*	-0.300 (-0.560, -0.040)	0.024*	-1.050 (-1.894, -2.206)	0.015*
	Placebo	60	3.52±1.70	3.22±1.71						
Language	Treatment	60	1.00 (0.00, 1.00)	1.00 (0.00, 1.00)	-0.017 (-0.230,0.196)	0.877	0.017 (-0.134, 0.167)	0.827	0.083 (-0.341, 0.508)	0.698
	Placebo	60	1.00 (0.00, 1.00)	1.00 (0.00, 1.00)						
Abstraction	Treatment	60	0.00 (0.00, 0.75)	0.00 (0.00, 1.00)	0.050 (-0.126, 0.226)	0.574	0.133 (0.009, 0.258)	0.036*	-0.033 (-0.342, 0.275)	0.831
	Placebo	60	0.00 (0.00, 0.75)	0.00 (0.00, 1.00)						
Delayed recall	Treatment	60	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	-0.117 (-0.281,0.048)	0.164	0.083 (-0.033, 0.200)	0.159	0.250 (-0.079, 0.579)	0.135
	Placebo	60	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)						
ADAS-Cog	Treatment	24	24.50±13.79	23.23±12.80	-4.242 (-9.688, 1.204)	0.123	2.967 (-1.306, 7.239)	0.168	7.848 (-2.858, 18.554)	0.146
	Placebo	15	20.89±9.83	23.86±14.84						
Registration	Treatment	24	16.46±8.14	16.10±7.55	-1.392 (-4.681, 1.898)	0.397	1.033 (-1.547, 3.614)	0.422	2.157 (-4.449, 8.872)	0.519
	Placebo	15	15.69±7.08	16.73±9.21						
Language	Treatment	24	4.50 (1.00,8.00)	3.50 (1.00,7.75)	-2.258 (-4.522, 0.006)	0.051	1.800 (0.024, 3.576)	0.047*	4.633 (0.555, 8.712)	0.027*
	Placebo	15	2.00 (0.00,5.00)	4.00 (1.00,6.00)						
Executive abilities	Treatment	24	1.00 (1.00,3.00)	1.50 (1.00,3.00)	0.083 (-0.675, 0.842)	0.825	0.000 (-0.595, 0.595)	1.000	-0.117 (-1.653, 1.419)	0.879
	Placebo	15	2.00 (0.00,2.00)	2.00 (1.00,2.00)						
Attention	Treatment	24	1.00 (0.00,1.00)	0.00 (0.00,0.00)	-0.675 (-1.162, -0.188)	0.008*	0.133 (-0.248, 0.515)	0.483	1.175 (0.219, 2.131)	0.017*
	Placebo	15	0.00 (0.00,1.00)	0.00 (0.00,1.00)						

Variables are presented as median (P25, P75) or mean±SD. The cognitive function was analyzed by using linear mixed models for repeated measures.* P<0.05 compared with placebo group.

plasma SAH level (P <0.001) indicates that there was a difference between individuals in the intervention and placebo groups, and the significance of the time effect in plasma SAH level (P <0.001) indicates that there was a difference between time points. Significance of interaction effect in plasma SAH level (β [95% CI]:-29.380[-36.534, -22.225], P<0.001) indicates that a decrease in plasma SAH level was associated with treatment over time. Significance of group effect in plasma SAM/SAH (P <0.001) indicates that there was a difference between individuals in the intervention and placebo groups. The significance of the interaction effect in plasma SAM/SAH (β [95% CI]:1.000 [0.587, 1.414], P<0.001) indicates that an increase in plasma SAM/SAH was associated with treatment over time.

Levels of peripheral inflammatory cytokines and biomarkers in Alzheimer's dementia

The concentrations of inflammatory cytokines and biomarkers in Alzheimer's dementia were evaluated for six months in the current trial (Table 4). According to the linear mixed models for repeated measures, the significance of group effect in serum TNFα level (P=0.003) indicates that there was a difference between individuals in the intervention and placebo groups. The significance of the time effect in serum TNFα level (P =0.043) indicates that there was a difference between time points; significance of interaction effect in serum TNFα level (β [95% CI]:-37.105[-56.811, -17.398], P<0.001) indicates that a decrease in serum TNFα level was associated with treatment over time. The significance of the effect of time on serum IL2 levels (P=0.036) indicates that there was a difference between the time points.

