Article

Short-Term Combined Intake of Vitamin B2 and Vitamin E Decreases Plasma Homocysteine Concentrations in Female Track Athletes

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Abstract: Female athletes who endure physical strain on the same bone area are prone to stress fractures. Preventing these improves their quality of life. Blood vitamin B2 (V.B2) and E (V.E) levels influence blood homocysteine (Hcy) levels, which, when elevated, increase the risk of stress fractures. We aimed to determine the effects of V.B2 and V.E supplementation on plasma Hcy concentrations in these athletes carrying the C677T polymorphism in methylenetetrahydrofolate reductase (MTHFR). This study is a before–after study in 16 female athletes. It was divided into three intervention periods of V.B2 (28 mg/day), V.E (60 mg/day), and V.B2 + V.E intake, and each period was 14 days. Blood samples were collected before and after each period, and plasma Hcy concentration and each blood vitamin concentration were measured. Plasma Hcy concentration significantly decreased in all periods, but the maximum lowering effect was obtained when V.B2 and V.E were used in combination. The administration of V.B2 might have lowered the plasma Hcy level by maximizing the catalytic activity of MTHFR; V.E might have suppressed cell oxidation, increasing the efficiency of folate in lowering the plasma Hcy level. We propose the combined intake of V.B2 and V.E as effective nutrients to reduce plasma Hcy concentrations in female athletes with MTHFR polymorphisms.

Keywords: vitamin B2; vitamin E; homocysteine; methylenetetrahydrofolate reductase; stress fractures; female athletes

1. Introduction

There has been an increasing interest in sports in recent years, resulting in an upsurge of health issues. Health problems associated with sports have led to the development of a concept called the "female athlete triad" (FAT), which comprises disordered energy availability, menstrual problems, and weak bones. This concept was devised by the American College of Sports Medicine in 2007 [1,2]. FAT refers to the interrelationship between energy availability, menstrual function, and bone mineral density that may have clinical manifestations such as eating disorders, functional hypothalamic amenorrhea, and osteoporosis. Prevention of FAT is important, as the condition leads to poor athletic conditioning, resulting in a loss of strength and endurance. Furthermore, in 2014 the International Olympic Committee presented the "Relative Energy Deficiency in Sport (RED-S)". RED-S is more comprehensive and broader, including the concept of FAT, revealing it to be a syndrome that affects many aspects of metabolic rate, immunity, and physiological function [3]. In particular, female track athletes are often reported to have a high prevalence of low energy availability. In addition, as a characteristic of track and field sports, sub-maximal stress

loads are repeatedly applied to bones, increasing the risk of stress fractures, which deteriorates their competitive lives [4]. These findings suggest that female track athletes may be at a significantly higher risk of stress fractures.

Homocysteine (Hcy), a non-protein amino acid, has been reported to be an independent risk factor for abnormal bone metabolism and is also implicated in stress fractures [5–10]. It is an intermediate metabolite of methionine, which is mainly metabolized through two pathways: (1) remethylation to methionine, which is dependent on folate, vitamin B12 (V.B12), and riboflavin (vitamin B2 [V.B2]), and (2) transsulfuration, which is dependent on vitamin B6 (V.B6). In the remethylation pathway, methionine is formed by the addition of a methyl group from 5-methyltetrahydrofolate, which is derived from dietary folate catalyzed by the enzyme 5,10-methyltetrahydrofolate reductase (MTHFR). In the gene encoding MTHFR, there is a C-to-T polymorphism at position 677 (MTHFR C677T), which causes alanine to be replaced by valine. Homozygotes and heterozygotes for C677T have been reported to have reduced MTHFR enzymatic activity by 60–70% and 30–35%, respectively [11–13]. Moreover, the single nucleotide polymorphism (SNP) C677T has been shown to decrease the enzymatic activity of MTHFR, resulting in decreased production of 5-methyltetrahydrofolate, which inhibits the remethylation pathway from Hcy to methionine, leading to an increase in blood Hcy concentration [14–17]. Mutations in the C677T polymorphism of MTHFR have been identified as an independent risk factor for stress fractures associated with hyperhomocysteinemia-mediated stress fractures [18]. V.B2, a precursor of flavin adenine dinucleotide (FAD), which is a cofactor of MTHFR, is an independent determinant of plasma Hcy levels [19]. However, low levels of V.B2 supplementation (<1.6 mg/day) failed to reduce blood Hcy levels [20]. Therefore, high V.B2 intake should be studied to determine the effect of V.B2 supplementation on plasma Hcy levels.

