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ORIGINAL CONTRIBUTION

Effects of low-dose B vitamins plus betaine supplementation on lowering homocysteine concentrations among Chinese adults with hyperhomocysteinemia: a randomized, double-blind, controlled preliminary clinical trial

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Abstract

Purpose To test the hypothesis that daily supplementation with low-dose B vitamins plus betaine could significantly reduce plasma homocysteine concentrations in Chinese adults with hyperhomocysteinemia and free from background mandatory folic acid fortification.

Methods One hundred apparently healthy adults aged 18–65 years with hyperhomocysteinemia were recruited in South China from July 2019 to June 2021. They were randomly assigned to either the supplement group (daily supplementation: 400 µg folic acid, 8 mg vitamin B_6, 6.4 µg vitamin B_{12} and 1 g betaine) or the placebo group for 12 weeks. Fasting venous blood was collected at baseline, week 4 and week 12 to determine the concentrations of homocysteine, folate, vitamin B_{12} and betaine. Generalized estimation equations were used for statistical analysis.

Results Statistically significant increments in blood concentrations of folate, vitamin B_{12} and betaine after the intervention in the supplement group indicated good participant compliance. At baseline, there were no significant differences in plasma homocysteine concentration between the two groups ($P=0.265$). After 12-week supplementation, compared with the placebo group, there was a significant reduction in plasma homocysteine concentrations in the supplement group (mean group difference − 3.87; covariate-adjusted $P=0.012$; reduction rate 10.1%; covariate-adjusted $P<0.001$). In the supplement group, the decreased concentration of plasma homocysteine was associated with increments of blood concentrations of both folate ($\beta=-1.680$, $P=0.004$) and betaine ($\beta=-1.421$, $P=0.020$) after 12 weeks of supplementation.

Conclusions Daily supplementation with low-dose B vitamins plus betaine for 12 weeks effectively decreased plasma homocysteine concentrations in Chinese adults with hyperhomocysteinemia.

Trial registration This trial was registered at clinicaltrials.gov as NCT03720249 on October 25, 2018. Website:https://clinicaltrials.gov/ct2/show/NCT03720249 .

Keywords Homocysteine · B vitamins · Folic acid · Betaine · Hyperhomocysteinemia · Randomized controlled trial · China

Abbreviations

ALP	Alkaline aminotransferase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BMI	Body mass index
CI	Confidence interval
COVID-19	Coronavirus disease 2019
CRFs	Case report forms
CVDs	Cardiovascular diseases
DRIs	Dietary reference intakes
GEEs	Generalized estimation equations

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HDL-C	High-density lipoprotein cholesterol
HPLC–FLD	High-performance liquid chromatography with fluorimetric detector
HPLC–MS/MS	High-performance liquid chromatography–tandem mass spectrometry
Hs-CRP	High sensitivity C-reactive protein
ITT	Intention-to-treat
LDL-C	Low-density lipoprotein cholesterol
METs	Metabolic equivalent tasks
MTHFR	Methylenetetrahydrofolate reductase
RCTs	Randomized controlled trials
SBD-F	7-Fluorobenzofurazan-4-sulfonic acid ammonium salt
SE	Standard error
TCA	Trichloroacetic acid
TCEP	Tris-(2-chloroethyl)-phosphate
USDA	The United States department of agriculture

Introduction

Homocysteine is an essential intermediate metabolite in the one-carbon cycle. According to the American Heart Association, hyperhomocysteinemia is defined as plasma concentrations of homocysteine higher than 15 μmol/L [1]. Numerous studies have reported that elevated homocysteine levels are positively associated with increased risks of several diseases, including cardiovascular diseases (CVDs), neurodegenerative diseases and cancers [2–4]. Therefore, attention has been paid to finding effective, safe, and inexpensive strategies to reduce plasma levels of homocysteine, such as nutritional interventions.

The homocysteine-lowering effects of B vitamins, including folic acid, vitamin B_6, and B_{12}, have been well-documented in previous clinical trials [5, 6]. A meta-analysis of 25 randomized controlled trials (RCTs) has demonstrated that daily supplementation with folic acid at doses of 0.2–5 mg (with or without additional B vitamins) reduces blood homocysteine concentrations by 13–25% [7]. Up to date, majority of nutritional interventional studies aimed at alleviating hyperhomocysteinemia have been conducted in patients or high-risk individuals [8, 9]. Although observational studies have revealed that higher dietary intakes of B vitamins are associated with lower risks of CVDs [10], most interventional studies that administrated B vitamins supplements in CVD patients have reported no apparent benefits for the CVD prognosis [6, 11]. In addition, it has been concerned that utilization of folic acid may prevent cancer in healthy individuals, but may potentially promote tumor growth in patients with established cancers [12, 13]. Collectively, nutritional interventions for reducing evaluated homocysteine concentrations may provide more potential benefits in primary prevention rather than treatment of homocysteine-related diseases. There is currently a lack of studies on the effects of nutritional interventions on reducing homocysteine concentrations in the generally healthy populations, especially in Asian countries [14, 15].

