Original Research

Effect of Powdered Fermented Milk with *Lactobacillus helveticus* on Subjects with High-Normal Blood Pressure or Mild Hypertension

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Key words: antihypertensive effects, high-normal blood pressure, mild hypertension, *Lactobacillus helveticus*, tripeptide

Objective: Two tripeptides (Val-Pro-Pro and Ile-Pro-Pro) that have inhibitory activities for angiotensin I-converting enzyme are produced in milk fermented with *Lactobacillus helveticus*. In this study we evaluated the effect and safety of powdered fermented milk with *L. helveticus* CM4 on subjects with high-normal blood pressure or mild hypertension.

Methods: A randomized, placebo-controlled, double-blind study was conducted using 40 subjects with high-normal blood pressure (HN group) and 40 subjects with mild hypertension (MH group). Each subject ingested 6 test tablets (12 g) containing powdered fermented milk with *L. helveticus* CM4 daily for 4 weeks (test group) or the same amount of placebo tablets for 4 weeks (placebo group).

Results: During treatment, the decrease in systolic blood pressure (SBP) and diastolic blood pressure (DBP) in the test group tended to be greater than in the placebo group for both blood pressure groups. At the end of treatment (week 4), a significant decrease in DBP in the HN group was observed (i.e. 5.0 mm Hg (0.1, 9.9; $p = 0.04$) compared with the placebo group). There was no significant change in SBP (3.2 mm Hg (95% CI −2.6, 8.9; $p = 0.27$). In the MH group, SBP decreased by 11.2 mm Hg (4.0, 18.4; $p = 0.003$) and there was a statistically non-significant decrease in DBP of 6.5 mm Hg (−0.1, 13.0; $p = 0.055$) compared with the placebo group. No marked changes were observed in other indexes, including pulse rate, body weight and blood serum variables, and no adverse effects attributed to the treatment was found in each group.

Conclusions: Daily ingestion of the tablets containing powdered fermented milk with *L. helveticus* CM4 in subjects with high-normal blood pressure or mild hypertension reduces elevated blood pressure without any adverse effects.

INTRODUCTION

Hypertension is a known risk factor for cardiovascular diseases, including heart disease and stroke, and the risk of these diseases can be lowered by the treatment of hypertension [1]. Lifestyle modifications such as weight reduction, moderation of alcohol consumption, reduction in salt intake, increase in physical activity, cessation of smoking and healthy eating patterns are recommended for treatment of mild hypertension without risk factors for cardiovascular diseases [2]. Furthermore, approaches like these can contribute to the primary prevention of hypertension [3]. With respect to the dietary approach, large-scale clinical trials have been carried out on subjects with mild hypertension, high-normal and normal blood pressure to determine the relationship between lifestyle modification and antihypertensive effects [4,5].

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Abbreviations: VPP = Val-Pro-Pro, IPP = Ile-Pro-Pro, ACE = angiotensin I-converting enzyme, SHR = spontaneously hypertensive rat, SBP = systolic blood pressure, DBP = diastolic blood pressure, HN = high normal blood pressure, MH = mild hypertension, BMI = body mass index, ANOVA = analysis of variance.

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Functional foods with antihypertensive effects could be used as a non-pharmacological method in a dietary approach to reduce the risk of hypertension. Food containing angiotensin converting enzyme (ACE) inhibitory peptides is one example of a dietary approach with an expected antihypertensive effect [6]. ACE, the key enzyme in the renin-angiotensin system which plays an important role in regulating blood pressure, generates vasopressor peptide angiotensin II from angiotensin I, and inactivates vasodilator peptide bradykinin. At present, ACE inhibitors (drugs that inhibit ACE) are widely used in the treatment of hypertension and heart failure. Nakamura et al. showed that two tripeptides (Val-Pro-Pro, VPP and Ile-Pro-Pro, IPP) with an inhibitory effect on ACE were produced in milk fermented by the lactic acid bacterium, *Lactobacillus helveticus* [7]. They also showed that *L. helveticus*-fermented milk had an antihypertensive effect after oral administration to spontaneously hypertensive rats (SHR) [8,9]. VPP and IPP are associated with the antihypertensive effect of *L. helveticus*-fermented milk [10]. The efficacy of *L. helveticus*-fermented milk, containing VPP and IPP, have been reported in the trial by Hata et al. [11] in which hypertensive patients with antihypertensive medication ingested a fermented milk drink on a daily basis for 8 weeks. It is thought to be more effective to consume the fermented milk as part of the diet during the early stages of a therapeutic plan for hypertension, or for prevention of the incidence of hypertension. To confirm this hypothesis, we have previously studied the efficacy and safety of powdered *L. helveticus* fermented milk in different blood pressure categories [12,13]. Kajimoto et al. [12] reported that SBP and DBP decreased significantly, by 12 mm Hg and 10 mm Hg, respectively, after 8 weeks daily ingestion of 2 tablets of powdered *L. helveticus* fermented milk in subjects with mild to moderate hypertension without antihypertensive medicine. In contrast, in normotensive subjects, no significant changes in blood pressure were observed after daily ingestion of 6 tablets of powdered *L. helveticus* fermented milk [13]. The present study evaluates the efficacy and safety of powdered *L. helveticus* fermented milk tablets on subjects with mild hypertension and high-normal blood pressure, using a placebo-controlled, double-blind, parallel group comparative design.