Folate plays a key role as a one-carbon carrier, facilitating the conversion of Hcy to methionine during methylation. Folate antioxidation has been explored as an attractive method to increase circulating levels of folate and reduce Hcy levels. Diet supplementation with antioxidants such as beta-carotene, V.C, and vitamin E (V.E), have been reported to reduce blood Hcy levels [21]. However, there are few studies on V.E intake alone and plasma Hcy concentrations. Thus, we hypothesized that the concurrent administration of antioxidant vitamins may potentiate the effects of group B vitamins on elevated plasma Hcy levels and aimed to determine the effects of V.B2 and/or V.E supplementation on blood Hcy levels in female track and field athletes with homozygous (TT) and heterozygous (CT) types of the C677T polymorphism. The findings of this study may provide scientific evidence for developing nutritional management support by considering genetic polymorphisms as a decision-making factor for the healthcare of female athletes.

2. Materials and Methods

2.1. Ethics of Human Research

This study followed the guidelines of the Declaration of Helsinki for research on human subjects. The study was approved by the Committee for the Ethical Guidelines for Medical and Health Research Involving Human Subjects at Toyo University (approval number: TU2018-16). Written consent was obtained from all participants for their participation after they were informed of the risks, discomforts, and benefits of the study.

2.2. Participants and Procedure

2.2.1. Participants

In this study, 16 female collegiate track and field athletes (age: 19.8 ± 0.8 years, height: 160.2 ± 5.5 cm, weight: 47.1 ± 5.2 kg) were enrolled. Participants taking medication were excluded. Of these, three participants who were unable to participate during all periods were excluded, and the remaining 13 were analyzed for TT or CT genotypes of the MTHFR C677T polymorphism. Eleven female track and field athletes had TT or CT genotypes in the MTHFR C677T polymorphism. Therefore, the final number of participants in the

experiment was 11. The sample size was calculated at a power of 80% using $\alpha = 0.05$ and $\beta = 0.20$. The effect size of Hcy was 1.0 µmol/L [22]. Hence, the number of participants in this study met the calculated values.

