



Research article

Effect of heat-moisture treated brown rice crackers on postprandial flow-mediated dilation in adults with mild endothelial dysfunction



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ABSTRACT

Background: Endothelial dysfunction is an early pathophysiological feature and independent predictor of a poor prognosis in most forms of cardiovascular disease. We evaluated the effect of brown rice crackers (BR-C) on endothelial function.

Methods: Effect of heat-moisture treated (HMT) -BR-C on postprandial flow-mediated dilation (FMD) in adults with mild endothelial dysfunction was compared with that of BR-C and white rice crackers (WR-C) in 12 adults with mild endothelial dysfunction (less than 7.0% of FMD) by a randomized, single-blind, three-treatment three-period crossover trial (UMIN 000034898). Since we considered that the FMD increase was associated with the treatment of HMT-BR-C, we examined the effect of three possible factors: postprandial glucose levels, polyphenol content, and polyphenol release from the food matrix.

Results: Mean pre-intake baseline FMD values of HMT-BR-C, BR-C, and WR-C were 4.9%, 5.1%, and 4.9%, respectively, and those values 1 h post-intake were 6.3%, 5.1%, and 4.8%, respectively. There was no difference in intergroup comparisons of FMD using Dunnett's multiple comparison test. There was a significant increase in FMD only in HMT-BR-C in intragroup comparisons ($P = 0.042$ by paired- t test). In comparison with BR-C, no significant difference was noted in the postprandial glucose level nor in the content of total polyphenols and ferulic acid derivatives in HMT-BR-C. However, the 70% ethanol extracted from HMT-BR-C contained a significantly larger amount of free and bound ferulic acids than from BR-C.

Conclusion: HMT-BR-C intake increased the postprandial FMD response.

1. Introduction

Endothelial dysfunction is an early pathophysiological feature and an independent predictor of a poor prognosis in most forms of cardiovascular disease (CVD) [1, 2, 3]. Assessment of the degree of flow-mediated dilation (FMD) of the brachial artery can reveal endothelial dysfunction [4, 5]. Food intake was reported to affect the FMD response according to

components of the ingested food [6]. FMD could be mediated by dietary polyphenols depending on their active components, the duration and dosage of intake, and bioavailability including bioaccessibility and absorption [7, 8, 9, 10, 11, 12].

Rice (*Oryza sativa* L.) is a major staple of the Japanese diet. Brown rice (BR) contains not only starch as the main nutrient but also many minor nutrients with potential health benefits, such as dietary fiber,

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polyphenols, amino acids, and vitamins. These minor nutrients can be altered depending on the variety of rice, methods of processing, and cooking conditions [13]. The intake of a white rice (WR) meal was reported to decrease postprandial FMD but not the consumption of a BR meal [14]. An elevation of blood glucose should be shown after intake of both meals. These results suggested that other ingredients in BR might induce the beneficial effect of increasing FMD as opposed to the elevation of blood glucose to decrease FMD.

Heat-moisture treatment (HMT) is a hydrothermal process that alters the physicochemical properties of starch by changing its molecular structure [15, 16]. HMT of starches changes thermal stability and retrogradation resulting in differences from those of native starches. However, its effect on the properties of endogenous polyphenols in BR has been obscured because thermal treatment of polyphenols in food matrices has been reported to become a double-edged sword with contradictory effects such as an increased release of polyphenols through softening or disruption of cell walls and decreased release through degradation and oxidation of polyphenols [17]. High-pressure cooking is more effective than normal pressure cooking in terms of retention of free phenolic compounds and a significant increase in two predominant bound phenolic compounds (ferulic acid and isoferulic acid). In addition, with increases in cooking pressure, the gamma-aminobutyric acid (GABA) content decreased [18]. In our preliminary trial, intake of rice crackers (C), a traditional Japanese confectionery prepared from heat-moisture treated brown rice flour only, was found to increase FMD values at 1 h post-intake [19, 20].

The aims of this study were two fold. First, we compared FMD responses after intake of rice crackers, that is heat-moisture treated brown rice crackers (HMT-BR-C), brown rice crackers (BR-C), and WR-C, prepared from three kinds of rice flour (HMT-BR-F, BR-F, and WR-F) produced by different procedures for post-harvest processing of Koshiibuki (Japonica normal-quality rice cultivar) in adults with mild endothelial dysfunction by a randomized, single-blind, three-treatment three-period crossover trial (experiment [Exp]-1). Then we examined two possible factors affecting FMD responses: postprandial glucose and insulin levels (Exp-2) and polyphenol release from the food matrix (Exp-3). Study participants in this randomized, single-blind, three-treatment three-period crossover trial were adults with mild endothelial dysfunction.