MATERIALS AND METHODS

Supplementary Tablets

The test tablet and the placebo tablet were produced by Calpis Co., Ltd (Tokyo, Japan) using the following procedures: reconstituted skimmed milk (9%, w/w) was prepared from powdered skimmed milk, then pasteurized and fermented with *L. helveticus* CM4 at 37°C for 22 hours. Casein was removed by centrifugation and lactic acid was eliminated from the supernatant by electrodialysis. Residual supernatant was converted to fermented milk whey powder using maltodextrin as a bulking agent and then spray dried. The fermented milk whey powder was mixed with sorbitol, cornstarch, cellulose, citric acid, sucrose fatty acid ester, aspartame and flavors, and then compressed to obtain tablets 2 cm in diameter and 2 g in weight. The placebo control contained a mixture of lactose, maltodextrin, calcium caseinate, citric acid, milk calcium, magnesium sulfate, and skimmed milk, and had the same nutritional content as the fermented milk whey powder. The placebo tablet was prepared by mixing the placebo powder, sorbitol, cornstarch, cellulose, citric acid, sucrose fatty acid ester, aspartame and flavors as described above for the test tablet. The nutritional content of the test and the placebo tablet are shown in Table 1. The amount of VPP and IPP peptides in the supplementary tablet was determined as follows: the tablet (2 g per tablet) was crushed to powder, and was accurately weighed out into a 25 ml volumetric flask. The powder was dissolved in 0.28% (w/v) trifluoroacetic acid (TFA) and the solution made up to 25 ml. The solution was centrifuged at 15,000 rpm for 10 minutes, and the supernatant was collected. 0.3 ml of the supernatant fraction was applied to a Sep-pak Plus tC18 column (Waters, Milford, MA) and washed with 7 ml of distilled water. The adsorbed substances on the Sep-pak Plus tC18 column were eluted with 2.5 ml of 30% (w/v) methanol, concentrated and dried with a centrifugal concentrator. The dried fraction was dissolved in 0.5% (w/v) TFA solution containing 0.3 M NaCl, and subjected to HPLC analysis using a gel filtration column (Shodex Asahipak GS-320 7G; Showa Denko, Tokyo, Japan) at a flow rate of 0.5 ml/min. The tripeptides were monitored at 215 nm and quantified using chemically synthesized peptides as standards.

Table 1. Daily Intake from Placebo Test Tablet[1]

	Placebo (6 placebo tablets)	Test group (6 test tablets)
Calories (kcal)	44	44
Moisture (g)	0.5	0.4
Protein (g)	0.6	0.6
Fat (g)	0.3	0.2
Carbohydrate (g)	10.2	10.4
Fiber (g)	0.3	0.3
Ash (g)	0.1	0.1
Sodium (mg)	1.8	1.4
Potassium (mg)	3.2	2.4
Calcium (mg)	31.2	27.6
Magnesium (mg)	7.9	7.1
Phosphorus (mg)	16.8	13.2
Iron (mg)	0.0	0.0
Val-Pro-Pro (VPP) peptide[2] (mg)	ND	8.3
Ile-Pro-Pro (IPP) peptide[2] (mg)	ND	4.7

[1] Composition per 6 tablets.

[2] Content of the angiotensin I-converting enzyme (ACE) inhibitory peptides VPP and IPP were determined by the method described in the text.

ND = not detectable.