2.2.2. Procedure

This study process is shown in Figure 1. This study is a before–after study. Participants were continuously administered a prescribed amount of V.B2 and/or V.E for 14 days. For the intake period, we referred to studies that had used V.B2 and V.E supplementation and studies that confirmed the Hcy level by supplement intake [21,23]. The study period was divided into three periods: V.B2 intake period (V.B2 period), V.E intake period (V.E period), and combined intake period (V.B2 + V.E period), including a 14-day washout period between each experimental period. Throughout the study, participants were blinded to the presence, type, and amount of vitamin intake. All participants stayed in the same dormitory and consumed the same meals in the morning and at night during the experimental period. Meals were prepared and served by registered dietitians in the dormitory kitchen. In accordance with the Dietary Reference Intakes for Japanese (2015 edition) [24] and previous studies on oral intake of vitamin B2 and Vitamin E, the daily loading level was set to 28 mg/day for V.B2 and 60 mg/day for V.E [24–27]. In the previous study [25], 27 mg of V.B2 was reported to be the maximum level for absorption into the human body as a water-soluble vitamin. V.B2 is not susceptible to overdose due to high doses, and even in a previous study in which 400 mg of V.B2, which greatly exceeds the loading dose in this study, was taken continuously daily for 3 months [26], no health problems were reported. In addition, V.E is a fat-soluble vitamin, which could be harmful for the human body with an overdose of more than 800 mg/day [24]. Therefore, we carefully targeted the level at 60 mg/day, which a previous study [27] had confirmed resulted in an increase in plasma V.E concentration and no health problems. The same amounts were also used for the combined intake. Participants ingested the powdered vitamin twice a day, at breakfast and at dinner. This intake frequency was based on the half-life of the vitamin in the blood. Each vitamin was administered by mixing it with a meal (yogurt or fruit gelatin). The V.B2 and V.E (α-tocopherol) supplements were prepared by Mitsubishi Chemical Foods Corp., Japan. Daily energy and nutrient intakes were assessed using a brief-type self-administered diet history questionnaire (BDHQ) consisting of approximately 80 questions. The BDHQ is a reliable questionnaire for adults living in Japan that checks the amount of nutrients habitually ingested from ordinary foods (excluding supplements) for the past month and has approximately the same as or slightly higher validity than similar questionnaires [28–30].
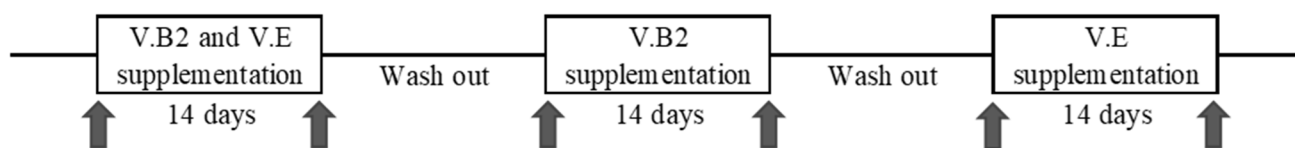
Figure 1. Process of this study. Three ingestion periods of V.B2 + V.E, V.B2, V.E were provided, and each ingestion period was 14 days. Blood sampling was performed at the point of the arrow.

2.3. Analysis of Saliva

Saliva was collected after no food or drink for 30 min. Saliva samples were collected, and DNA was extracted using the Oragene® Dis-cover kit OGR-600 (DNA Genetek Inc., Ottawa, ON, Canada) as per the manufacturer's instructions. MTHFR was amplified using primers MTHFR C677T Primer-S, 5′-TGAAGGAGAAGGTGTCTGCGGGA-3′, MTHFR C677T Primer-AS, 5′-CCTCACCTGGATGGGAAAGATCC-3′, and DNA polymerase Premix Taq™ version 2.0 (Takara Bio Inc., Shiga, Japan). The DNA amplification program comprised initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 60 °C for 60 s, and extension at 72 °C for 60 s which was repeated for 50 cycles and followed by a final extension at 72 °C for 60 s, and incubation at 4 °C thereafter. HinfI (New

England Biolabs, Inc., Rowley, MA, USA) was the restriction enzyme, and DNA was cleaved using the polymerase chain reaction (PCR)-restriction fragment length poly-morphism method (PCR-RFLP). The cleaved PCR products were separated on a 3% agarose gel to differentiate the wild-type, C677T (heterozygote), and T677T (homozygote) polymorphisms of the MTHFR gene. The analysis of the MTHFR C677T polymorphism described above was performed with reference to previous studies [31,32].

2.4. Analysis of Blood Components

Fasting morning blood samples were collected from the participants before (pre) and after (post) each vitamin intake period. Plasma was separated from the blood samples within an hour of collection. Plasma samples were stored at $-80\ °C$ until further analysis. The plasma V.E and Hcy concentrations were measured using high-performance liquid chromatography (HPLC). Analysis of the serum V.B2 concentration was out-sourced to LSI Medience Corporation, Tokyo, Japan.