2. Methods

2.1. Rice cracker preparation

BR and WR of the normal-quality rice cultivar “Koshiibuki” were purchased from a local market in Niigata, Japan in 2018. HMT-BR was prepared at Niigata-Seifun Co., Ltd., (Niigata, Japan) according to our previous report [20]. BR samples (2000 g) with an initial moisture content of 12.0% (wet basis) were put in a stainless steel sieve within a stainless steel vessel with a glass lid. The device made use of a vacuum steam heating apparatus (tank volume 15 L) with a flow-through boiler (SZ60; Miura Co., Ltd., Matsuyama Ehime, Japan). After reducing the pressure using a vacuum pump, pressure was then applied with steam at 0.2 MPa (added water vapor pressure) and 134 °C for 3 min after which there was a rapid reduction to atmospheric pressure. BR-F with an average particle size of 100 microns or less was prepared using a cyclone mill (PG-1; Nara Machinery Co., Ltd., Tokyo, Japan).

HMT-BR-C, BR-C, and WR-C, respectively, were prepared from the corresponding rice flour, HMT-BR-F, BR-F, and WR-F, at Bourbon Corp (Kashiwazaki Niigata, Japan). Rice flour (4000 g) was mixed with water (2200–2700 g), and the mixture was kneaded well to get a homogeneous dough. Dough was rolled out into 1.3 mm thick sheets, which were converted into cracker dough 30 mm in diameter. Drying (70 °C) followed by roasting (240–270 °C, 2.5 min) provided rice crackers.

Nutrient analysis of rice crackers was conducted by Bourbon Corp (Niigata, Japan. <https://www.bourbon.co.jp>) using the following methods standard in Japan: energy, modified Atwater method; protein,

Kjeldahl nitrogen determination (conversion factor: 6.25) method; fat, ether extraction method; ash, direct ashing method; carbohydrate, calculated by difference; dietary fiber, modified Prosky method; moisture, oven drying at 105 °C for 16 h; and salt equivalent calculated from sodium measured by inductively coupled plasma atomic emission spectrometry (conversion factor: 2.54). The analysis of non-nutrient components in rice crackers was performed by Japan Food Research Laboratories (Tokyo, Japan. <http://www.jfrrl.or.jp/>) using the following procedures: total polyphenols, Folin-Ciocalteu method; bound and free ferulic acid, high-performance liquid chromatography (HPLC); γ -oryzanol, HPLC; GABA, automatic amino acid analysis; and α -tocopherol, HPLC.

2.2. Exp. 1. postprandial FMD measurements

2.2.1. Study population

This study was approved by the ethics committee of Niigata University (Niigata, Japan; approved number 2018–0268) and Niigata Bio-research Center (Niigata, Japan; approved number IRB 2018-BPD-004) and was performed according to the Declaration of Helsinki. It was registered as UMIN 000034898 in the University Hospital Medical Information Network (UMIN) Clinical Trials Registry, Japan.

We calculated the sample size for the primary analysis based on differences observed in our previous study [20] of 6 healthy participants who received 60 g HMT-BR-C and had a mean increase in FMD of 1.18% (0.99% SD) 1 h after intake. Assuming a similar effect size, power calculations showed that a sample size of 11 individuals would be sufficient to reach a power of 80% at a significance level of 0.05. To allow for the possibility of one participant dropping out, 12 healthy adults were recruited from 20 applicants at Niigata Bio-research Center. Inclusion criteria were: (i) ≥ 18 years of age and < 70 years of age, (ii) a low fasting FMD value $< 7.0\%$ (5), and (iii) screening test and questionnaire results judged to be appropriate for this study by an investigator. Exclusion criteria were: (i) diabetes mellitus under medical treatment, (ii) having serious diseases (e.g., liver, kidney, heart, or blood diseases, and infections requiring notification), (iii) a history of gastrectomy, enterectomy, or gastro-intestinal diseases judged to be inappropriate for this study, and (iv) pregnancy, possibility of pregnancy, or lactation.

2.2.2. Study design

This was a randomized, single dose, single-blind, three-treatment three-period crossover trial conducted from November 2018 to February 2019 at Niigata Bio-research Center (Niigata, Japan). Since we could not perform Exp. 1 and Exp. 2 on the same day, these experiments were done with different participants. The protocol consisted of a screening visit, first FMD study phase, 1-week wash-out phase, second FMD study phase, 1-week wash-out phase, and third FMD study phase. After providing written informed consent and after completion of the screening procedures, participants entered the first FMD study phase.

Participants were randomly assigned to group A, group B, and group C, and the order of test rice cracker intake was as follows: group A, HMT-BR-C followed by WR-C and BR-C; group B, WR-C followed by BR-C and HMT-BR-C; and group C, BR-C followed by HMT-BR-C and WR-C. All participants were instructed to avoid lifestyle changes during the study including those of diet, sleep, and exercise, donating blood, or starting intake of new health foods. Beginning the day before testing and continuing throughout the study period, participants were prohibited from drinking alcohol, strenuous exercise and overeating. From 20:00 on the day before testing, eating and drinking anything except water were prohibited.