Subjects

Subjects were recruited from a pool of volunteers from the Institute of General Medical Science, and from adult men and women who had undergone a medical examination for this study at the Soiken Clinic, affiliated with the Institute of General Medical Science, for 3 months before the trial began. Criteria for selection were: those who had blood pressure categorized in the high-normal (systolic blood pressure, SBP, 130–139 mm Hg and diastolic blood pressure, DBP, 85–89 mm Hg), and those who had blood pressure categorized to mild hypertension (SBP, 140–159 mm Hg and DBP, 90–99 mm Hg) at low risk according to the definition of the World Health Organization/International Society of Hypertension (WHO/ISH) Hypertension Guidelines from 1999 [2]. Exclusion criteria included those: with allergy to milk proteins; taking medication that might influence blood pressure; with lactose intolerant symptoms; considered unsuitable by a medical doctor in charge of the study. Ninety-five subjects who fulfilled the eligibility criteria for the study agreed to participate. However, 15 subjects were excluded before the randomization: 6 subjects whose blood pressure levels were outside the categories shown above prior to the experimental period, and 9 subjects whose blood pressure levels showed a marked change in the measurements at two and one day before treatment (i.e. blood pressure levels outside the criteria for selection). Thus, a total of 80 subjects consisting of 40 with high-normal blood pressure (HN group) and 40 with mild hypertension (MH group) were enrolled and randomized. All 80 subjects were enrolled into the study before random allocation. They completed the study and were followed up during the study period. They all had the intended blood pressure measurements and all 80 subjects were included in the statistical analysis. The allocation of test or placebo group was concealed from the investigator who enrolled the subjects. Furthermore, the nurses and the medical doctor in charge were blind to the treatment assignments.

This study was approved by the Institutional Review Board of the Institute of General Medical Science according to an ethic principle and experimental plan based on the Helsinki Declaration. Prior to the trial, a full explanation concerning the purpose of the study and its methodology was given to the subjects by the medical doctor. The 95 subjects who agreed to participate in the study gave fully informed consent.

Trial Design

This study was a randomized, double-blind, placebo-controlled design, and the experimental periods were divided into: 1 week of observation before treatment, 4 weeks of treatment, and 1 week of observation after treatment. The 40 subjects in the HN or MH group were randomly and blindly assigned into two groups of 20. An independent, blinded investigator conducted the randomization and checked there were no differences in the age, gender and SBP/DBP values between the two groups. The characteristics of the subjects are shown in Table 2. Baseline values were measured at one day before treatment. There were no significant differences between the test and placebo groups in gender, age, BMI, blood pressure or pulse rate ($p > 0.2$). One group ($n = 20$) was given the test tablets (test group), and the other group ($n = 20$) was given the placebo tablets (placebo group). The subjects were instructed to ingest 6 tablets (12 g total) every morning by chewing. They were also instructed to check their health condition, and instructed not to change their dietary and exercise habits.

Measurements

Blood pressure, pulse rate, body weight, height, blood test, urinalysis, and other medical examinations/inquiries of a subject about his/her condition were all carried out at the Soiken Clinic. Subjects were required to fast before he/she visited the Institute on the test dates, except for 5 days (day 1 and week 1, 2, 3, 4) when he/she was allowed to take a supplementary tablet

Table 2. Baseline Characteristics of Subjects

	High-normal blood pressure (HN)		Mild hypertension (MH)	
	Placebo group (n = 20)	Test group (n = 20)	Placebo group (n = 20)	Test group (n = 20)
Male	13	13	16	16
Female	7	7	4	4
Age[1] (years)	52.8 ± 10.9	49.9 ± 12.4	51.5 ± 11.8	51.9 ± 10.3
Body weight[1] (kg)	64.1 ± 12.4	64.8 ± 12.9	60.5 ± 7.7	58.7 ± 12.2
Height[1] (cm)	161.9 ± 7.8	163.7 ± 8.1	166.4 ± 5.5	164.5 ± 8.9
Body mass index (BMI)[1] (kg/m^2)	24.3 ± 3.6	24.0 ± 3.0	25.1 ± 3.5	24.9 ± 2.8
Systolic blood pressure (SBP)[1,2] (mm Hg)	136.8 ± 6.1	137.4 ± 5.1	146.6 ± 8.7	148.8 ± 7.2
Diastolic blood pressure (DBP)[1,2] (mm Hg)	84.8 ± 11.0	85.1 ± 4.9	92.1 ± 8.7	92.6 ± 11.9
Pulse rate[1,2] (beats/min)	71.2 ± 8.0	71.9 ± 6.7	73.4 ± 7.3	73.4 ± 6.3

There were no significant differences between the test and placebo groups ($p > 0.2$; Student's t test).

[1] Data are expressed as means ± SD.

[2] Baseline values were measurements taken 1 day before treatment (Day −1).

2 to 3 hours before arrival. All measurements were carried out under the doctor's supervision.

Blood pressure and pulse rate were measured before treatment at day 7, 3, 2 and 1, and after treatment commenced at day 1 and week 1, 2, 3, 4 and 5 (10 times in total). Blood pressure was measured using a mercury sphygmomanometer and was carried out by the same registered nurse for every subject. The subjects were instructed to visit the clinic in a fasted condition between 8:30 and 11:30 a.m.; after relaxing for over 20 minutes at the clinic, blood pressure was measured three times (each time by a different trained registered nurse) in the sitting position on the left upper arm at one minute intervals, clothed but without shoes. The median systolic blood pressure value was counted as the blood pressure of the day. In addition, the pulse rate was measured using the UDEX-SUPER automatic sphygmomanometer (UEDA Avancer Corporation, Chiba, Japan).