2.4.1. Plasma Homocysteine Analysis

The plasma sample for Hcy analysis was prepared by adding 205 µL of 0.3 M PBS (pH 7.4) and 25 µL of 10 µM N-Acetyl-L-cysteine (Sigma-Aldrich Japan K.K., Tokyo, Japan) to 25 µL of plasma. After the sample was stirred, 10 µL of 60 mM Tris (2-carboxyethyl) phosphine hydrochloride (Nacalai Tesque, Inc., Kyoto, Japan) was added. After the sample was stirred again the mixture was incubated at 20–24 $°C$ for 30 min with stirring. Next, 90 µL of 100 g/L trichloroacetic acids (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing 1 mM EDTA-2Na (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was added, and the mixture was stirred and centrifuged (20 $°C$, 13,000× *g*, 10 min). The supernatant (90 µL) was transferred to a sample vial, 7.5 µL of 1.55 M NaOH was added, and the mixture was stirred again.

The mobile phase was prepared by adding 12 g of disodium hydrogen phosphate to 1000 mL of MilliQ water, and the sample was stirred again. The pH of the solution was adjusted to pH 2.5 by adding phosphoric acid. Further, 10% methanol, 170 mg anhydrous sodium dihydrogen phosphate (Kanto Chemical Co., Inc., Tokyo, Japan), and 5 mg of EDTA-2Na were added. The samples were analyzed by HPLC (JASCO Co., Tokyo, Japan). The analytical conditions are shown in Table 1.

Table 1. Analytical conditions for plasma Hcy and V.E concentrations by HPLC.

Items	Equipment, Reagents, and Conditions for Hcy	Equipment, Reagents, and Conditions for V.E.
Pump	EP-700 (LIQUID CHROMATOGRAPH PUMP/Eicom)	EP-700 (LIQUID CHROMATOGRAPH PUMP/Eicom)
Auto Sampler	M-514 (Eicom)	AS-4050 (Eicom)
Detector	Eicom ECD-700 (Eicom)	FP-2025 Plus intelligent fluorescence detector (Jasco)
Working Electrode	Eicom WE-AU (Eicom)	Eicom WE-AU (Eicom)
Precolumn	COSMOSIL Guard Cartridge 5PFP 4.6ID × 10 mm (COSMOSIL)	Eicom PC-04 4.0 mmφ × 5 mm (Eicom)
Column	Eicom-3OSD 3.0φ × 150 nm (Eicom)	COSMOSIL Packed column 5PFP 4.6 mm I.D. × 250 mm (nacalai tesque)
Column Temp	25 $°C$	40 $°C$
Buffer	99% 0.1 M Sodium phosphate buffer (pH 2.5), 1% Methanol, 170 mg/L Sodium octansulfonate, 5 mg/L EDTA-2Na	Methanol:MilliQ (*v/v*) = 9:1
Flow rate	500 µL/min	700 µL/min

Hcy, homocysteine; HPLC, high-performance liquid chromatography; V.E, vitamin E.

2.4.2. Plasma V.E Analysis

The plasma sample for V.E analysis was prepared by adding 390 µL of 100% ethanol and 10 µL of 100 µM dl-Tocol (Tama Biochemical Co., Ltd., Tokyo, Japan) as an internal

standard to 100 μL of plasma, and the mixture was centrifuged (4 °C, 15,000 rpm, 15 min). Then, the supernatant was collected with a 1 mL syringe and passed through a 0.2 μm filter, and the precipitate was discarded. Subsequently, 100 μL was dispensed into a sample vial and substituted with argon.

The mobile phase for the analysis was 90% methanol (prepared using HPLC-grade methanol and MilliQ water). The samples were analyzed using HPLC (JASCO Co., Tokyo, Japan). The analytical conditions are shown in Table 1.

2.4.3. Plasma Folate Analysis Using Enzyme-Linked Immune Sorbent Assay (ELISA) Kit

Plasma folate concentrations were quantitatively estimated using a folate ELISA kit (Cell Biolabs, Inc., San Diego, CA, USA) as per the manufacturer's instructions. After the enzymatic reaction, the absorbance of each microwell was read on a spectrophotometer (Sunrise™, Tecan Japan Co., Ltd., Kanagawa, Japan) at 450 nm, and the folate standard curve was used to determine plasma folate concentrations.

