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Effects of long-term intake of iron-enriched beverage containing L-ascorbic acid 2-glucoside on iron nutrition status and condition of female college athletes

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Abstract In female athletes, iron deficiency and anaemia have marked effects on performance and could also impair health. Female athletes often limit their diet to control their weight, and thus may not obtain sufficient iron. Iron is difficult to absorb, so it is necessary to devise an efficient intake method. Absorption of non-haem iron seems to increase when taken with L-ascorbic acid (vitamin C [VC]). L-Ascorbic acid 2-glucoside (VCG), a food additive that binds glucose and thus improves its stability, is often added to beverages. Therefore, the effects of simultaneous intake of iron and VCG on iron nutrition were examined in female athletes. The subjects were 41 female college student athletes, and a placebo test was conducted in which a drink containing 200 mg VCG and 6 mg iron was consumed twice daily for 2 months. The placebo group consumed only the iron without VCG. Iron nutrition status was evaluated by blood tests, dietary surveys and subjective symptoms before and after the 2 months. Blood tests were also performed to determine the effects of iron intake on liver function and hepcidin levels. After 2 months, the VCG/iron drink had increased serum iron and blood VC levels in the VCG group compared to the placebo group. By contrast, aspartate aminotransferases (AST) tended to decrease in the VCG group, and no increase in hepcidin was observed in either group. This suggests that long-term iron intake through beverages can improve iron nutritional status in female athletes, and that VCG may enhance this status by suppressing the oxidative damage to the liver associated with iron intake.

Keywords : L-ascorbic acid 2-glucoside, iron-nutrition, female college athlete, hepcidin

Introduction

Athletes reportedly often have iron deficiencies due to poor iron intake from their diets and may suffer intense inflammation[1)]. Lack of iron reduces oxygen-carrying capacity, which affects endurance capacity[2)]. And such cases can lead to sports anaemia, which is a major disadvantage for athletes. All athletes should be aware that an iron deficiency may not only reduce endurance, but also reduce muscle metabolism and slow recovery from fatigue. Therefore, even if the haemoglobin (Hb) level is clinically normal, athletes need to continuously monitor their iron nutritional status, such as by measuring their serum ferritin (Fer) levels, to maintain good iron nutritional status[3)]. Low Fer levels can indicate latent iron deficiency, so athletes with low Fer levels need iron supplementation[4)].

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The absorption of iron in the intestinal tract is controlled, because excessive iron oxidises cells and causes liver dysfunction[5)]. The expression of hepcidin, that inhibits iron absorption, increases after excess iron intake and inflammation[6,7)]. Hepcidin inhibits the ferroportin export pathway on the surface of the small intestine and macrophages, inhibiting the intestinal absorption of dietary iron taken from food and suppressing iron release by macrophages[8)]. Iron supplements and high intensity exercise could increase inflammatory cytokine release and hepcidin in long-distance runners. Athletes should be supplemented while paying attention to liver function and the degree of inflammation.

The absorption efficiency of iron is 15–35% for haem iron and 2–5% for non-haem iron, a nutrient whose absorption efficiency from the gastrointestinal tract is very poor[9)]. Thus, it is necessary to devise ways to consume iron efficiently. Non-haem iron, which has particularly

poor absorption efficiency, needs to be reduced in the gastrointestinal tract. Ingesting an acid at the same time seems to help absorption[10]. The absorption of non-haem iron in the diet is thought to increase when it is taken together with L-ascorbic acid (vitamin C, VC)[11]. Furthermore, VC has strong antioxidant, anti-inflammatory and immunostimulatory effects, and is recommended for athletes to improve their physical condition and prevent fatigue[12]. However, a few reports of human interventional studies are unclear whether VC enhances iron absorption and improves iron nutrition in athletes. In addition, VC is water-soluble and is susceptible to light and oxidation and its effect on functional performance in the body when used as a food is unknown. L-Ascorbic acid 2-glucoside (VCG) is a food additive that enhances the stability of VC via the addition of glycosides[13]. Unlike VC, VCG is not reduced in aqueous solution, so does not taste of iron and can be added to beverages. Therefore, VCG thus has potential for use in drinks to improve sports performance.

This study investigated the effects of the simultaneous intake of iron and VCG on iron absorption from the intestinal tract and iron nutrition in the blood of female athletes with poor iron nutrition, low Hb and Fer levels and suspected latent iron deficiency. Furthermore, we observed the effects of iron intake on liver function and the inflammatory condition, and evaluated the inhibitory effects of VCG on these side effects.