The dosages of B vitamins widely used in previous studies have ranged from 0.4 to 5 mg for folic acid, 5–50 mg for vitamin B_6 and 0.8–1 mg for vitamin B_{12} [7], most of which are higher than the recommended nutrient intakes of dietary reference intakes (DRIs) [16]. An earlier study by Wang et al. has reported that the homocysteine-reduction effects decline and steadily return back to baseline concentrations following supplementation cessation, suggesting a requirement for persistent supplementation to maintain the associated benefits [17]. However, side effects such as vascular events may exist after long-term supplementation with high dosages of B vitamins [18]. Similar to folate, betaine is another key methyl donor, which transfers the methyl group to homocysteine and reduces the circulating homocysteine concentration through one-carbon metabolic pathways [19]. A meta-analysis of five supplementation trials with betaine has reported that daily supplementation with at least 4 g betaine between 6 and 24 weeks reduces blood homocysteine concentration by 1.23 μmol/L [14]. A recent RCT in Gambia has demonstrated that a novel nutritional supplement combining B vitamins and betaine has enhanced efficacy in reducing blood homocysteine concentration compared to a conventional supplement of B vitamins without betaine [20]. This finding indicates that B vitamins administrated at a safer dosage plus betaine might be an alternative and effective strategy for the long-term management of evaluated homocysteine concentration.

Compared to people in western countries, Chinese people have been reported to have lower concentrations of circulating folate [21, 22] and a higher prevalence of hyperhomocysteinemia [23, 24]. According to a meta-analysis covering 19 provinces and municipalities with 60,754 subjects aged 3–97 years, the prevalence of hyperhomocysteinemia has been reported to be 27.5% in the Chinese population [23]. The higher homocysteine concentrations may be partially attributed to the diverse dietary habits and genetic polymorphisms among various ethnic groups. Folic acid fortification in foods is not mandatory in China, which may explain the lower circulating folate concentrations in Chinese residents compared to populations accessible to folic acid-fortified foods [21, 22]. In Chinese population, the higher mutation rate of methylenetetrahydrofolate reductase (MTHFR), a key enzyme in forming a bioactive form of folate, may also restrict the use of dietary folate to reduce homocysteine concentration in several individuals [25]. In addition, the results of studies in other ethnic groups may not be directly applied to Chinese population.

Therefore, in the current study, we aimed to conduct a randomized, double-blind, controlled trial to evaluate the efficacy of a novel supplementation with low-dose B vitamins plus

betaine in lowering blood homocysteine concentration among Chinese adults with hyperhomocysteinemia and free from background mandatory folic acid fortification. We hypothesized that daily supplementation with low-dose B vitamins plus betaine for 12 weeks could reduce plasma homocysteine concentrations in the generally healthy population with hyperhomocysteinemia, which may provide additional choices for the long-term management for hyperhomocysteinemia.

Methods

Study design and participants

The randomized, double-blind, placebo-controlled trial was conducted from July 2019 to January 2020 and from October 2020 to June 2021. Individuals were recruited from the First Affiliated Hospital of Sun Yat-sen University and the Third Affiliated Hospital of Sun Yat-sen University (Guangzhou, China) via posters or invitations from physicians and/or researchers. The inclusion criteria were: (1) aged 18–65 years; (2) concentrations of blood homocysteine were between 15 and 100 μmol/L; (3) blood aspartate aminotransferase (AST) concentration < 120 U/L, blood alanine aminotransferase (ALT) concentration < 105 U/L, and blood creatinine concentration < 116.0 μmol/L; (4) free from any supplements or drugs that may decrease blood homocysteine concentrations for at least 1 month prior to the beginning of study; (5) were willing to participate in the study. Individuals were excluded if they met any of the following criteria: (1) pregnant or lactating women; (2) had thyroid diseases or any types of cancers; (3) had any diseases that required hospitalization in the next 3 months; (4) were unable to complete the questionnaire. All procedures of the trial were conducted in accordance with the declaration of Helsinki and its later amendments. Written informed consent was obtained from all participants at enrollment. This study was registered at clinicaltrials.gov as NCT03720249 on October 25, 2018. It was approved by the Ethics Committee of the School of Public Health at Sun Yat-sen University with an approval number of 006 on February 28, 2019.

Randomization and blinding

Participants were randomized into the supplement or the placebo groups according to a computer-generated randomization procedure and randomization was stratified by sex. Two hundred serial numbers were assigned to one of the two arms in a 1:1 ratio using block randomization with a block size of four, stratified by sex. The researcher printed these serial numbers and labeled them onto an opaque box containing either the supplement or the placebo according to the group allocation. A researcher who was not involved in recruitment, data collection, laboratory measurement and statistical analysis was responsible for the randomization scheme. Participants, investigators and data analysts were all blind to the allocation.

Preparation of supplementation

Four 800-mg tablets were provided to the supplement or the placebo group per day. Supplement tablets included folic acid, vitamin B_6, vitamin B_{12} and betaine, while placebo tablets contained fillers without nutrients. The nutrient compositions of the supplements are shown in Table 1. As suggested in a previous study, zinc citrate (9.6 mg/d) was added to the supplement tablets to avoid the possible influences of zinc deficiency on the absorption and metabolism of dietary folate [26]. Previous studies have demonstrated that zinc supplementation does not modify plasma homocysteine concentrations [27]. Dosages of all nutrients were confirmed according to the requirements of the State Administration for Market Regulation (China) and studies reported previously [28]. Both intervention tablets with identical appearance, weight and taste were manufactured and packed by By-Health Corporation (Zhuhai, Guangdong, China).