2.5. Statistical Analysis

All statistical analyses were performed using SPSS version 26 (IBM Corp., Armonk, NY, USA). The normality of pre-and-post-intervention data was compared by the Shapiro–Wilk test. Normally distributed data were compared using the paired *t*-test, and non-normally distributed data were analyzed using Wilcoxon's signed-rank test. For the change in concentrations, significance was determined by one-way analysis of variance using the one-way non-parametric Kruskal–Wallis test based on the normality of variables. For all statistical analyses, values of $p < 0.05$ were considered significant.

3. Results

3.1. The C677T Polymorphism Status and Physical Characteristics of the Participants

Of the 16 healthy participants, 11 had the C677T polymorphism and were recruited for subsequent analyses. The age of these 11 participants ranged from 19 to 21 years, with an average age of 19.9 ± 0.9 years. The mean number of competing years was 7.5 years. The participants' body mass index (BMI) was 17.7 ± 1.3 and monthly running distance was 476 ± 128 km. Physical characteristics of the participants are listed in Table 2.

Table 2. Physical characteristics of female track and field athletes with C677T genotype and their competition performance.

ID	Sex	Age (Years)	Height (cm)	Weight (kg)	BMI	Number of Stress Fractures	SNPs (MTHFR C677T)	Monthly Running Distance (km)	Competing in the Track and Field (Year)	Best Record (3000 m Race) (min)
A	F	21	156.0	45.0	18.5	2	CT	425	9	9.34
B	F	21	165.2	49.5	18.1	1	CT	392	9	9.57
C	F	21	162.8	42.4	16.0	2	CT	416	10	9.43
D	F	21	150.0	42.0	18.7	0	CT	519	12	9.47
E	F	20	160.0	42.5	16.6	0	CT	804	5	9.25
F	F	20	160.0	46.5	18.2	0	TT	418	8	9.48
G	F	19	163.0	47.5	17.9	0	CT	500	7	9.36
H	F	19	158.3	38.5	15.4	0	CT	252	4	9.45
I	F	19	157.3	45.9	18.6	0	CT	517	7	9.49
J	F	19	163.5	53.0	19.8	0	CT	467	4	10.08
K	F	19	171.0	49.0	16.8	0	CT	525	7	9.29
(Mean ± S.D.)		19.9 ± 0.9	160.6 ± 5.2	45.6 ± 3.9	17.7 ± 1.3	0.5 ± 0.8		476 ± 128	7.5 ± 2.4	9.47 ± 21

ID, identification; BMI, body mass index; MTHFR, methylenetetrahydrofolate reductase; SNP, single nucleotide polymorphism. The number of stress fractures was defined as the number of times a fracture had occurred since beginning training for track.

3.2. Dietary Nutrient Intake

The energy intake of the participants was 2145 ± 572 kcal. The intakes of V.B2 and V.E were 0.9 ± 0.2 mg/1000 kcal and 5.2 ± 1.5 mg/1000 kcal, respectively. Protein and

carbohydrate intakes were 2.2 g and 6.2 g per kg of body weight, respectively. Dietary energy and nutrient intake of the participants are shown in Table 3.

Table 3. Dietary energy and nutrient intake of female athletes.

	Calculated Values (Mean ± S.D.)	DRIs	
Energy (kcal)	2145 ± 572		
Protein (%)	18.6 ± 2.4	13~20%	
Fat (%)	28.5 ± 6.0	20~30%	PFC energy ratio
Carbohydrates (%)	52.1 ± 7.6	50~65%	
V.B2 (mg/1000 kcal)	0.9 ± 0.2	0.6	RDA
V.B6 (mg/1000 kcal)	1.0 ± 0.2	0.55	RDA
V.B12 (μg/1000 kcal)	9.1 ± 2.5	1.2	RDA
Folic acid (μg/1000 kcal)	252 ± 78	120	RDA
V.C (mg/1000 kcal)	95 ± 38	50	RDA
V.A (μgRAE/1000 kcal)	825 ± 310	325	RDA
V.E (mg/1000 kcal)	5.2 ± 1.4	2.5	RDA

Dietary protein, fat, and carbohydrate intakes are shown by the PFC energy ratio. V.B2 and V.E are shown as intakes per 1000 kcal of energy. DRI, dietary reference intakes; PFC, protein, fat, and carbohydrates; RDA, recommended dietary allowances.