Materials and Methods

Participants and study design. Subjects were recruited from among female college students who belonged to a competitive sports club. The potential subjects were those who often had subjective symptoms, such as headache, shortness of breath, or fatigue, which are characteristic of anaemia, and were interested in treating their anaemia. Among the applicants, there were 41 athletes (age, 20.2 ± 0.2 years; height, 160.2 ± 0.9 cm; weight, 54.7 ± 1.3 kg) with a blood Hb level ≤ 13.5 g/dL. Applicants with an Hb of ≤ 10 g/dL who required medical treatment based on a doctor's diagnosis were excluded from the study. The subjects were engaged in 14 sports: fencing (n = 6), athletics, gymnastics, lacrosse, triathlon (n = 5 each), basketball (n = 4), cross-country skiing, handball, volleyball (n = 2), alpine skiing, futsal, swimming, tennis, and ultimate (frisbee) (n = 1). The subjects were national-level athletes who trained more than 5 days a week. During the intervention period, 75% of the subjects were preparing for events or were in the competitive period. The subjects were randomised into two groups using a double-blind, placebo-controlled design. The VCG group consumed iron beverages containing VCG (n = 21), while the control group consumed only iron beverages (n = 20). During the study period, the subjects were asked to refrain from taking iron and VC supplements and fortified beverages other than the test beverage. The test drink was consumed for 2 months, and blood tests, body composition measurements, changes in subjective symptoms and dietary intake surveys were performed before and after the experiment (Fig. 1).

Preparation and intake of test beverage. A beverage containing 6 mg iron per bottle (100 mL) and 200 mg VCG (108 mg VC; Hayashibara, Japan) was used as the VCG drink, and 6 mg iron was used as a control-placebo beverage (Table 1). Iron pyrophosphate (Taiyo Chemical, Japan) was the form of iron added to the beverages. The test and placebo drinks used the same flavouring, adjusted so that there were no differences in taste, sealed in an aluminium bottle, and mailed to the subject. All test beverages were manufactured at a factory in accordance with HACCAP and were used as approved by safety tests. The subjects drank two test drinks per day: one after breakfast and one after dinner. During the intervention period, the VCG group received 12 mg of iron and 216 mg of VC daily.

Measurements. Blood sampling and physical condition interviews were conducted where measurements were taken in the morning between 9:00 and 11:00 am, following a 3 h fast. The subjects were told to refrain from drinking alcohol and exercising strenuously on the day

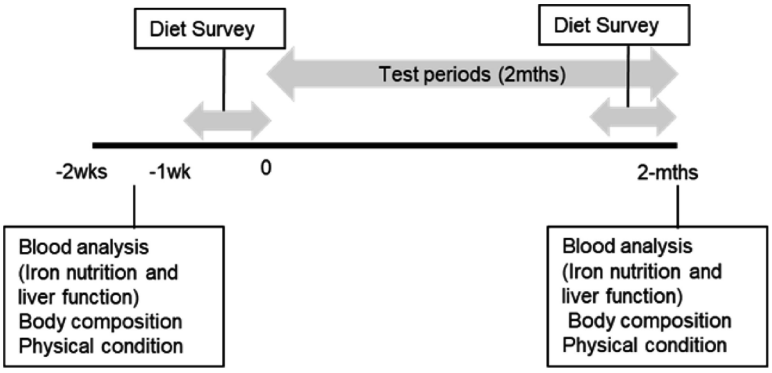

Fig. 1 Test period and measurement schedules.

Table 1. Nutrient composition of test and control beverages with VCG per 100 ml.

Ingredients (g)	Control	VCG
Fructose glucose liquid sugar	0.1	0.1
Erythritol	3.0	3.0
Acesulfame potassium	0.02	0.02
Sucralose	0.008	0.008
Trehalose	1.0	1.0
Citric acid (anhydrous)	0.15	0.06
VCG	-	0.2
Trisodium citrate	0.1	0.1
Ferric pyrophosphate (Fe)	0.6	0.6
Flavoring	0.2	0.2

The blending amount is shown in w / v %.

before taking the measurements, and the measurements were taken after a 3-hour fasted rest period. The initial blood collection date was adjusted as necessary so that it did not overlap with the menstrual period, and subsequent blood samples were not collected within 2~3 days of the menstrual period. Body composition was measured using the InBody instrument (ver. 3.2; InBody Japan, Tokyo, Japan) based on bio-electrical impedance analyses. Blood analyses included measurements of albumin (Alb), serum Fe, total iron binding ability, unsaturated iron binding capacity (UIBC), Fer, creatine kinase, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GTP), serum VC, zinc (Zn), red blood cell count, Hb, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), platelet count, reticulocyte count, and hepcidin. In total, 4 mL of blood was collected from a cubital fossa vein using two vacuum tubes (one with and one without EDTA). After collection, the vacuum tubes were allowed to stand for 30 minutes and then transported at 4°C. The samples were analysed in a clinical laboratory (SRL, Tokyo, Japan).

In the dietary survey, all meals and beverages consumed in the past week were recorded and photographed, and energy and nutrient intakes were estimated and checked by a registered dietitian. Food intake frequency was indexed using the Excel Eiyokun Food Frequency Questionnaire (FFQ; ver. 6.0, Kenpakusha, Japan) in conjunction with the Food Frequency Questionnaire Based on Food Groups (FFQg; ver. 3.5, Kenpakusha, Japan). Total intake of calories, carbohydrates, protein, fat, VC, Fe, and Zn and food groups, such as grains, meat and vegetables, was compared among the groups.