Intervention procedures

At the baseline visit, participants were invited to the research center (School of Public Health, Sun Yat-sen University, Guangzhou, China) for a questionnaire survey, venous blood collection and anthropometric assessments. They were

Table 1 Nutrient compositions of the interventional products (per daily dose)

	Placebo group	Supplement group
Nutrients		
Folic acid	–	400 μg
Vitamin B_6	–	8 mg
Vitamin B_{12}	–	6.4 μg
Betaine	–	1 g
Zinc citrate	–	9.6 mg
Other fillers	Microcrystalline cellulose, maltodextrin, hydroxypropyl cellulose, silica, magnesium stearate, coating powder, etc.	

invited back to the research center for a midline visit at week 4 and an endline visit at week 12. Tablets for 6 weeks were provided at baseline and the remaining 6-week tablets were offered at the midline visit. At the endline visit, participants were instructed to return all the remaining tablets. Participants were encouraged to maintain their lifestyle and dietary habits as usual and to avoid the intake of supplements containing nutrients in our tablets during the entire course of the trial. Withdrawals, adverse events or other situations that needed to be recorded were documented in case report forms (CRFs). Compliance during the intervention was evaluated by determining the blood concentrations of folate, vitamin B_{12} and betaine as well as counting the returned remaining tablets.

Outcome measurement and sample size calculation

The primary outcome was the effects of low-dose B vitamins plus betaine supplementation on plasma homocysteine concentrations among participants with hyperhomocysteinemia. According to a previous study [29] with a 10% mean difference in homocysteine concentrations and a standard deviation of 1.26, sample size calculation was conducted with a two-tailed α level of 0.05 and 90% power. Result of the sample size calculation demonstrated that a total of 45 subjects were required per group. Considering a 10% dropout rate, we included 50 subjects in each group.

Blood sample collection and laboratory measurements

After an overnight fasting for at least 8 h, peripheral venous blood was drawn at baseline, week 4 and week 12 of the trial. Blood samples were centrifuged at 3000 × g for 15 min. Serum (in uncoated vacuum tubes) and plasma (in EDTA-coated vacuum tubes) were isolated and stored at − 80℃ immediately for subsequent measurement. All samples were measured and analyzed in one batch at the end of the trial.

Plasma homocysteine concentration was quantified by high-performance liquid chromatography with fluorimetric detector (HPLC–FLD) (Agilent Technologies 1260 Infinity, Agilent Technologies 1260 FLD Spectra, USA) [30]. Plasma was reduced by tris-(2-chloroethyl)-phosphate (TCEP), deproteinized by trichloroacetic acid (TCA), and derivatized by 7-fluorobenzofurazan-4-sulfonic acid ammonium salt (SBD-F) for the pretreatment of samples. Supernatants were then collected and injected into an HPLC C18 column (250 mm × 4.6 mm, 5 μm, Welch, Xtimate) directly coupled to the fluorimetric detector for separation and detection. The mobile phase consisted of methanol: 0.1 mol/L acetate buffer (pH = 4.5, 3.5:96.5, v/v) at a flow rate of 1 mL/min with equivalent elution. Excitation and emission wavelengths of the fluorimetric detector were set at 385 nm and 515 nm, respectively. The intraassay coefficient of variance was 10.24%.

Serum folate and vitamin B_{12} concentrations were measured using a chemiluminescent microparticle immunoassay at the KingMed Diagnostics Laboratory (Guangzhou, Guangdong, China). Plasma betaine concentration was determined by high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) (Agilent 6400 Series Triple Quad LCMS, CA, USA) as previously described [31, 32] and the intraassay coefficient of variance was 10.31%.

Glucose in capillary blood was tested with the glucose dehydrogenase method using the Gold-Accu series of Sinocare. Other routine laboratory indices, including AST, ALT, alkaline aminotransferase (ALP), cholesterol, triglyceride, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), high sensitivity C-reactive protein (hs-CRP) and creatinine were measured at the KingMed Diagnostics Laboratory (Guangzhou, Guangdong, China).

Sociodemographic data collection and anthropometric assessments

Sociodemographic information was collected at baseline using a structured questionnaire, including sex, race, date of birth, education levels (secondary school or below, high school or equivalent, and college or above), smoking status, alcohol consumption, dietary habits, physical activities, multivitamins use and medical history. At baseline and endline visits, blood pressures, heights and weights were measured by trained researchers using the same calibrated equipment with standard methods. Body mass index (BMI) was calculated as weight/height squared (kg/m^2). Alcohol drinkers were defined as participants who drank alcohol at least once a week consecutively for more than 6 months in their entire life, and smokers were those who smoked more than 100 cigarettes in their entire life. Dietary habits and physical activities over the past year and during the trial were collected by a validated 79-item semi-quantitative food frequency questionnaire [33] and a 19-item physical activity questionnaire [34], respectively. Dietary betaine intakes were estimated additionally based on the betaine contents of common foods [35] and the United States Department of Agriculture (USDA) Food Composition Database [36], while dietary intakes of energy and other nutrients were estimated based on the China Food Composition Database 2009 [37]. Data from 24-h physical activities were converted into the metabolic equivalent for activities including physical exercise and activities conducted during sedentary time, occupation, transportation and housework [34].