3.3. Serum Vitamin B2, Plasma Vitamin E, Folate, and Homocysteine Concentrations

The concentrations of serum V.B2, plasma V.E, and folate are shown in Table 4. Serum V.B2 levels increased significantly during the period of V.B2 intake (from 20.3 ± 3.4 to 24.0 ± 2.6 in the V.B2 + V.E period, $p < 0.01$; from 17.4 ± 4.1 to 22.4 ± 2.4 in the V.B2 period, $p < 0.001$) and decreased significantly during the period of V.E intake (from 23.5 ± 4.5 to 19.5 ± 2.5 in the V.E period, $p < 0.05$). Plasma V.E concentration decreased significantly during the period of ingestion of V.B2 (from 25.2 ± 4.4 to 20.6 ± 3.9 in the V.B2 period, $p < 0.001$) and increased significantly during the period of ingestion of V.E (from 18.1 ± 3.1 to 20.7 ± 1.5 in the V.B2 + V.E period, $p < 0.05$; from 19.5 ± 4.1 to 23.6 ± 4.0 in the V.E period, $p < 0.05$). Plasma folic acid concentration did not show a significant difference ($p > 0.05$). In contrast, plasma Hcy concentrations significantly decreased in all periods after the supplements were administered ($p < 0.05$). However, the reduction was maximum in the V.B2 + V.E period (2.2 μmol/L), which was significantly higher than in the V.E period (2.0 μmol/L, $p < 0.05$; Figure 2).

Table 4. Serum V.B2, plasma V.E and folate concentrations pre and post V.B2 and/or V.E administration.

(*n* = 11)	V.E + V.B2 Period		V.B2 Period		V.E Period	
	Pre	Post	Pre	Post	Pre	Post
Serum V.B2 (μg/dL) [#]	20.3 ± 3.4	24.0 ± 2.6 **	17.4 ± 4.1	22.4 ± 2.4 ***	23.5 ± 4.5	19.5 ± 2.5 *
Plasma V.E (μmol/L) [#]	18.1 ± 3.1	20.7 ± 1.5 *	25.2 ± 4.4	20.6 ± 3.9 ***	19.5 ± 4.1	23.6 ± 4.0 *
Plasma folate (ng/mL) [†]	9.5 ± 5.0	10.7 ± 3.7	8.0 ± 4.1	6.8 ± 3.8	7.7 ± 4.7	7.2 ± 4.2