Weight was measured 1 day before treatment and at week 4 and 5 after treatment started. Subjects wore a shirt/blouse and trousers/skirt but no jewelry. The same subject was guided to measure body weight wearing the same dress. Height was measured 1 day before treatment only, and body mass index (BMI) was calculated based on this measurement.

Blood samples were taken from each subject 1 day before treatment and at week 4 and 5 after treatment started. Subjects were told to fast overnight prior to measurement days. Blood examination was carried out at Sakai Biochemistry Research Lab Co., Ltd (Osaka, Japan). Biochemical markers measured were as follows: white blood cell, red blood cell, hemoglobin, hematocrit, platelet, glucose, glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, γ-glutamyltranspeptidase, lactate dehydrogenase, total protein, triacylglycerol, total cholesterol, HDL cholesterol, blood urea nitrogen, uric acid, creatine, sodium, potassium, chloride, calcium and plasma renin activity. The urinalysis was carried out using appropriate test paper at the clinic.

A doctor carried out medical examinations and inquiries 1 day before treatment, and day 1 and week 1, 2, 3, 4 and 5 after treatment started (7 times in total). The subjects were asked about and examined for adverse effects such as dry cough, exanthema, skin itching, dysgeusia, headache, dizziness/drift, and abdominal symptoms such as inappetence, diarrhea, constipation, flatulence and abdominal discomfort. The subject recorded the presence of any of these symptoms during the trial.

Statistical Analysis

On the basis of information from a previous study [12], a sample size of 20 mild hypertensive subjects per group was deemed sufficient to give 80% power to detect a 9 mm Hg difference between the test and placebo group at $\alpha = 0.05$, from baseline SBP. This assumed a standard deviation (SD) of 10 mm Hg for the change from baseline SBP.

All measured values are expressed as mean ± SD unless otherwise noted. Statistical analyses were carried out using "SPSS Advanced model" (SPSS Inc., Chicago, IL). The student *t* test was used to compare the test and placebo groups and Dunnett's test was used for multiple comparisons between baseline and treatment period in SBP, DBP and pulse rate. Changes from baseline values in blood tests and BMI values were evaluated using a paired *t*-test. Two-way analysis of variance (ANOVA) for repeated measures was used in the assessment of treatment results of SBP, DBP and pulse rate values. A $p < 0.05$ with two-tail test was considered as statistically significant.

RESULTS

Blood Pressure and Pulse Rate

Changes in blood pressure are shown in Fig. 1 and Fig. 2. During the pre-treatment observation period, no significant

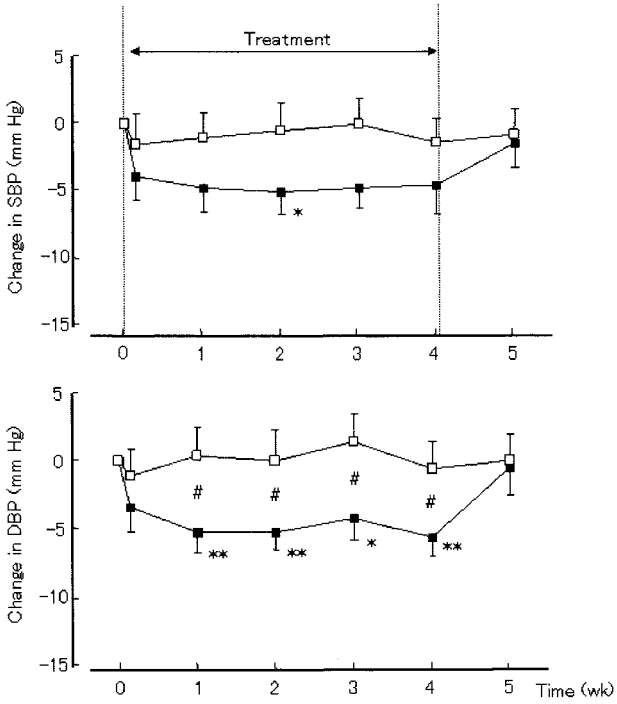

Fig. 1. Change in systolic blood pressure (SBP) and diastolic blood pressure (DBP) from baseline in subjects with high-normal blood pressure during the 4 wk intake and 1 wk follow-up period in the test group (■; n = 20) and placebo group (□; n = 20). The subjects ingested 6 test tablets containing powdered fermented milk with *Lactobacillus helveticus* or 6 placebo tablets, daily for 4 weeks. After treatment started, SBP and DBP were measured at day 1 and week 1, 2, 3, 4 and 5. Data are expressed as mean ± SEM. Mean values were significantly different from baseline values (Dunnett's test): $*p < 0.05$, $**p < 0.01$, and also significantly different between two treatments (Student's t test): $\#p < 0.05$.