Statistical analysis

All the statistical analyses were conducted according to the intention-to-treat (ITT) principle. Data that were not under normal distribution were analyzed after natural logarithm or reciprocal transformation, if appropriate. Dietary intakes of nutrients were adjusted for energy intake using the residuals method before analysis [38]. Differences in baseline characteristics, changes in blood indices and dietary intakes at the same timepoints (△), were analyzed by one-way ANOVA, Kruskal–Wallis tests or Pearson's Chi-squared tests as appropriate for continuous and categorical variables, respectively. Generalized estimation equations (GEEs), Fisher's least significant difference test and repeated measures analysis of variance were used to compare the changes in concentrations of homocysteine, folate, vitamin B_{12} and betaine between groups (supplement group and placebo group) over time (baseline, week 4, and week 12). Linear regression analysis without and with adjustment for covariates, including sex, age, energy intake, BMI, smoking status, alcohol consumption, physical activities, blood folate, vitamin B_{12}, betaine and homocysteine concentrations at baseline, was performed to assess associations between changes in concentrations of folate, vitamin B_{12}, betaine and changes in concentrations of homocysteine after intervention in the supplement group. To eliminate the effects of dimension, data on blood concentrations of folate, vitamin B_{12} and betaine were z-score transformed before linear regression analysis. Stratified analysis was performed to explore whether the changes in plasma homocysteine concentrations in the supplement and placebo groups were different in various folate, vitamin B_{12}, betaine and homocysteine status at baseline. Interactions were subsequently evaluated by including the multiplicative interaction terms in the GEE models, which were calculated by multiplying plasma homocysteine concentrations and the stratification variables mentioned above.

Statistical analysis was performed using SPSS (version 25.0, IBM Corp., Armonk, NY, USA) and Stata (version 15.0, StataCorp., College Station, TX, USA). All statistical analyses were two-sided and $P < 0.05$ was considered as statistically significant.

Results

Baseline characteristics

As shown in Fig. 1, from July 2019 to January 2020 and from October 2020 to June 2021, one hundred eligible participants were enrolled and randomized to either supplement or placebo groups in the trial. Due to the occurrence of coronavirus disease 2019 (COVID-19), eight participants did not start the trial. Finally, a total of 92 participants attended baseline visits and started the interventions (44 in the supplement group and 48 in the placebo group). In the follow-up period, six (6.5%) and eleven (12.0%) participants withdrew from the trial at week 4 and week 12, respectively. Eighty-six

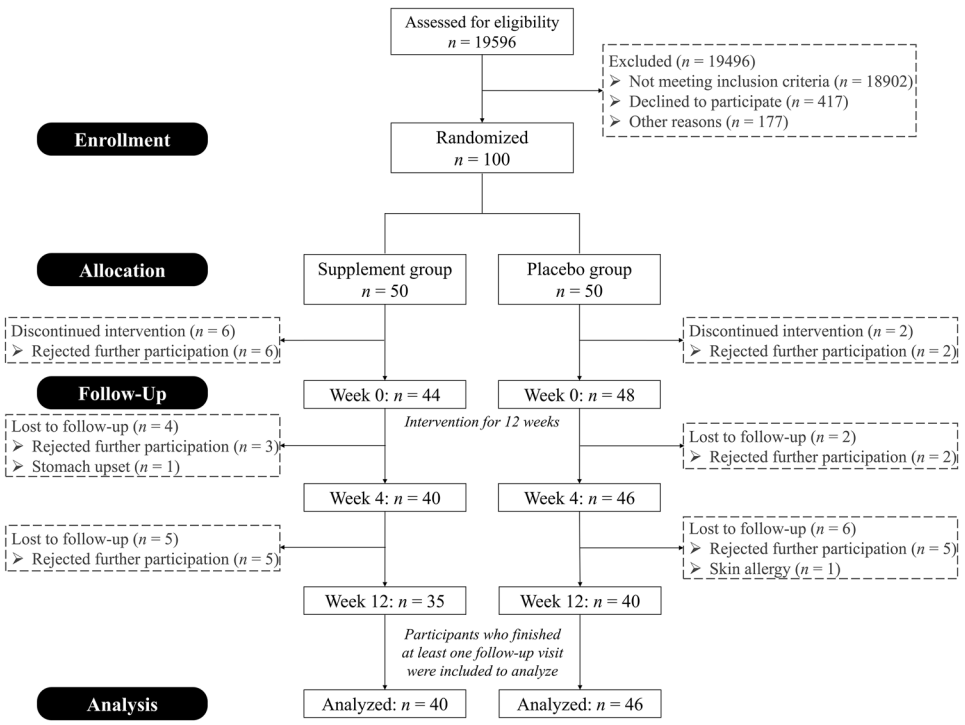

Fig. 1 Flow diagram of a randomized, double-blind, controlled trial investigating the effects of low-dose B vitamins plus betaine supplementation on plasma homocysteine concentrations among Chinese adults with hyperhomocysteinemia

participants completed at least two visits and blood samples were collected during the trial visits.