Amount of endurance, technical, and strength training were recorded in a diary. The calculation was done as follows: amount of physical activity (kcal) = body weight (kg) × duration of activity (h) × metabolic equivalents (METs) × 1.05. Body weight was derived from the InBody measurement, and the METs value was calculated as previously described[14].

Using the same physical condition assessment applied in our past research, the current physical condition, including "defecation status" and "menstrual cycle" (5 items), was recorded. In addition, "headache" and "vertigo" (5 items) were recorded as chronic anaemia symptoms along with "irritability" and "swelling of the face and limbs" (8 items) as premenstrual symptoms[15]. "Abdominal pain" and "back pain" (5 items) were considered as types of menstrual pain, while "shortness of breath" and "muscle pain" (5 items) during training were considered to reflect fatigue. The frequencies of the symptoms were measured using a visual analogue scale.

Statistical analyses. The data were subjected to repeated-measures two-way ANOVA. Multiple comparisons were performed using the Bonferroni test in cases of significant interaction effect. In addition to the homoscedastic f-number, the partial η^2 value (when performing a two-way ANOVA using repeated measures) was calculated to determine the effect size (ES)[16]. After confirming the normality of the distribution of the AST and ALT data, the nonparametric Mann-Whitney U test was performed. The statistical analyses were done using IBM SPSS Statistics software (ver. 27.0; IBM Corp., Armonk, NY, USA). Data are shown as mean and standard deviation. The significance level was set at 5%.

Ethical considerations. After obtaining approval from the Ethics Review Committee of the Faculty of Humanities and Sciences, Nihon University, we explained the significance, purpose, method, confidentiality of personal information and the results of the study to the participants

in writing and verbally, and they gave their consent (approval number 29–37). The study was conducted in accordance with the Declaration of Helsinki.

Results

Nutrition intake status. Dietary energy, protein, lipid, carbohydrate, iron, Zn, VC and food groups intake did not differ before and after the intervention (Table 2). The total iron intake, including the test drink, increased after the intervention in both groups, but the total VC intake in the VCG group was significantly higher than in the control (Table 3).

Body composition and physical activity. Both groups showed significant increases in body weight and fat after the intervention, and a tendency toward decreased training time (Table 4).

Table 2. Energy and nutrient intake from the diet pre and post test in the control and VCG groups.

	Control group		VCG group		2-way ANOVA				
	Pre	Post	Pre	Post	TIME	GROUP	TIME×GROUP	η2	F
Energy (kcal)	1405.3 ± 282.0	1345.8 ± 339.7	1409.2 ± 271.4	1395.0 ± 264.7	0.453	0.731	0.643	0.01	0.22
Protein (g)	45.6 ± 8.9	43.6 ± 12.6	45.6 ± 10.9	44.4 ± 12.4	0.387	0.896	0.812	<0.01	0.06
Protein (g/kg weight)	0.8 ± 0.19	0.8 ± 0.22	0.9 ± 0.25	0.8 ± 0.25	0.180	0.545	0.791	<0.01	0.07
Fat (g)	46.2 ± 7.5	42.3 ± 11.7	46.0 ± 10.8	43.1 ± 12.3	0.086	0.910	0.788	<0.01	0.07
Carbohydrate (g)	190.6 ± 48.0	188.2 ± 56.5	191.4 ± 38.0	198.1 ± 37.6	0.759	0.665	0.529	0.01	0.40
Calcium (mg)	312.0 ± 73.3	276.3 ± 133.7	310.6 ± 113.4	285.8 ± 97.3	0.097	0.887	0.762	<0.01	0.09
Iron (mg)	4.3 ± 0.3	3.9 ± 1.1	3.9 ± 1.1	3.9 ± 1.1	0.168	0.603	0.205	0.04	1.66
Zine (mg)	5.6 ± 1.2	5.3 ± 1.6	5.7 ± 1.5	5.5 ± 1.5	0.263	0.741	0.747	<0.01	0.11
Copper (mg)	0.67 ± 0.14	0.65 ± 0.20	0.66 ± 0.16	0.66 ± 0.17	0.744	0.883	0.739	<0.01	0.11
Vitamin B_1 (mg)	0.64 ± 0.16	0.58 ± 0.17	0.61 ± 0.14	0.59 ± 0.17	0.187	0.886	0.527	0.01	0.41
Vitamin B_2 (mg)	0.71 ± 0.13	0.62 ± 0.23*	0.71 ± 0.19	0.65 ± 0.18*	0.028	0.762	0.746	<0.01	0.11
Niacin (mg)	8.8 ± 2.6	9.1 ± 2.3	9.2 ± 2.9	9.1 ± 3.1	0.867	0.853	0.676	<0.01	0.18
Vitamin B_6 (mg)	0.56 ± 0.13	0.53 ± 0.19	0.56 ± 0.16	0.52 ± 0.19	0.268	0.858	0.885	<0.01	0.02
Vitamin B_{12} (μg)	2.4 ± 1.1	2.6 ± 1.4	2.6 ± 1.2	2.4 ± 1.5	0.866	0.942	0.518	0.01	0.43
Folic Acid (μg)	117.6 ± 22.4	105.8 ± 38.8	110.9 ± 32.6	108.2 ± 42.7	0.144	0.825	0.358	0.02	0.87
Vitamin C (mg)	38.4 ± 14.3	36.5 ± 29.3	33.4 ± 20.2	32.4 ± 26.8	0.698	0.460	0.913	<0.01	0.01

The value is mean ± SD (n = 20 or 21). The *P*-value was estimated by two-way ANOVA. Effect size values and homoscedasticity are shown in η^2 and F.