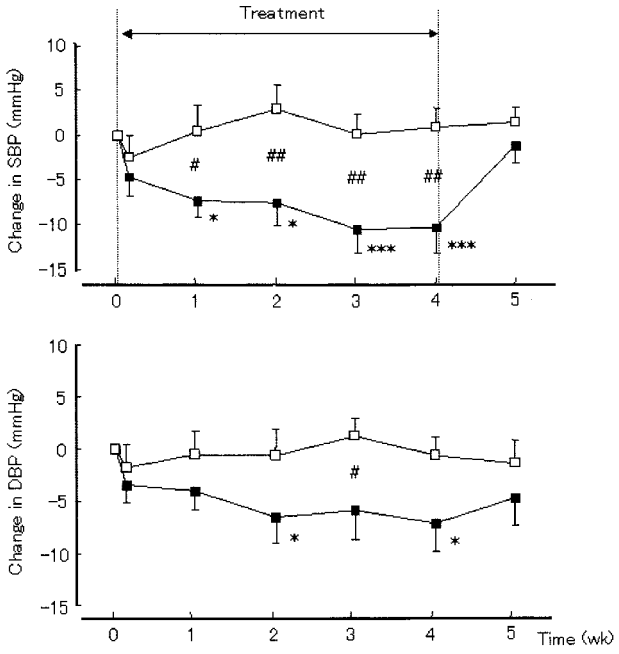

Fig. 2. Change in systolic blood pressure (SBP) and diastolic blood pressure (DBP) from baseline in subjects with mild hypertension during the 4 wk intake and 1 wk follow-up period in the test group (■; n = 20) and placebo group (□; n = 20). The subjects ingested 6 test tablets containing powdered fermented milk with *Lactobacillus helveticus* or 6 placebo tablets, daily for 4 weeks. After treatment started, SBP and DBP were measured at day 1 and week 1, 2, 3, 4 and 5. Data are expressed as mean ± SEM. Mean values were significantly different from baseline values (Dunnett's test): $*p < 0.05$, $***p < 0.001$, and also significantly different between two treatments (Student's *t* test): $\#p < 0.05$, $\#\#p < 0.01$.

changes in SBP, DBP and pulse rate were observed for both the test and placebo groups in either the high-normal blood pressure (HN) group or mild hypertension (MH) group. However, SBP and DBP in the test group tended to decrease more than that in the placebo group during treatment in both groups.

In the HN group, SBP and DBP in the test group changed from 137.4 ± 5.1/85.1 ± 4.9 mm Hg (baseline; SBP/DBP) to 132.7 ± 6.2/79.9 ± 4.8 mm Hg at week 1, 132.4 ± 4.8/79.9 ± 4.3 mm Hg at week 2, 132.7 ± 5.6/80.9 ± 6.2 mm Hg at week 3, and 132.9 ± 7.0/79.5 ± 6.8 mm Hg at week 4. At the end of treatment (week 4), the change in SBP in the test group was 3.2 mm Hg (95% CI −2.6, 8.9; $p = 0.27$) lower than that in the placebo group, whereas DBP decreased more in the test group than in the placebo group during treatment, by: 5.7 mm Hg (0.6, 10.7; $p = 0.029$), 5.3 mm Hg (0.0, 10.5; $p = 0.049$), 5.6 mm Hg (0.4, 10.8; $p = 0.037$) and 5.0 mm Hg (0.1, 9.9; $p = 0.045$) at weeks 1, 2, 3 and 4, respectively (Fig. 1). According to two-way ANOVA, a significant main effect of supplementary tablets for SBP and DBP values was found ($p = 0.006$ and 0.045, respectively).

In the MH group, SBP and DBP in the test group changed from 148.8 ± 7.2/92.6 ± 11.9 mm Hg (baseline; SBP/DBP) to 141.6 ± 7.3/88.6 ± 12.3 at week 1, 141.4 ± 7.9/86.1 ± 14.4 mm Hg at week 2, 138.5 ± 10.2/86.8 ± 15.7 mm Hg at week 3, and 138.6 ± 10.1/85.6 ± 16.5 mm Hg at week 4. SBP showed a greater decrease in the test group than in the placebo group during treatment (at weeks 1, 2, 3 and 4), by: 7.8 mm Hg (95% CI 0.8, 14.8; $p = 0.03$), 10.5 mm Hg (3.0, 18.0; $p = 0.007$), 10.6 mm Hg (3.6, 17.6; $p = 0.004$) and 11.2 mm Hg (4.0, 18.4; $p = 0.003$), respectively (Fig. 2). DBP decreased by 7.1 mm Hg (95% CI 0.4, 13.7; $p = 0.039$) more in the test group than in the placebo group at week 3, and tended to decrease by 6.5 mm Hg (−0.1, 13.0; $p = 0.055$) more in the test group than in the placebo group at the end of treatment (week 4) (Fig. 2). According to two-way ANOVA, an interaction between supplementary tablets and treatment period for SBP values was significant ($p = 0.008$). For DBP values a significant main effect of supplementary tablets was not found.