Of the 92 participants at baseline, there were 81 (88.0%) males and 11 (12.0%) females. The mean age was 43.3 ± 10.6 years. Baseline characteristics of participants were provided in Table 2. There were no significant differences in sex, age, height, weight, BMI, blood pressure, and physical activities between the supplement and the placebo groups. The use of minerals or vitamins and serum biochemical indices such as liver functions were also comparable between the two groups. There were also no significant differences in dietary intakes of total energy, macronutrients as well as folate, vitamin $B_{6,}$ vitamin B_{12} and betaine during the intervention period between the two groups (Supplementary Table 1). No adverse event was reported and the intervention procedure was well-followed throughout the trial period.

Blood concentrations of folate, vitamin B_{12} and betaine during the intervention

Circulating concentrations of folate, vitamin B_{12} and betaine between the two groups were comparable at baseline (Fig. 2). In the supplement group, blood folate, vitamin B_{12} and betaine concentrations increased by 1.5 ng/mL, 57.0 µmol/L and 20.1 µmol/L at week 4, and 3.0 ng/mL, 83.0 µmol/L and 23.7 µmol/L at week 12, respectively. In the placebo group, blood folate, vitamin B_{12} and betaine concentrations increased by − 0.1 ng/mL, 22.0 µmol/L and 0.3 µmol/L at week 4, and 0.1 ng/mL, 0 µmol/L and 2.2 µmol/L at week 12, respectively (Fig. 2). Supplementation with B vitamins plus betaine resulted in significantly higher increases in blood folate, vitamin B_{12} and betaine concentrations from baseline in comparison with intervention with placebo after

Table 2 Baseline characteristics of participants[a]

	Overall ($n=92$)	Placebo group ($n=48$)	Supplement group ($n=44$)	*P*
Age, years	43.3 ± 10.6	43.7 ± 11.1	42.8 ± 10.1	0.696
Male, *n* (%)	81 (88.0)	42 (87.5)	39 (88.6)	0.867
Education levels, *n* (%)				0.346
Secondary school or below	14 (15.2)	5 (10.4)	9 (20.5)	
High school or equivalent	14 (15.2)	7 (14.6)	7 (15.9)	
College or above	64 (69.6)	36 (75.0)	28 (63.6)	
Smoker, *n* (%)	29 (31.5)	15 (31.3)	14 (31.8)	0.526
Alcohol drinker, *n* (%)	33 (35.9)	17 (35.4)	16 (36.4)	0.925
Multivitamins user, *n* (%)	15 (16.3)	6 (12.5)	9 (20.5)	0.302
Height, cm	169.4 ± 6.5	169.9 ± 7.1	168.8 ± 5.7	0.387
Weight, kg	70.1 ± 10.4	69.9 ± 10.9	70.4 ± 9.9	0.813
BMI, kg/m^2	24.4 ± 3.2	24.1 ± 3.2	24.7 ± 3.2	0.408
SBP, mmHg	122.7 ± 16.5	123.8 ± 16.7	121.6 ± 16.4	0.539
DBP, mmHg	84.5 ± 11.0	85.3 ± 11.2	83.6 ± 10.8	0.473
Physical activities, MET × hours/day	42.7 ± 12.3	43.7 ± 14.0	41.7 ± 10.1	0.419
Serum biochemical indices				
Glucose, mmol/L	5.5 ± 0.8	5.5 ± 1.0	5.5 ± 0.7	0.983
AST, U/L	21.9 ± 5.5	21.8 ± 5.8	22.1 ± 5.3	0.801
ALT, U/L	24.3 ± 12.5	23.4 ± 11.2	25.2 ± 13.8	0.509
ALP, U/L	72.4 ± 16.7	72.6 ± 17.4	72.1 ± 16.1	0.890
Cholesterol, mmol/L	5.2 ± 1.2	5.4 ± 1.4	5.1 ± 1.1	0.217
Triglyceride, mmol/L	1.4 ± 0.7	1.5 ± 0.6	1.4 ± 0.8	0.365
HDL-C, mmol/L	1.3 ± 0.3	1.3 ± 0.4	1.2 ± 0.3	0.798
LDL-C, mmol/L	3.4 ± 1.0	3.4 ± 1.0	3.3 ± 1.0	0.505
Hs-CRP, mg/L	1.2 ± 1.4	0.9 ± 0.9	1.4 ± 1.8	0.099
Creatinine, µmol/L	79.9 ± 16.4	80.2 ± 18.3	79.5 ± 14.3	0.850

ALP alkaline aminotransferase, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *BMI* body mass index, *DBP* diastolic blood pressure, *HDL-C* high-density lipoprotein cholesterol, *Hs-CRP* high sensitivity C-reactive protein, *LDL-C* low-density lipoprotein cholesterol, *METs* metabolic equivalent tasks, *SBP* systolic blood pressure

[a]Values were presented as mean ± standard deviation or *n* (%). One-way ANOVA and Pearson's Chi-square tests were used for comparing continuous and categorical variables between the supplement and the placebo groups, respectively