Table 3. Total intake of iron and VC in diet and beverages pre- and post-test in the control and VCG groups.

	Control group		VCG group		2-way ANOVA				
	Pre	Post	Pre	Post	TIME	GROUP	TIME×GROUP	η2	F
Iron (mg)	4.3 ± 0.3	15.9 ± 1.1*	3.9 ± 1.1	15.9 ± 1.1*	<0.001	0.603	0.205	0.04	1.66
Vitamin C (mg)	38.3 ± 14.3	36.5 ± 29.3	33.4 ± 20.2	240.3 ± 24.8*	0.371 <0.001	0.728 <0.001	<0.001	0.95	800.73

The value is mean ± SD (n = 20 or 21). The *P*-value was estimated by two-way ANOVA. Effect size values and homoscedasticity are shown in η^2 and F. The data with the interaction was given a p-value for multiple comparison in the columns of TIME (former: between Pre and Post in Control group and latter: between Pre and Post in VCG group) and GROUP (former: between Control and VCG in Pre and latter: between Control and VCG in Post).

Table 4. Body weight and body fat and muscle mass pre- and post-test in the control and VCG groups.

	Control group		VCG group		2-way ANOVA				
	Pre	Post	Pre	Post	TIME	GROUP	TIME×GROUP	η2	F
Weight (kg)	55.9 ± 6.7	56.7 ± 6.8*	54.4 ± 7.9	55.1 ± 8.1*	<0.001	0.516	0.551	0.01	0.36
Muscle mass (kg)	40.7 ± 3.6	40.9 ± 3.4	39.5 ± 3.9	39.4 ± 3.8	0.735	0.237	0.186	0.04	1.81
Body fat (%)	22.1 ± 5.6	22.8 ± 5.6*	22.4 ± 4.9	23.4 ± 5.4*	<0.001	0.783	0.531	0.01	0.40
Physical activity (kcal)*	1166 ± 436.7	878 ± 521.3	1152 ± 1033.3	1075 ± 638.7	0.068	0.645	0.284	0.03	1.18

The value is mean ± SD (n = 20 or 21). The *P*-value was estimated by two-way ANOVA. Effect size values and homoscedasticity are shown in η^2 and F.

Blood test results. The pre- and post-test values are shown in Table 5. In both groups, mean corpuscular haemoglobin (MCH), MCHC, Hb, Fe, and total iron binding capacity (TIBC) increased significantly after the intervention, while γ-GTP and UIBC were significantly reduced in both groups. AST changed –9.7 ± 4.0% and ALT changed –5.2 ± 6.5% in the VCG group, whereas these values were 3.7 ± 6.0% and 20.1 ± 12.9%, respectively, in the control group (Fig. 2). For the other parameters, there was no change before and after the intervention in either group.

Results of physical condition interview. Concerning subjective symptoms of anaemia, the "Frequency of giddiness" was significantly reduced in both groups after the intervention (Table 6). And for subjective symptoms during training, "Endurance training feels painful" and "Muscle fatigue" were significantly reduced (Table 6). Premenstrual symptoms were also reduced in both groups. For all other symptoms, there were no significant effects of the iron drink and VCG combination (Table 7). No subject experienced constipation or a gastrointestinal disorder due to ingestion of the iron-enriched beverage.

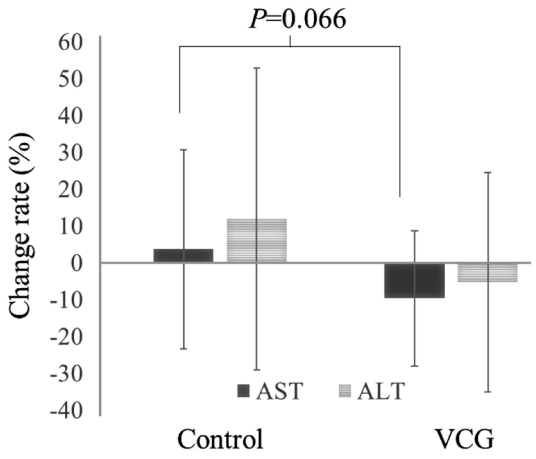

Fig. 2 Rate of change in concentration of blood AST and ALT in control group and VCG group at Pre-Post. Values are mean ± SD (n = 20 or 21). The rates of change for Control and VCG groups were compared using the Mann-Whitney U Test (*p*-value).