Pulse rate did not change significantly during the experimental period in the test or placebo groups, and no significant difference between groups was observed. In the HN group, pulse rate (beats/min) at baseline were 71.9 ± 6.7/71.2 ± 8.0 (test group/placebo group), and 72.5 ± 5.5/69.9 ± 6.4 at the end of treatment (week 4). In the MH group, pulse rate at baseline were 73.4 ± 6.3/73.4 ± 7.3, and 72.7 ± 5.8/73.9 ± 6.1 at the end of treatment (week 4).

BMI, Blood Test Values and Urinalysis

BMI did not change significantly in the test and placebo groups of the HN group. BMI in test and placebo groups were 24.1 ± 3.2 and 24.3 ± 3.8, respectively, at week 0, and 24.0 ± 2.9 and 24.3 ± 3.6, respectively, at week 4. In both groups within the MH group, BMI was 24.9 ± 2.8 and 25.1 ± 3.5 at week 0, and 25.1 ± 2.8 and 25.1 ± 3.4 at week 4.

Changes in blood test values after treatment were as follows: in the HN group, the increase from baseline was statistically significant in triacylglycerol, creatinine, and sodium in the test group, and creatinine in the placebo group. The decrease from baseline was statistically significant in hemoglobin, HDL-cholesterol, potassium, chloride, and plasma renin activity in the test group, and red blood cell, hemoglobin, hematocrit and chloride in the placebo group. In the MH group, the increase from baseline was statistically significant in creatinine in the test and placebo group. The decrease from baseline was statistically significant in red blood cell, hemoglobin, hematocrit and chloride in the test group, and chloride in the placebo group. All these changes were, however, within normal range, and even the result of each subject showed no change that might develop to an abnormal range (data not shown). There were no statistically significant changes in any of the other biochemical markers, and no significant difference between the test group and the placebo group was observed.

There were no abnormal changes observed for uric glucose, uric protein, uric urobilinogen between the pre-intake and the post-intake periods for the test and placebo group. Two subjects

in the HN group were positive for uric protein and one subject in the MH group was positive for uric glucose at the pre-intake period, but no abnormal changes were observed between the intake and no-intake periods.

Medical Examinations and Inquiries

During the study, adverse effects such as dry cough, exanthema, skin itching, dysgeusia, headache and dizziness/drift were not observed. In addition, abdominal symptoms such as inappetence, diarrhea, constipation, flatulence or abdominal discomfort were not observed in the test group. Although transient diarrhea (four subjects in the placebo group and two subjects in the test group) and constipation (two subjects in the placebo group and one subject in the test group) were observed, the doctor judged that the symptoms were not caused by intake of supplementary tablets. The symptoms were equally distributed between test and placebo group.

DISCUSSION

We observed a decrease in elevated blood pressure without any adverse effects in subjects with high-normal blood pressure or mild hypertension without antihypertensive medication after ingestion of tablets containing powdered *L. helveticus* fermented milk during a 4 week period. Regarding the report associating cardiovascular complication with high-normal, normal and optimal blood pressures [14] and the report concerned with the development from normotension to hypertension [15], it may be important to investigate dietary therapy as the primary prevention of hypertension for high-normal blood pressure subjects with a high risk for incidence of hypertension. In the Dietary Approaches to Stop Hypertension (DASH) trial [4], which examined the effects of dietary therapy, the blood pressure of nonhypertensive subjects was significantly decreased by ingesting foods rich in fruit, vegetables, and low-fat dairy products with reduced saturated and total fat, compared with a control. A significant decrease in blood pressure has also been shown in subjects with high-normal blood pressure by reducing body weight and restricting salt intake over the long term [5]. Other reports did not show that daily intake of particular food components might cause a significant decrease in blood pressure in subjects with high-normal blood pressure. The results of the present study suggest that ingestion of tablets containing powdered *L. helveticus* fermented milk might lead to a decrease in blood pressure in subjects with high-normal blood pressure to the normal range, and furthermore, prevention of the incidence of hypertension.