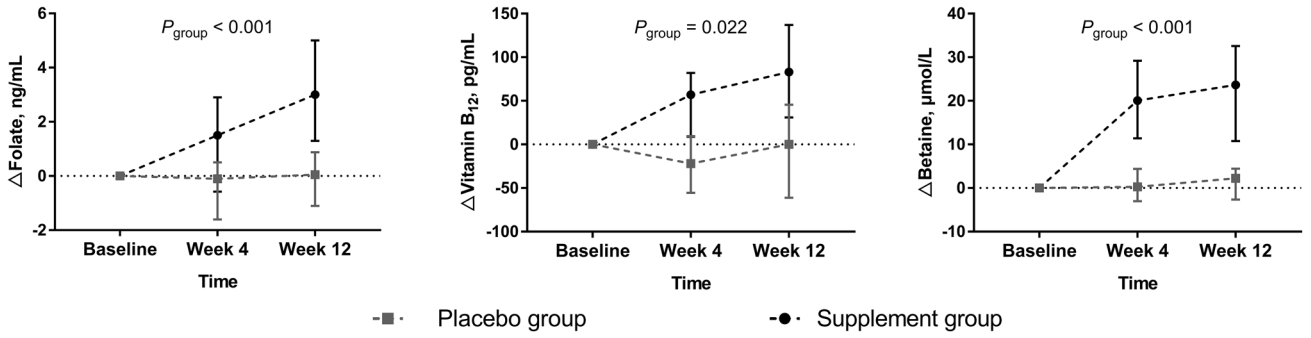

Fig. 2 Changes in blood concentrations of folate, vitamin B_{12} and betaine during the intervention in the placebo group and the supplement group, respectively. Data are presented as the median with an interquartile range. Generalized estimation equations were used to compare the group difference in changes in blood concentrations of folate, vitamin B_{12} and betaine from baseline at week 4 and week 12 in the placebo group (gray dotted line with square) and the supplement group (black dotted line with circle), with adjusting for covariates, including age, sex, energy intake, BMI, smoking status, alcohol consumption and physical activities, and P_{group} was calculated accordingly

adjustment of potential covariates (all $P_{group} < 0.05$) (Fig. 2). These results indicated good compliance in the two groups to the intervention.

Effects of supplementation with B vitamins plus betaine on plasma homocysteine concentrations

Plasma concentrations of homocysteine in the two groups during the intervention are presented in Table 3. At baseline, there were no significantly differences in plasma homocysteine concentrations between the two groups ($P = 0.265$). In the supplement group, plasma concentrations of homocysteine decreased from 15.57 μmol/L at baseline to 13.29 μmol/L at week 4, and further decreased to 13.26 μmol/L at week 12, both of which were significant reductions from baseline (both $P < 0.001$). In the placebo group, plasma concentrations of homocysteine decreased slightly from 16.96 μmol/L at baseline to 16.53 μmol/L at week 4, and increased slightly to 17.05 μmol/L at week 12. There were no significantly differences in plasma homocysteine concentrations among baseline, week 4 and week 12 in the placebo group ($P = 0.493$). As for the group difference, supplementation with B vitamins plus betaine for 12 weeks resulted in a significant reduction in plasma homocysteine concentrations from baseline compared to administration with placebo, (mean group

Table 3 Plasma concentrations of homocysteine during the intervention in the placebo group and the supplement group

	n, p/s[a]	Placebo group	Supplement group	*P*
Homocysteine, μmol/L[b]				
Baseline	48/44	16.96 (14.19, 21.14)	15.57 (13.65, 17.25)	0.265
Week 4	46/40	16.53 (14.66, 21.20)	13.29 (12.00, 15.93)*	< 0.001
Week 12	40/35	17.05 (14.31, 20.36)	13.26 (11.21, 15.10)*	< 0.001
Change in homocysteine at week 12, μmol/L				
Group difference crude[c]	46/40	–	− 4.33 (− 7.69, − 0.97)	0.012
Group difference adjusted[c]	46/40	–	− 3.87 (− 6.90, − 0.84)	0.012
Reduction rate at week 12, %				
Group difference crude[c]	46/40	–	− 10.0 (− 14.2, − 5.7)	< 0.001
Group difference adjusted[c]	46/40	–	− 10.1 (− 14.4, − 5.9)	< 0.001

[a]Sample sizes are presented as the numbers in the placebo and the supplement group, respectively

[b]Values are presented as median (P_{25}, P_{75}) and *P* values for Kruskal–Wallis tests are presented, unless otherwise stated

[c]Group difference between the placebo and the supplement group at week 12 was estimated by the crude and adjusted generalized estimating equations models and the Fisher's least significant difference test. The adjusted model included sex, age, energy intake, BMI, smoking status, alcohol consumption, and physical activities. Values of the group difference are presented as estimated marginal means (95% CIs)

*$P < 0.001$ versus baseline in the supplement group. No statistically significant differences were found at week 4 and week 12 compared with baseline in the placebo group

difference − 4.33; 95% confidence interval (CI) − 7.69, − 0.97; $P=0.012$), with a reduction rate of 10.0% (95% CI − 14.2, − 5.7; $P<0.001$). Similar results were observed after additionally adjusting for potential covariates (mean group difference − 3.87; 95% CI − 6.90, − 0.84; $P=0.012$; reduction rate 10.1%; 95% CI − 14.4, − 5.9; $P<0.001$).