Table 5. Concentrations of blood iron and hepcidin as well as liver function pre and post 2 months in each group.

	Control group		VCG group		2-way ANOVA				
	Pre	Post	Pre	Post	TIME	GROUP	TIME×GROUP	η2	F
WBC	6080.0 ± 1251.8	6445.0 ± 1193.2	6728.6 ± 2185.9	6842.9 ± 1453.1	0.170	0.265	0.469	0.01	0.53
RBC	450.2 ± 25.0	454.9 ± 27.5	449.3 ± 25.3	454.8 ± 22.4	0.123	0.950	0.900	<0.01	0.02
HT (%)	42.2 ± 1.6	42.5 ± 1.7	41.9 ± 2.3	42.2 ± 2.1	0.452	0.514	0.926	<0.01	0.01
MCV (fl)	93.9 ± 3.7	93.6 ± 3.7	93.3 ± 3.0	92.7 ± 3.2	0.100	0.461	0.599	0.01	0.28
MCH (pg)	30.0 ± 1.0	30.3 ± 1.2	29.7 ± 1.0	30.0 ± 1.2	0.008	0.364	0.946	<0.01	<0.01
MCHC (%)	32.0 ± 0.7	32.4 ± 0.6	31.9 ± 0.6	32.4 ± 0.6	0.001	0.664	0.683	<0.01	0.17
Platelet count (10000/μl)	29.3 ± 4.8	27.8 ± 5.3	27.8 ± 4.3	27.4 ± 4.9	0.103	0.503	0.319	0.03	1.02
Alb (g/dl)	4.7 ± 0.2	4.7 ± 0.2	4.7 ± 0.2	4.8 ± 0.2	0.272	0.747	0.576	0.01	0.32
RET	10.9 ± 2.8	11.7 ± 3.0	11.7 ± 3.7	12.4 ± 3.8	0.079	0.431	0.916	<0.01	0.01
Hb (g/dl)	13.5 ± 0.5	13.8 ± 0.5	13.3 ± 0.7	13.6 ± 0.7	0.019	0.421	0.909	<0.01	0.01
Fe (μg/dl)	90.1 ± 35.6	114.1 ± 38.6	93.5 ± 56.9	121.3 ± 44.0	0.013	0.626	0.812	<0.01	0.06
TIBC (μg/dl)	347.1 ± 40.6	358.5 ± 38.6	349.8 ± 44.0	354.1 ± 37.4	0.849	0.507	0.417	0.01	0.45
UIBC (μg/dl)	256.1 ± 56.8	235.8 ± 52.1	265.1 ± 77.3	232.8 ± 52.1	0.015	0.851	0.569	0.01	0.33
Fer (ng/ml)	29.9 ± 20.2	31.6 ± 19.2	26.5 ± 24.9	27.7 ± 19.3	0.449	0.564	0.896	<0.01	0.02
Hepcidin (ng/ml)	13.5 ± 10.0	20.4 ± 12.2	17.1 ± 20.7	19.1 ± 11.3	0.110	0.750	0.371	0.02	0.82
AST (U/l)	22.1 ± 5.0	22.4 ± 6.1	24.9 ± 12.0	20.9 ± 5.6	0.159	0.744	0.102	0.07	2.80
ALT (U/l)	14.9 ± 4.9	16.2 ± 6.6	17.3 ± 10.3	14.6 ± 4.0	0.585	0.808	0.136	0.06	2.32
γ-GTP (U/l)	15.4 ± 5.2	14.3 ± 4.2	17.6 ± 6.8	15.6 ± 5.8	0.025	0.280	0.504	0.01	0.46
CK (U/l)	173.1 ± 125.1	170.0 ± 128.5	164.0 ± 76.2	139.5 ± 58.1	0.528	0.392	0.626	0.01	0.24
Zn (μg/dl)	84.6 ± 12.3	89.3 ± 15.4	85.8 ± 13.7	90.7 ± 10.9	0.052	0.690	0.975	<0.01	<0.01
VC (μg/ml)	11.8 ± 0.6	12.5 ± 0.7	12.8 ± 0.8	14.8 ± 0.7	0.022	0.045	0.281	0.03	1.19

The value is mean ± SD (n = 20 or 21). The *P*-value was estimated by two-way ANOVA. Effect size values and homoscedasticity are shown in η^2 and F.

Table 6. Chronic physical condition and physical condition during training on the pre- and post-test values in the control and VCG groups.