Values represent the mean ± standard deviation of fasting blood samples taken 14 days pre and post vitamin treatment. The number of asterisks in a row indicates the significant difference between the pre- and post-treatment data (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Reference ranges for blood tests are serum V.B2: 12.8–27.6 μg/dL, plasma V.E: 17.4–32.7 μmol/L; and plasma folate: 3.6–12.9 ng/mL. All blood data were within the reference ranges. One of plasma folate levels in the V.E group was hemolyzed; therefore, it was excluded from the data and is not shown in the table. The statistical analysis method is specified with symbols ([#]: paired *t*-test, [†]: Wilcoxon's signed-rank test).
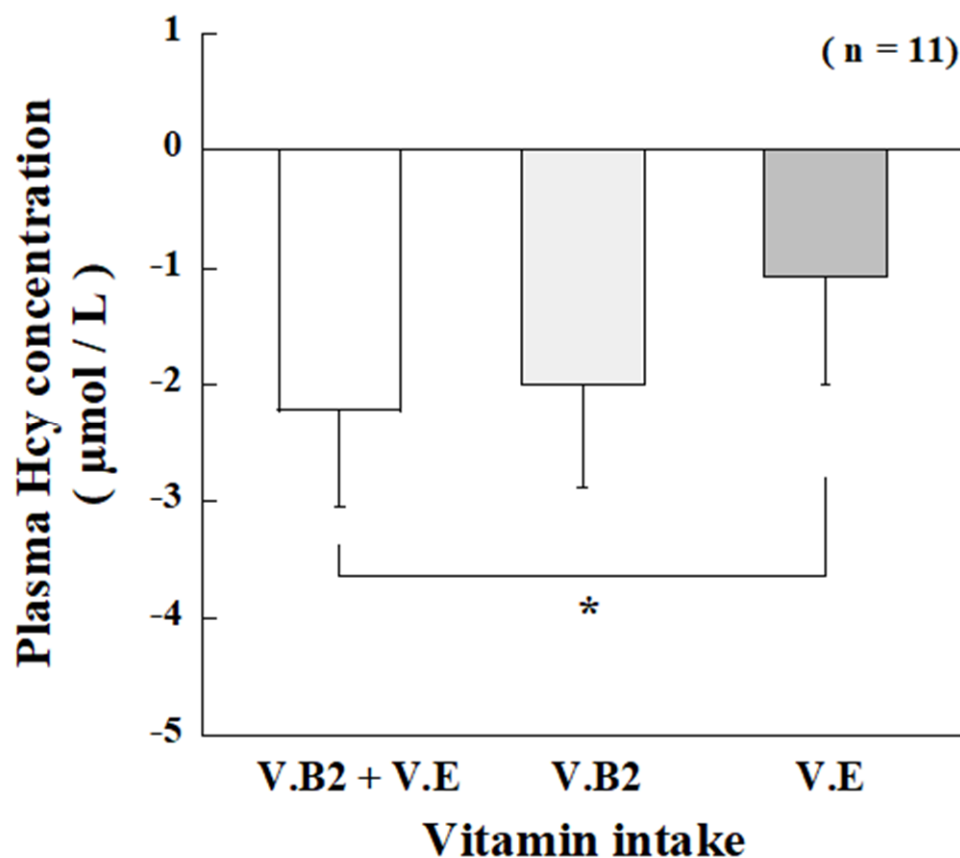
Figure 2. Comparison of the reduction in plasma homocysteine (Hcy) concentration after vitamin (V.B2) and/or vitamin E (V.E) administration. There was a significant difference between the vitamin B2 (V.B2) + vitamin E (V.E) period and the V.E period (* $p < 0.05$). The lowest plasma homocysteine (Hcy) concentration was in the V.B2 + V.E period. Plasma Hcy concentration decreased (pre−treatment to post−treatment [μmol/L] from 6.4 ± 1.0 to 4.2 ± 1.1 in the V.B2 + V.E period ($p < 0.001$), and from 10.1 ± 0.7 to 8.1 ± 1.0 in the V.B2 period ($p < 0.001$), and in the V.E period it also decreased from 8.7 ± 1.1 to 7.7 ± 1.2 ($p < 0.05$). In Japan, the reference value of plasma Hcy concentration is 3–15 μmol/L. In this study, all fluctuations were within the standard value. V.B2, vitamin B2; V.E, vitamin E; Hcy, homocysteine.

4. Discussion

This study examined female track and field athletes with the C677T SNP associated with elevated Hcy, and therefore with stress fractures. The purpose of this study was to determine the effect of V.B2 and antioxidant V.E supplementation on plasma Hcy levels. The results of this study showed that administration of V.B2 and/or V.E for 14 days significantly reduced plasma Hcy concentrations in all periods, with the greatest reduction effect in the V.B2 + V.E period. Reduction in plasma Hcy concentrations may prevent stress fractures. Prevention of stress fractures can improve athletes' quality of life.