Discussion

This was a single-blind, randomized, placebo-controlled trial of daily oral folic acid (1.2 mg/d) and vitamin B12 (50 μg/d) supplementation in individuals with AD conducted over six months. In this trial, we found that the MoCA total scores, naming, and orientation increased in individuals in the treatment group compared to individuals supplemented with placebos. The variation in total ADAS-Cog score did

Table 3. The concentrations of vitamin cofactors in blood at the baseline and 6 months

Item	Group	n	Baseline	6 months	Interaction effect		Time effect		Group effect	
					β (95%CI)	P	β (95%CI)	P	β (95%CI)	P
Folate (nmol/L)	Treatment	60	14.37 (9.52, 19.32)	53.57 (29.22, 68.91)	29.690 (21.315,38.066)	<0.001*	-0.969 (-6.891, 4.953)	0.746	-27.013 (-42.339, -11.688)	<0.001*
	Placebo	60	16.08 (11.35, 24.58)	15.95 (11.33, 22.77)						
Vitamin B12 (pmol/L)	Treatment	60	280.73 (182.97, 422.02)	489.90 (345.11, 618.65)	110.632 (56.070,165.193)	<0.001*	33.115 (-5.466, 71.696)	0.092	-83.149 (-186.735, 20.437)	0.115
	Placebo	60	267.45 (208.06, 427.00)	284.42 (208.06, 458.91)						
Hcy (μmol/L)	Treatment	60	13.75 (11.70, 15.96)	11.70 (10.33, 13.52)	-3.101 (-4.351,-1.852)	<0.001*	0.503 (-0.380, 1.387)	0.262	3.367 (-0.901, 7.635)	0.121
	Placebo	60	13.21 (11.40, 16.79)	14.07 (12.00, 17.18)						
SAM (nmol/L)	Treatment	57	154.47±35.88	200.75±39.92	52.707 (37.555,67.858)	<0.001*	-6.423 (-17.430, 4.584)	0.250	-61.654 (-86.591, -36.717)	<0.001*
	Placebo	51	163.42±38.02	156.99±40.34						
SAH (nmol/L)	Treatment	57	133.81±51.51	114.75±56.63	-29.380 (-36.534,-22.225)	<0.001*	10.323 (5.125, 15.520)	<0.001*	51.949 (34.179, 69.720)	<0.001*
	Placebo	51	111.24±28.16	121.56±28.97						
SAM/SAH	Treatment	57	1.18 (0.96, 1.42)	1.91 (1.33, 2.68)	1.000 (0.587,1.414)	<0.001*	-0.126 (-0.426, 0.174)	0.406	-1.314 (-1.810, -0.819)	<0.001*
	Placebo	51	1.37 (1.24, 1.89)	1.25 (1.04, 1.51)						

Variables are presented as median (P25, P75) or mean±SD. Variables were analyzed by using linear mixed models for repeated measures.* P<0.05 compared with placebo group.

Table 4. The concentration of inflammatory cytokines and biomarkers in Alzheimer's dementia at baseline and 6 months in the trial