		Control group		VCG group		2-way ANOVA				
		Pre	Post	Pre	Post	TIME	GROUP	TIME×GROUP	η2	F
Chronic physical condition	Feel languid	3.6 ± 3.0	3.9 ± 2.8	4.3 ± 2.3	3.2 ± 2.5	0.331	0.975	0.140	0.06	0.69
	Hardships of morning	4.4 ± 3.5	4.1 ± 3.1	4.6 ± 3.1	4.8 ± 3.2	0.962	0.604	0.589	0.01	0.30
	Head feels heavy	2.5 ± 2.7	2.5 ± 2.3	3.9 ± 2.2	2.7 ± 1.9	0.196	0.168	0.185	0.04	1.82
	Headache	2.4 ± 2.9	2.1 ± 2.2	4.1 ± 2.5	2.8 ± 2.2	0.030	0.086	0.157	0.05	2.08
	Giddiness	4.9 ± 3.4	3.4 ± 3.2	5.7 ± 2.4	3.3 ± 3.0	<0.001	0.667	0.319	0.03	1.02
	Nausea	0.7 ± 0.3	0.6 ± 0.7	1.4 ± 0.3	1.0 ± 0.2	0.281	0.037	0.675	<0.01	0.18
Physical condition in training	Shortness of breath	4.7 ± 2.8	4.5 ± 2.7	5.1 ± 2.4	4.0 ± 2.2	0.064	0.987	0.190	0.04	1.78
	Tired feeling	4.4 ± 3.0	3.9 ± 2.6	5.0 ± 2.7	4.2 ± 2.1	0.212	0.495	0.843	<0.01	0.04
	Endurance training feels painful	4.7 ± 3.0	4.9 ± 3.0	5.7 ± 2.4	4.7 ± 2.5	0.174 (0.209 and 0.781)	0.601 (0.566 and 0.014)	0.033	0.11	4.89
	Muscular fatigue	4.4 ± 3.1	4.6 ± 3.1	5.2 ± 2.5	3.7 ± 2.4	0.126 (0.392 and 0.319)	0.954 (0.746 and 0.014)	0.049	0.10	4.12
	Headache due to lack of oxygen	3.2 ± 2.7	2.2 ± 2.9	3.2 ± 2.4	2.8 ± 2.7	0.083	0.676	0.410	0.02	0.69

The value is mean ± SD (n = 20 or 21). The *P*-value was estimated by two-way ANOVA. Effect size values and homoscedasticity are shown in η^2 and F. The data with the interaction was given a p-value for multiple comparison in the columns of TIME (former: between Pre and Post in Control group and latter: between Pre and Post in VCG group) and GROUP (former: between Control and VCG in Pre and latter: between Control and VCG in Post).

Table 7. Premenstrual syndrome and physical condition during menstruation on the pre- and post-test values in the control and VCG groups.

		Control group		VCG group		2-way ANOVA				
		Pre	Post	Pre	Post	TIME	GROUP	TIME×GROUP	η2	F
Premenstrual syndrome	Frustrated	4.6 ± 3.5	5.2 ± 3.2	4.7 ± 3.0	5.1 ± 2.7	0.177	0.995	0.802	<0.01	0.06
	Abdominal pain	4.5 ± 3.2	5.7 ± 3.4	5.3 ± 3.3	5.7 ± 2.9	0.084	0.697	0.387	0.02	0.76
	Head feels heavy	2.5 ± 3.0	2.5 ± 2.9	3.5 ± 2.8	2.3 ± 1.7	0.119	0.588	0.108	0.07	2.71
	Headache	2.2 ± 3.0	2.1 ± 2.7	3.6 ± 3.0	3.2 ± 2.7	0.287	0.172	0.532	0.01	0.40
	Back pain	4.0 ± 3.7	3.6 ± 3.6	4.2 ± 3.4	4.9 ± 2.9	0.827	0.433	0.220	0.04	1.55
	Swelling	2.7 ± 3.2	1.9 ± 2.8	3.9 ± 3.0	2.3 ± 2.6*	0.005	0.332	0.363	0.02	0.85
	Joint pain	1.5 ± 2.5	1.6 ± 2.1	3.4 ± 2.8	2.5 ± 2.3*	0.171	0.048	0.149	0.05	2.17
	nausea	1.3 ± 2.0	0.8 ± 1.1	1.7 ± 2.5	1.4 ± 2.1	0.326	0.271	0.805	<0.01	0.06
	Dullness	4.1 ± 3.8	4.6 ± 3.7	5.5 ± 3.4	5.0 ± 3.0	0.945	0.352	0.282	0.03	1.19
Menstrual pain	Abdominal pain	5.4 ± 3.7	7.0 ± 3.5*	6.6 ± 2.9	5.9 ± 3.0	0.252 (Control) 0.260 (VCG)	0.006 (Control) 0.232 (VCG)	0.006	0.18	8.57
	Head feels heavy	2.0 ± 0.5	1.9 ± 0.5	4.0 ± 0.7*	3.0 ± 0.6*	0.212	0.035	0.316	0.03	1.03
	Headache	1.7 ± 0.6	2.0 ± 0.6	4.1 ± 0.8*	3.4 ± 0.7 *	0.540	0.029	0.262	0.03	1.29
	Back pain	4.6 ± 3.8	4.4 ± 4.0	4.9 ± 3.4	4.2 ± 3.0	0.291	0.972	0.487	0.01	0.49

The value is mean ± SD (n = 20 or 21). The *P*-value was estimated by two-way ANOVA. Effect size values and homoscedasticity are shown in η^2 and F. The data with the interaction was given a p-value for multiple comparison in the columns of TIME (former: between Pre and Post in Control group and latter: between Pre and Post in VCG group) and GROUP (former: between Control and VCG in Pre and latter: between Control and VCG in Post).