We studied the efficacy and safety of powdered *L. helveticus* fermented milk in normotensive and hypertensive subjects. In normotensive subjects (mean blood pressure: 114/68 mm Hg), 6 tablets containing powdered *L. helveticus* fermented milk did not affect blood pressure [13]. The net changes of SBP and DBP were 0/2 mm Hg at 2 weeks after the daily intake of 6 tablets [13]. In the present study, we observed a mild decrease in SBP and DBP in subjects with high-normal blood pressure (137/85 mm Hg) after daily intake of 6 tablets. The net reduction of SBP and DBP was 5/5 mm Hg at 2 weeks ($p = 0.11$ and $p = 0.049$, respectively) and 3/5 mm Hg at 4 weeks ($p = 0.27$ and $p = 0.045$, respectively). In subjects with mild hypertension (148/92 mm Hg), the net reduction of SBP and DBP was 11/6 mm Hg at 2 weeks ($p = 0.007$ and $p = 0.1$, respectively) and 11/6 mm Hg at 4 weeks ($p = 0.003$ and $p = 0.055$, respectively). In subjects with mild to moderate hypertension (150/93 mm Hg), a significant decrease in blood pressure was observed after daily intake of 2 tablets [12]. The net reduction was 8/7 mm Hg and 9/6 mm Hg at 2 and 4 weeks, respectively, after daily intake of 2 tablets [12]. Although the doses in the present trial were higher than those used in the previous study [12], we did not observe an abrupt or excessive decrease in blood pressure. These results suggest that the magnitude of decrease in blood pressure after ingestion of tablets containing powdered *L. helveticus* fermented milk may be affected by the hypertensive status (i.e. greater effect in hypertensives than nonhypertensives). In the DASH trial, the net reduction in blood pressure (SBP/DBP) of hypertensive and nonhypertensive subgroups were 11.4/5.5 mm Hg and 3.5/2.1 mm Hg, respectively, which supports the result in the present study.

VPP and IPP peptides contained in the test tablet have ACE inhibitory activity. It has been reported that ingestion of these tripeptides in SHR caused an antihypertensive effect equivalent to the fermented milk that had corresponding ACE inhibitory activity of the tripeptides [8]. In addition, after administration of the fermented milk to SHR, ACE activity in extracts from arterial endothelium tissues of the SHR rat was significantly decreased, and both tripeptides were detected in these tissues [10]. From these results, the antihypertensive effect of the fermented milk may result from these two ACE inhibitory peptides, and these peptides may contribute to the blood pressure lowing effect in the present study and the clinical studies previously reported [11,12,16–20]. The test tablet may have contained components other than VPP and IPP that might have a blood pressure lowering effect: the test tablet contained calcium, magnesium and potassium derived from skimmed milk. An inverse relationship between these minerals and blood pressure has been reported [21–23]. However, in the present study, the placebo tablet did not exhibit antihypertensive effects even though the daily intake of potassium and calcium from the placebo tablet was 0.6 mg and 3.6 mg higher, respectively, than that from the test tablet, which indicates that potassium and calcium were not involved in the observed antihypertensive effect. It has been suggested that cell wall fragments from lactic acid bacteria may cause an antihypertensive effect [24]. However, the cell debris fraction was removed by centrifugation during the preparation of the test tablet in the present study.

Regarding the baseline characteristics of the subjects, no significant differences were observed between test and placebo

groups (Table 2). However, the mean age of subjects in the test group of the high-normal blood pressure (HN) group was approximately three years younger than those in the placebo group. Also, there was a 2 cm difference in the mean height between the test and placebo groups. The possible effects of the difference in the age and height at baseline on the change in blood pressure were analyzed using regression analysis. The change in blood pressure was not significantly related to these factors, which suggests that these differences did not influence the results.

Some of the blood test values were significantly different within the test or the placebo group during the 4 week period. However, the changes in the blood test values were in the same direction in both groups and no significant difference between the test and the placebo groups was observed. Therefore, the changes were probably not attributable to the study, but to the seasonal variation that has been reported for several hematological and serum biochemical variables [25–27]. Although the treatment period of the present study was relatively short (4 weeks) the study was conducted from February to March: the transition from winter to spring in Japan.