Item	Group	n	Baseline	6 months	Interaction effect		Time effect		Group effect	
					β (95%CI)	P	β (95%CI)	P	β (95%CI)	P
TNFα (pg/ml)	Treatment	41	44.83 (26.03, 103.22)	38.11 (22.73, 62.97)	-37.105 (-56.811,-17.398)	<0.001*	14.779 (0.492, 29.067)	0.043*	52.612 (18.508, 86.716)	0.003*
	Placebo	37	48.44 (29.56, 62.56)	54.54 (33.68, 84.58)						
MCP1 (pg/ml)	Treatment	41	616.70±186.03	600.12±188.33	-39.175 (-92.969,14.620)	0.151	22.599 (-16.402, 61.601)	0.252	72.788 (-46.254, 191.831)	0.227
	Placebo	37	583.09±214.51	605.68±206.75						
IL2 (pg/ml)	Treatment	24	0.13 (0.03, 0.41)	0.13 (0.03, 0.44)	-0.189 (-0.380,0.001)	0.051	0.153 (0.010, 0.295)	0.036*	0.162 (-0.183, 0.506)	0.348
	Placebo	19	0.16 (0.13, 0.46)	0.35 (0.13, 0.80)						
IL6 (pg/ml)	Treatment	36	4.25 (0.76, 19.58)	5.29 (1.40, 13.26)	-5.509 (-12.783,1.766)	0.135	3.658 (-1.486, 8.802)	0.161	13.478 (-2.873, 29.828)	0.105
	Placebo	36	1.16 (0.00, 6.34)	3.35 (1.37, 25.05)						
IL10 (pg/ml)	Treatment	30	1.32 (0.62, 2.33)	1.54 (0.79, 2.74)	-0.789 (-4.825,3.247)	0.697	0.975 (-1.788, 3.738)	0.483	-0.944 (-5.147, 3.258)	0.655
	Placebo	34	2.69 (1.64, 4.62)	1.79 (1.04, 3.01)						
Aβ40 (pg/ml)	Treatment	36	247.22 (101.53, 845.47)	535.73 (101.53, 1164.70)	245.300 (-323.818,814.419)	0.392	18.782 (-401.540, 439.104)	0.929	-364.437 (-1223.997, 495.123)	0.400
	Placebo	30	445.57 (151.60, 856.14)	665.86 (101.53, 1160.70)						
Aβ42 (pg/ml)	Treatment	41	0.96±0.61	0.95±0.64	-0.062 (-0.200,0.077)	0.377	0.049 (-0.051, 0.149)	0.334	0.178 (-0.112, 0.468)	0.225
	Placebo	37	0.84±0.34	0.89±0.36						

Variables are presented as median (P25, P75) or mean±SD. Variables were analyzed using linear mixed models for repeated measures.* P<0.05 compared with placebo group.

not differ between individuals in these two groups, but the ADAS-Cog domain score of attention decreased significantly in subjects in the treatment group compared to subjects in the placebo group. This study also demonstrated that vitamin B supplements decreased serum Hcy, serum TNFα and plasma SAH levels and increased plasma SAM levels and SAM/SAH.

Outcome of folic acid and vitamin B12 supplementation on cognition decline

This trial revealed the significance of the interaction effect in MoCA total, naming, and orientation scores, indicating that an increase in the respective scores was associated with treatment over time. We also found that the ADAS-Cog domain score of attention decreased significantly in subjects in the treatment group compared to subjects in the placebo group. A previous study showed that a tHcy concentration between 10.0 to 18.0 μmol/L in the blood of AD patients had a significant positive correlation with the rate of cognitive decline (13). Another study demonstrated that the incidence rate of dementia in the elderly with blood tHcy concentrations>15.0 μmol/L was almost 5 times higher than that in the elderly with tHcy<10.1μmol/L (14). Here, the treatment and placebo groups had 91.67% and 85% of the subjects, respectively, with Hcy levels greater than 10μmol/L. Of note, China does not have a folate-fortified food policy, which implies that the outcomes of this study were undoubtedly due to the effect of vitamin B supplementation, highlighting the significantly beneficial effect of this therapy.

The outcomes of this study are in line with those of previous studies on AD patients with mild dementia

(15, 16). The combination of folic acid and vitamin B12 decreased Hcy levels and mitigated cognitive decline in subjects with mild cognitive impairment (MCI) (17). In a randomized trial, 140 participants with mild to moderate AD or vascular dementia received 1 mg and 5 mg of folic acid and methylcobalamin, respectively, or placebo, once a day for 24 months. No apparent differences were observed in global cognition between the two groups, but the decline in the Chinese Mattis Dementia Rating Scale (construction domain) was lower in subjects in the treatment group than in subjects inthe placebo group, which contained patients with elevated total Hcy plasmalevels (>13 μmol/L) (18). Increased levels of Hcy are associated with a high risk of coronary stroke, heart diseases, and cognitive impairment, which commonly accompany AD and dementia (19, 20). In a study in MCI, vitamin B supplementation demonstrated efficacy as it reduced Hcy levels and delayed cognitive decline (21). Moreover, we found that folic acid could delay cognitive decline in patients with AD in previous studies (22). As folic acid and vitamin B12 interact with each other, this study explored the therapeutic potential of the combined effect of folic acid and vitamin B12 supplementation in AD patients.