Discussion

Changes in iron intake and nutritional status. The diets of the subjects in this study were not sufficient in terms of the energy or nutrients required by athletes[17,18]. Specifically, the iron intake was lower than that recommended for women[19], and the blood tests and nonspecific physical complaints suggested latent iron deficiency[4]. Subjects consumed a drink containing 6 mg iron pyrophosphate twice a day for 8 weeks; thus, including iron from the diet, both groups consumed about 16 mg iron per day. The blood parameter levels related to iron nutrition status, other than Fer, increased in both groups. A previous study reported a significant increase in Fer in female athletes

who took 22 mg iron per day for 4 months, together with 14 mg haem iron supplements and dietary iron[15]. There may also have been an increase in Hb due to the extended period of supplementation. Matsumoto et al. reported that nutritional counselling once every 2 weeks by a sports dietician, along with iron supplements, causes an increase in dietary iron intake in the diet[15]. In this study, no special dietary education or nutritional consultation was provided. It has been suggested that it is difficult to improve iron levels within 2 months only using an iron-fortified drink. Guidance from a sports dietitian, along with iron drinks and supplements, may enhance iron levels, and prevent latent iron deficiency and anaemia.

VCG intake to increase iron absorption efficiency. Vitamin C promotes iron solubilisation and increases absorption in the gastrointestinal tract, but the appropriate balance between iron and VC taken simultaneously in human experiments is unknown. In this study, we used a beverage containing 108 mg of VC, but no significant increase in serum iron or improvement in iron nutrition was observed in the VCG group relative to the control, *i.e.* no iron absorption-promoting effect was detected. However, as iron is strictly controlled and not excessively absorbed by the digestive tract, the effects of VC on promoting iron absorption may be transient and faint[11]. Food components such as tannins inhibit iron absorption. In our experiment, the iron drink was provided after meals, so it is possible that other food ingredients inhibited iron absorption. In addition, other food ingredients may have had a negative impact on the ability of VC to promote iron solubilisation in the gastrointestinal tract. In the future, it will be necessary to devise a monitoring scheme to examine the effects of VC on promoting iron absorption and the recommended intake amount.

Effect of VCG on physical condition. In a previous study involving the same questions on physical condition used herein, when haem iron supplementation was combined with nutritional counselling, iron nutritional status (Fer) and nonspecific physical complaints, improved[15]. Although no significant effect of VCG on iron absorption was observed in this study, the nonspecific physical complaints improved, especially during training. VC has strong antioxidant properties and has been reported to relieve fatigue[20]. We recruited subjects with vague complaints such as headache, dizziness, and fatigue, which are symptoms of latent iron deficiency. They also complained of menstrual problems. Latent iron deficiency is also associated with dysmenorrhea and premenstrual syndrome, and prevents female athletes from performing well during training and competition[21]. Although evidence that VC improves vague menstrual complaints is lacking, it is plausible that its inhibitory effect on inflammation and oxidative damage would improve these symptoms[22]. The estimated dietary intake of VC at the start of this study was very low, i.e. below the recommended amount[19]. There is no consensus on the amount of VC that athletes require. Intervention studies using 1,000 mg daily reported no significant effect on maximal oxygen uptake, and high intakes may not necessarily improve athletic performance[23]. VC may be important for athletes because of its positive effects on immunity[12,21]. Comparative studies are need to determine the optimal VC intake amount, and to determine if VCG is superior for improving subjective symptoms compared to VC.

Relationship between iron intake and liver function. Excessive iron intake is harmful to the body and can induce oxidative damage, cause deposits in the liver and endocrine organs and lead to dysfunction[24]. Therefore, excessive or long-term intake of an iron supplement requires careful attention to avoid deteriorating liver function. This is particularly true for athletes who tend to become fatigued during daily training[25]. In the present study, there were no complaints of illness due to iron intake during the study period, and there was no apparent deterioration in liver function. However, a close observation of the liver function indices showed that the VCG group tended to have lower rates of change in AST than controls. This finding suggests that VCG may have improved the indicators of liver function. This is the first report on the effects of simultaneous VCG (including VC) and iron intake on liver function. VC improves liver function in patients with fatty liver, and VC reportedly has an antioxidant effect in that organ[26,27]. Furthermore, in animal studies, VCG has been reported to accelerate the recovery of hepatectomised rats better than VC. The VCG used in this study is a food additive in which one molecule of glucose is bound to VC by an enzymatic reaction to stabilise it in aqueous solution and improve its structure so that it is not easily degraded. As it was a non-reducing VC derivative, it maintains its stability in food, and is hydrolysed by α-glucosidase in the small intestine and absorbed into the blood as VC[28]. However, there is little evidence that it has stronger antioxidant activity in humans than ascorbic acid, and the effect remains unclear. Detailed studies are needed to determine whether this effect is a general effect of VC or a specific effect of VCG and its mechanism.