Associations between changes in blood concentrations of folate, vitamin B_{12} and betaine with changes in plasma concentrations of homocysteine

In the supplement group, associations between changes in blood concentrations of folate, vitamin B_{12} and betaine with changes in plasma homocysteine concentrations are presented in Table 4. At week 4, there were no significant associations between changes in blood concentrations of folate, vitamin B_{12} and betaine with changes in plasma homocysteine concentrations (all $P>0.05$). At week 12, changes in serum folate concentrations were inversely associated with changes in plasma concentrations of homocysteine in the fully adjusted model (model 3: $\beta=-1.680$, $P=0.004$). Similar inverse associations were also observed between changes in plasma betaine concentrations and changes in plasma homocysteine concentrations in the fully adjusted model (model 3: $\beta=-1.421$, $P=0.020$). However, no significant associations were observed between changes in serum vitamin B_{12} concentrations and changes in plasma homocysteine concentrations (model 3: $\beta=-1.253$, $P=0.062$). There was no evidence of significant interactions between treatment effects and blood concentrations of folate, vitamin B_{12}, betaine and homocysteine at baseline on changes in plasma homocysteine concentrations within groups (all $P_{interaction}>0.05$, Supplementary Table 2).

Discussion

In this randomized, double-blind, placebo-controlled trial in Chinese adults with hyperhomocysteinemia free from background mandatory folic acid fortification, plasma homocysteine concentrations were markedly decreased after daily supplementation with a low dosage of folic acid, vitamin B_6, vitamin B_{12} and betaine for 12 weeks. These findings were consistent regardless of the baseline folate, vitamin B_{12}, betaine and homocysteine concentrations. The reductions in plasma homocysteine concentrations were significantly associated with the increased concentrations of blood folate and betaine.

Majority of previous trials that investigated the homocysteine-lowering effects of B vitamins or betaine have been conducted in high-risk populations or patients with CVDs [5, 6, 39], such as hypertension [8, 9], coronary, cerebrovascular or peripheral vascular diseases [5, 6]. In generally healthy populations, although numerous observational studies have demonstrated inverse associations between dietary intakes or circulating B vitamins and betaine concentrations with circulating homocysteine concentrations, evidence from interventional studies is few and limited to western populations. In a RCT conducted with a large group of healthy Dutch adults, daily supplementation with 400 µg folic acid and 500 µg vitamin B_{12} for 2 years reduces homocysteine concentration by 4.2 µmol/L in comparison with the

Table 4 Associations between changes in blood folate, vitamin B_{12} and betaine concentrations and changes in plasma homocysteine concentrations after intervention in the supplement group[a]

Blood concentrations	Model 1[b]			Model 2[c]			Model 3[d]		
	β	SE	P	β	SE	P	β	SE	P
△Week 4 ($n=40$)									
Folate, ng/mL	− 0.525	0.583	0.374	− 0.617	0.658	0.355	0.068	0.522	0.897
Vitamin B_{12}, pg/mL	0.393	0.586	0.506	0.620	0.731	0.402	− 0.144	0.618	0.818
Betaine, µmol/L	− 0.177	0.589	0.765	− 0.170	0.604	0.780	0.243	0.500	0.630
△Week 12 ($n=35$)									
Folate, ng/mL	− 1.752	0.721	0.021	− 1.737	0.782	0.034	− 1.680	0.523	0.004
Vitamin B_{12}, pg/mL	− 0.723	0.772	0.356	− 0.788	0.900	0.388	− 1.253	0.638	0.062
Betaine, µmol/L	− 0.651	0.774	0.407	− 0.884	0.821	0.290	− 1.421	0.568	0.020

SE standard error

[a]Data of blood concentrations of folate, vitamin B_{12} and betaine were z-score transformed before analysis. Linear regression analysis was used

[b]Model 1, unadjusted

[c]Model 2, adjusted for sex and age

[d]Model 3, model 2 additionally adjusted for energy intake, BMI, smoking status, alcohol consumption, and physical activities. In the meanwhile, blood concentrations of folate, vitamin B_{12}, and betaine at baseline were also mutually adjusted for in the corresponding model 3

placebo group [40]. A meta-analysis with five betaine supplementation trials, which includes 206 healthy adults from European countries, has confirmed that daily supplementation with 4–6 g betaine for 6–24 weeks significantly lowered homocysteine concentration by 1.23 µmol/L (reduction rate of 11.8%) [14]. In agreement with these studies, the current study observed that supplementation with B vitamins plus betaine effectively reduced plasma homocysteine concentrations in relatively healthy adults.