Outcome of folic acid and vitamin B12 supplements on nucleotide derivatives of methionine

In this study, the significance of the interaction effect in serum folate level indicated that an increase in folate level was associated with treatment over time, and the significance of the interaction effect in serum vitamin B12 level indicates that an increase in vitamin B12 was associated with treatment over time. Moreover, the interaction effects showed that the significant decrease in Hcy and SAH level, and significant increase in SAM and SAM/SAH level, was associated with treatment over time. We found that after 6 months, the level of folate, vitamin B12, and SAM increased by 39.2 nmol/L, 209.17 pmol/L, and 46.28 nmol/L, respectively, as compared to individuals in the placebo group, in which folate and SAM levels decreased by 0.13 nmol/L and 6.43 nmol/L, respectively, and vitamin B12 increased by16.97 pmol/L.

Vitamin B12 acts together with folate to provide methyl groups for Hcy-mediated SAM formation. SAM provides a methyl group for the methylation of proteins, DNA, and RNA. Demethylation of SAM leads to its conversion to SAH, and SAH, in turn, is hydrolyzed to form homocysteine to continue the methionine cycle (23, 24). Deficiency of folate and vitamin B12 can not only affect the formation and transformation of nucleotides, but also deplete the methylation levels of DNA and protein and increase the Hcy levels (25). Hyperhomocysteinemia can damage vascular endothelial cells and increase the risk for cardiovascular, cerebrovascular, and neurodegenerative diseases (26, 27). A meta-analysis indicated that the effects of homocysteine-lowering on cognitive decline was inconclusive, as the trials typically did not include individuals who were experiencing such decline (28). Trials in high-risk subjects, which have taken into account the baseline B vitamin status, show a slowing of cognitive decline and atrophy in critical brain regions which is consistent with the modification of the AD process (29).

Outcome of folic acid and vitamin B12 supplementation on inflammatory cytokines

In this study, the daily oral intake of folic acid and vitamin B12 supplements decreased serum TNFα levels, which was associated with treatment over time. Previous studies have shown that the TNFα level in thecerebrospinal fluid (CSF) of AD and MCI patients is higher than in individuals with normal cognitive functions (30, 31). TNFα synthesized by peripheral immunocompetent cells enters the brain parenchyma and CSF through a well-structured blood-brain barrier (32). The increased levels of peripheral cytokines are associated with stem cell malfunction and decreased hippocampal volume and memory (33-35). A systematic review showed that the concentration of inflammatory markers significantly changed in AD and MCI subjects compared to healthy subjects (36). This suggests that the inflammatory markers observed in the periphery and CSF are associated with AD and MCI.

In this study, we found a significant expression of inflammatory factors, such as TNFα protein, in subjects in the vitamin B group compared to subjects in the placebo group. This indicates that folic acid, coupled with vitamin B12, significantly affects the inflammatory process in AD. In addition, this study has shown that inflammatory factors play a crucial role in AD. We propose that the underlying mechanism of the effects of folic acid plus vitamin B12 on AD is that folic acid, a methyl donor, and vitamin B12 as a cofactor, inhibits the expression of inflammation-related proteins in the TNFα pathway by DNA methylation; however, further in-depth investigation is required to verify this.

This study had certain limitations. The short duration of the study would have undermined the therapeutic effects of folate and vitamin B12. In addition, the methylation levels of the inflammatory factors were not evaluated, which should be examined in future studies.

Conclusion

In this study, we found that folic acid together with vitamin B12 improved cognitive performance, decreased Hcy and SAH levels, increased SAM levels, and inhibited the expression of inflammatory factors. Folic acid and vitamin B12 supplementation showed a certain therapeutic effect in AD subjects who were not on a folate-fortified diet. These findings have public health

management implications for the prevention of dementia. Moreover, the combined action of folic acid and vitamin B12 significantly affects the inflammatory process in patients with AD, which deserves future consideration in subsequent investigations.

Conflicts of interest: None declared.

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Ethical standards: The study was carried out in accordance with the Declaration of Helsinki and the International Conference on Harmonization Good Clinical Practice Guidelines.

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