Relationship among increased hepcidin, iron intake and training. The expression of hepcidin increases with inflammation and stress in the body, which suppresses iron absorption in the digestive tract. Increased muscle inflammation due to overtraining by athletes may increase hepcidin, which impairs iron absorption. Ishibashi et al. reported that hepcidin levels increase when elite long-distance athletes are given a 30 mg iron supplement daily while conducting a training camp[29]. In Roecker et al., an increase in hepcidin continued up to 24 h after high-intensity training, and iron supplementation during intense training may not have been fully effective as a result[30].

Furthermore, it is possible that excessive iron intake itself may increase hepcidin levels[31]. In this study, daily intake of 16 mg of iron in the control group continued for 2 months, and the hepcidin level was not elevated. Training time tended to decrease in both groups during this period, probably because 75% of the subjects during the intervention were preparing for competitive sports (and thus adjusted their training regimes). Our results suggest that continued iron intake and training may not be accompanied by elevated hepcidin levels, except during continuous high-intensity training[32]. Although no data is shown, we tested for a correlation between physical activity (kcal) and hepcidin level, but did not detect any. As the subjects were involved in a wide variety of sports, statistical analysis of the role of type of sport was not feasible. In future studies of the relationships among training intensity and duration, iron intake and hepcidin, it will be necessary to control for training type.

Effects of VCG on suppressing increase in hepcidin. Vitamin C has a strong antioxidant effect and suppresses intracellular inflammation[26]. Therefore, VC should supress increases in hepcidin in athletes, although this was not observed by Díaz et al.[33] and a consensus is lacking. We failed to observe an increase in hepcidin in association with iron intake and training, and no inhibitory effect on hepcidin of VCG was found. However, the hepcidin levels in our subjects varied widely, making statistical analysis difficult. Moreover, as changes in inflammatory cytokines were not observed in this study, it was not possible to verify whether this effect suppressed inflammation. Inflammatory cytokines, such as interleukin-6, should be measured to demonstrate that VCG suppressed inflammation due to iron intake and continued training, and thus suppressed hepcidin.

Study limitations and future directions. While this study showed that iron drinks can improve iron nutritional status, no enhancing effect of VCG was detected. This might be due to the low dose of VCG, and to any effect being obfuscated by the diet. The small number of study subjects and large interindividual variability may also explain why no effect was detected. In long observational studies of athletes' diets, the subjects tend to be engaged in training and it is difficult to control for the effects thereof. It is also difficult to enrol a sufficient number of elite athletes for meaningful statistical analyses, which was a limitation of this study. Future studies should aim to enrol more subjects.

All menstrual cycles were investigated during this study period, and blood was collected by selecting a day other than the menstrual period. This is because the days when there is bleeding may affect iron values. However, it is not fully understood how hepcidin changes during the menstrual cycle. Considering that hepcidin is greatly stimulated by inflammation, there may have been a change in the expression level of hepcidin during the premenstrual phase when prostaglandin increases. In the future, elucidating the changes in the menstrual phase and hepcidin may be useful for studying the appropriate intake timing of iron supplements for female athletes and the training method.

How the intensity and duration of training are related to liver function and increased hepcidin levels has not been clarified in detail, considering factors such as gender, athlete level and type of competition. Furthermore, it is unclear whether taking an iron supplement is effective when good iron nutrition is desired, such as during high-altitude or low-oxygen training. It would be very interesting to determine if antioxidants, such as VC, are effective in helping athletes stay healthy and safe. In this study, no blood parameters other than AST and ALT were examined, so it is difficult to determine the effect of VCG on liver function. In future studies, more liver function parameters should be analysed. Furthermore, in studies of the effects of VC in foods, forms with higher stability, such as VCG, should be evaluated.

Conclusions

Long-term iron beverage intake improved iron nutritional status in female athletes. Ingesting the VCG-containing beverage with iron tended to improve the metabolism associated with liver function compared to ingestion of iron alone. However, no increase in hepcidin due to iron intake or inflammation was observed, and the inhibitory effect of VCG could not be verified.

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Conflict of Interests

The authors have read the JPFSM policy concerning conflicts of interest and have the following conflict: SU is an employee of Hayashibara Co., Ltd., Japan. However, this company had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. There are no patents, products in development or marketed products to declare. The authors declare no competing interests.

Author Contributions

Conceptualisation was by MM, TS, SU and HK, and methodology by MM. Software was managed by MM and SA, valida-

tion by MM, SA and HK, formal analysis by MM and TS, and investigation by MM and SA. Resources were gathered by MM and SU, and data curation by MM and SA. MM and TS wrote the original draft, MM visualized, reviewed and edited the draft. MM and HK supervised the project and MM and SU provided further administrative functions. Finally, funding acquisition was carried out by MM. All authors read and agreed to the published version of the manuscript.

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