Of the 13 participants who participated in the entire study period, 11 had the MTHFR C677T polymorphism, 10 (76.9%) had the CT genotype and 1 (7.7%) had the TT genotype. The remaining 2 (15.4%) had the CC genotype. Previous studies in Japanese people have reported that 11% of the population have the TT type, 46.8–54% have the CT type, and 35–42.2% have the CC type [15,33]. The distribution of the participants in this study tended to include a lower proportion of CT type than in the general population, but it is considered that there is no big difference from previous studies. In addition, the assessment of the dietary energy and nutrient uptake of the participants, as obtained through the BDHQ analysis (Table 3), showed that each nutrient met the recommended dietary allowance in

Japan (2015 edition) [24]. Therefore, this study was considered to be a vitamin load test in a group of well-fed female athletes.

V.B2, the precursor of FAD (a cofactor for MTHFR), has been reported to influence plasma Hcy levels [34,35]. Ingestion of folate and V.B2 lowers blood Hcy levels, whereas low V.B2 levels are associated with elevated plasma Hcy levels [36]. Another study reported that riboflavin supplementation led to a reduction in Hcy, even when limited to participants with low blood folate levels and the MTHFR C677T genotype [37]. In the present study, the plasma Hcy concentration was reduced by V.B2 intake, as it was observed in participants with the MTHFR C677T genotype who had adequate folic acid intake. A study reported that the administration of 1.6 mg V.B2 daily for 12 weeks did not affect Hcy levels [19]. In this study, V.B2 at 28 mg per day for 14 consecutive days reduced plasma Hcy concentrations by maximizing the catalytic activity of MTHFR. In the present study, the effect of V.B2 was demonstrated in a study of only participants with the MTHFR gene polymorphism.

Some researchers have suggested that vitamins with antioxidant effects enhance folate utilization efficiency, promote the conversion of Hcy to methionine, reduce intracellular oxidative stress, and lower blood Hcy concentrations [21,38,39]. The present results indicate that V.E reduces plasma Hcy concentrations. The athletes were routinely managed by a dietician and received sufficient energy-rich nutrients (carbohydrates, fats, and proteins), folate, and antioxidant vitamins. However, despite adequate dietary intake of V.E, plasma concentrations of V.E were <20 μmol/L before supplementation. Mean values were within the reference range, but some participants were below the reference range. These results suggest that, within the range of daily dietary intake, it may be difficult for female athletes to obtain adequate amounts of V.E from their daily diet. In this study, adding 60 mg of V.E to the daily diet maintained V.E concentration at ≥20 μmol/L in female track athletes running 400 km per month. This suggests that V.E supplementation has the potential to reduce post-training oxidative stress [40,41] and contributes to a decrease in plasma Hcy concentrations.

In this study, the maximum effect was obtained by the combined intake of V.B2 + V.E. As mentioned above, V.B2 decreased plasma Hcy concentration by increasing the activity of MTHFR, and V.E decreased plasma Hcy concentration by enhancing folate utilization efficiency. Although the mechanisms of action of these vitamins are different, both vitamins work to promote the metabolism of Hcy. It is presumed that combined intake of V.B2 and V.E promoted turnover and decreased plasma Hcy concentration more than separate supplement consumption. In addition, Rajesh et al. [42] showed that there were significantly lower blood Hcy levels in the group that took antioxidant vitamins (V.C, V.E) and the vitamin B group (folic acid, V.B2, V.B6, V.B12) in combination than in the group that took only vitamin B. Similarly, we found that it was more effective to take V.E and V.B2 in combination than to take V.B2 alone, which was a result supporting the previous study.

This study, although successful, had some limitations. First, the effect of the participant's menstrual cycle on Hcy has not been eliminated. In the future, it is hoped that a FAT solution method will be constructed by conduction of research that takes the menstrual cycle into consideration. The second is the lack of measurement of body composition, including bone density. In the future, it is necessary to confirm the effects on bones through long-term interventions. Thirdly, there is no set control period. In order to more accurately determine the effect of vitamins, it is desirable to conduct the study using control conditions in the future.

5. Conclusions

The results of this study suggest that V.B2 and V.E supplementation for track and field athletes may prevent stress fractures via reduction in plasma Hcy concentrations. The results of these studies may be useful in building further scientific evidence for nutritional management support based on genetic polymorphisms.

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