A low dosage of B vitamins plus betaine (daily supplementation: 400 µg folic acid, 8 mg vitamin B_6, 6.4 µg vitamin B_{12} and 1 g betaine) was used in the supplement group in the present trial. A meta-analysis based on twelve RCTs has demonstrated that daily supplementation with 0.5–5 mg folic acid and 0.02–1 mg vitamin B_{12} reduces blood homocysteine concentrations by 2.95–3.93 µmol/L (reduction rate of 25–33%) [41]. Schwab et al. have reported that after daily supplementation with 6 g betaine for 16 weeks, serum homocysteine concentrations decreases by 0.83 µmol/L (reduction rate of 9%) compared to the controls [39]. Consistent with these findings, data from the present study demonstrated daily supplementation with B vitamins plus betaine at doses close to recommended nutrient intakes of DRIs for 12 weeks reduced plasma homocysteine concentration by 3.87 µmol/L (reduction rate of 10.1%). Our results indicated that low dosages of B vitamins plus betaine were as effective as higher dosages used in previous studies to reduce plasma homocysteine concentrations. Serious side effects may appear after long-term supplementations with high dosages of B vitamins [18]. Some studies have also suggested that high dosages of folic acid supplementations may promote cancers and impair fetal development under certain conditions [42]. The finding of satisfactory homocysteine-lowering effects of supplementations with low-dose B vitamins plus betaine may provide a potential new strategy for the long-term intervention for the management of hyperhomocysteinemia.

B vitamins and betaine play crucial roles in homocysteine transmethylation in the one-carbon cycle [19]. As methyl donors, folate and betaine transmit methyl groups to homocysteine to form methionine, leading to reducing homocysteine concentrations. Vitamin B_6 and B_{12} are cofactors of metabolic enzymes in the one-carbon cycle [19]. B vitamins are widely used as nutritional supplements to reduce homocysteine concentrations. However, there may be some deficiencies in supplementation with B vitamins alone. The prevalence of the common *MTHFR* mutation (*MTHFR* C677T) is 24.0–63.1% in Chinese population [25]. Being heterozygous or homozygous for *MTHFR* C677T may lead to 30–65% of normal enzyme function [43], which decreases concentrations of bioactive folate. A previous RCT conducted in Chinese people with hyperhomocysteinemia has reported that more than 40% of subjects do not reach the normal homocysteine concentrations (lower than 15 µmol/L) after supplementation with 5 mg/d folic acid for 3 months [44], which indicates that intervention with folic acid alone may be insufficient for reducing homocysteine concentrations in individuals with hyperhomocysteinemia. In the process of homocysteine transmethylation, independent of the folate cycle, betaine is not affected by either the vitamin B_{12} concentration or the activity of MTHFR [25]. Homocystinuria is an inherited genetic condition caused by the abnormal accumulation of homocysteine and its related metabolites in blood and urine [45]. Betaine or other one-carbon sources are recommended to be considered to combine with B vitamins supplementation to ameliorate homocystinuria [46]. However, only one RCT conducted in nonpregnant women in Gambia has evaluated the efficacy of supplementation with B vitamins plus betaine for 12 weeks on lowering homocysteine concentrations [20]. They have reported that the novel supplement (daily supplementation: 800 µg folic acid, 2.8 mg vitamin B_6, 5.2 µg vitamin B_{12} and 4 g betaine) reduces plasma homocysteine concentration by 2.32 µmol/L compared with the baseline and is more effective than a traditional supplement containing 15 micronutrients without betaine [20]. Results of this study indicate that the combination of B vitamins and betaine may be a more effective nutritional supplement to reduce homocysteine concentration. In accordance with this finding, the current study conducted in Chinese adults with hyperhomocysteinemia also demonstrated that a similar dosage of B vitamins plus betaine reduced plasma homocysteine concentration by 3.87 µmol/L.

To the best of our knowledge, this is the first randomized, double-blind, controlled trial exploring the efficacy of a novel supplement with low-dose B vitamins plus betaine on plasma homocysteine concentrations in a generally healthy population with hyperhomocysteinemia and free from background mandatory folic acid fortification. Blood concentrations of folate, vitamin B_{12}, betaine and homocysteine were determined in all three visits (baseline, week 4 and week 12) by HPLC–MS/MS and HPLC–FLD, making the values of circulating concentrations more precise.

Several limitations should also be noted in our study. Some participants rejected further participation in the study and withdrew during the trial due to various reasons. However, GEEs and repeated measures analysis of variance were used for statistical analysis to utilize the data fully, and consistent results were found between them (Supplementary Table 3). Furthermore, our study precluded assessment of the separate effects of B vitamins and betaine. We could only discuss the integrated effects of the four nutrients on homocysteine concentrations. Further studies, which include each of the 4 compounds administered separately for comparison with the placebo and the combination, are required to investigate their individual or synergistic effects.

Conclusion

Daily supplementation with low-dose B vitamins plus betaine for 12 weeks effectively reduced plasma homocysteine concentrations among Chinese adults with hyperhomocysteinemia.

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Author contributions X-TL, X-GZ, and H-LZ designed the research. X-TL, Y-NW, Q-WM, Y-FW, Z-HH, YL, WM, S-YL, R-ZH, M-TY, X-ZL, Z-YL, and SC conducted the research. T-TH created the randomization sequences and performed the masking procedure. X-TL, B-XH, and A-PF analyzed the data. X-TL. wrote the paper. X-TL, B-XH, and H-LZ revised the paper. H-LZ had primary responsibility for the final content. All authors have read and approved the final manuscript.

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Data availability Data described in the manuscript, code book, and analytic code will be made available upon request pending.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the Ethics Committee of the School of Public Health at Sun Yat-sen University and was registered at clinicaltrials.gov as NCT03720249.

Consent to participate Written informed consent was obtained from all participants at enrollment.

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