In the HN group, the magnitude of the reduction in SBP and DBP was very similar for both the treatment group (SBP and DBP reductions: 5 mm Hg from the baseline) and the control group (approximately 2 mm Hg for SBP and DBP) (Fig. 1). SBP reduction is usually greater than DBP, as shown in our previous studies [11,12,16–19]. A possible explanation is the relatively short treatment period. In the DASH trial, the net reduction of SBP was smaller than DBP at 1 week of treatment [4]. If the treatment period in the present study had been longer, the reduction in SBP may have been greater than the reduction in DBP. Some studies on the blood pressure lowering effect of calcium supplementation have shown larger DBP reductions compared with SBP [28–30]. Calcium supplementation is thought to be effective in those with low serum calcium and high parathyroid hormone levels, caused by high sodium intake and subsequent volume expansion in sodium sensitive, low renin hypertensives [31]. Although parathyroid hormone levels and high sodium intake were not taken into account in the present study, a greater DBP reduction, rather than SBP, could be attributed to a similar mechanism.

On the first day of treatment (day 1), in the HN group, the net reduction in SBP and DBP was 2.4 mm Hg (95% CI −3.4, 8.2; $p = 0.41$) and 2.3 mm Hg (−3.2, 7.8; $p = 0.40$), respectively (Fig. 1), and in the MH group, 2.2 mm Hg (−4.6, 8.9; $p = 0.52$) and 1.7 mm Hg (−4.0, 7.4; $p = 0.55$), respectively (Fig. 2). Although the reduction was not statistically significant and may be a placebo effect, the mean reduction in the test group was slightly larger than that in the placebo group. After single oral administration of fermented milk containing VPP and IPP peptides or VPP or IPP peptide administration to SHR, blood pressure gradually decreased 2 hours after administration and continued to be depressed for approximately 8 hours; 24 hours later, blood pressure levels had returned to their initial levels [8]. Since we did not study the effect of single oral administration in humans, we measured blood pressure at day 1 to confirm no acute effect on blood pressure. Our previous studies suggest that daily ingestion of the fermented milk product generates mild antihypertensive effects [11,12,16–19]. In addition, the significant interaction between supplementary tablets and treatment period for SBP values in the MH group supports this view. In the present study the dose of VPP and IPP peptides in the test product were approximately 0.13–0.14 mg VPP and 0.073–0.080 mg IPP/kg body weight. This dose seems insufficient for a blood pressure lowering effect in SHR by single oral administration [8]. However, taking the estimated body surface area into consideration, we suggest the dose may be sufficient for a blood pressure lowering effect in SHR [11]: a single dose of the test product may also lead to a blood pressure lowering effect in those with slightly higher blood pressure. The mean reduction in the HN test group continues with almost the same magnitude during the treatment period (Fig. 1). This may be because, during the treatment period, the blood pressure reduction in this group was relatively small.

It is known that treatment with ACE inhibitors increases the plasma renin activity [32]: this was not observed after ingestion of the supplementary tablet in the present study. However, chronic administration of ACE inhibitor in humans is not always accompanied by a change in plasma renin activity [33]. It is also reported that after administration of sour milk containing VPP and IPP to SHR, plasma ACE activity does not change but arterial tissue ACE activity is significantly reduced [10]: the same phenomenon may have occurred in the present study. Further studies are required to determine the precise mechanism of the observed antihypertensive effect mediated by VPP and IPP peptides.

Since the amount of VPP and IPP peptides in the supplementary tablet ingested in this study were relatively low, and their ACE inhibitory activities mild in comparison to ACE-inhibitor drugs, the tablets are considered safe with respect to adverse effects associated with ACE inhibitors. Dry cough is the most common adverse effect of ACE inhibitors [34,35], and is one of the causes for the decrease of Quality of Life or termination of administration. The occurrence of a dry cough associated with ACE inhibitors is thought to involve the accumulation of substances such as bradykinin and substance P [36]. Bradykinin promotes the production of prostaglandins and leucotrienes as well as the release of histamine, and these substances are thought to irritate the C fiber receptors of the bronchial tubes, resulting in a cough [36]. Since the dry cough observed with ACE inhibitors dissipates rapidly following discontinuation of administration or reduction in the dose level, regulating the drug concentration in the body is considered to be important in dealing with the cough. Coughs caused by ACE inhibitors usually occur within one month after the initiation of administration [35]. However, Hata et al. [11] and Seppo et al. [20] allowed hypertensive subjects to intake the fermented milk

containing VPP and IPP for 8 weeks and 21 weeks, respectively, and reported that adverse effects were not observed. Our results are entirely consistent with these observations. In addition, since VPP and IPP have been reported to exhibit antihypertensive effects by inhibiting arterial tissue ACE in animal studies [10], no incidence of dry cough can be attributed to the tissue-specific action. Further studies are necessary to clarify these hypotheses.

CONCLUSION

Administration of supplementary tablets containing powdered *L. helveticus*-fermented milk to subjects with high-normal blood pressure or mild hypertension resulted in a significant decrease in blood pressure without any adverse effects. This result suggests that foods containing powdered *L. helveticus*-fermented milk could be used in dietary approaches to the primary prevention of hypertension.

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