Before the FMD study phase, the baseline fasting FMD value was recorded. Subsequently, rice crackers (60 g) were consumed with 350 mL water over a 10-min period. The FMD value at 1 h post-intake was measured.

2.2.3. Physiological and biochemical variables of participants

The following physiological and biochemical variables were measured at the screening visit using standard methods: height, body weight, body mass index (BMI), body fat percentage (BFP), systolic and diastolic blood pressure (SBP and DBP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ -glutamyl transpeptidase (γ -GTP), high-density and low-density lipoprotein cholesterol (HDL-C and LDL-C), triglycerides (TG), blood urea nitrogen (BUN), creatinine (Cre), fasting blood glucose (BG), white blood cells (WBC), red blood cells (RBC), hematocrit (Hct), hemoglobin (Hb), and platelets. These measurements were performed by a clinical laboratory division of the Association of Occupational Health, Inc (Niigata, Japan).

2.2.4. Measurements of FMD

FMD values were measured in the brachial artery using a UNEX EF18G FMD device (Unex Corp., Nagoya, Japan) as described previously [20, 21]. Participants were examined while in a supine position after 30 min of rest in a quiet air-conditioned room (22–24 °C). Brachial artery diameter was measured by B-mode ultrasound imaging with a 7.5 MHz linear array transducer while an electrocardiogram was recorded simultaneously. The right brachial artery was scanned in longitudinal sections 1–10 cm above the elbow after at least a 5-min rest in the supine position. The skin surface was marked, and the arm was kept in the same position for subsequent measurements. After baseline measurement of brachial artery diameter, FMD was determined by scans during reactive hyperaemia. A pneumatic cuff placed around the forearm was inflated to 50 mmHg above systolic pressure and deflated after 5 min. The diameter of the brachial artery was scanned and recorded continuously from 30 s before to 2 min after cuff deflation to obtain the maximal diameter. After a 15-min rest, a second control scan of the diameter of the brachial artery was performed. FMD was calculated as the percentage change in diameter from the baseline value before cuff release to the peak value after cuff release.

2.3. Exp. 2. measurement of postprandial glucose and insulin levels

Exp. 2 was carried out using the same test foods as in Exp. 1 in 12 healthy study participants.

2.3.1. Study population

The procedures for Exp. 2 were approved by the ethics committee of Niigata University (Niigata, Japan; approved number 2018–0269) and Niigata Bio-research Center (Niigata, Japan; approved number IRB 2018-BPD-005) and was registered as UMIN 000035168. From among 23 applicants, 12 healthy adults were recruited at the Niigata Bio-research Center. They were screened for inclusion and exclusion criteria. Inclusion criteria were: (i) ≥ 18 years and <70 years of age, (ii) fasting BG < 126 mg/dL, and (iii) having results of the screening test and questionnaire judged to be appropriate for this study by an investigator. Exclusion criteria were the same as for Exp. 1.

2.3.2. Study design

Experiment 2 consisted of a screening visit, first BG study phase, 1-week wash-out phase, second BG study phase, 1-week wash-out phase, and third BG study phase. After providing written informed consent and after completion of the screening procedures, participants entered the first BG study phase. Enrollment in group A, group B, and group C (4 per group), the order of test rice cracker intake, and instructions for various activities during the study were similar to Exp. 1.

In the glucose study phase, antecubital venous blood was collected at baseline. Subsequently, participants ingested test rice crackers (60 g) with 350 mL water over a period of 10 min. Antecubital venous blood was collected at 30, 60, 90, and 120 min post-intake. Analysis of glucose and insulin levels in venous blood was performed by Niigata Association of Occupational Health Inc (Niigata, Japan).

2.3.3. Physiological and biochemical variables of study participants

Physiological and biochemical variables were measured at the screening visit using standard methods as described for Exp. 1 with the exception of measurements of FMD. The incremental areas under the curve (AUC) of blood glucose and insulin responses were calculated using the trapezoidal method for the period ranging from 0 to 180 min, ignoring the area below the fasting level.

2.4. Exp 3. efficiency of extraction of free and bound ferulic acids from test rice crackers by 70% ethanol

Efficiency of extraction was compared among three kinds of test rice crackers as a marker of polyphenol release from the food matrix. Extraction and analysis of free and bound ferulic acids were performed by the modified procedure of Nakano et al. [22] as follows.

2.4.1. Extraction

To finely pulverized rice crackers (5.0 g), 70% ethanol (50 mL) was added, and the resultant mixture was shaken for 10 min. After centrifugation (4000 rpm, 10 min) at room temperature, the supernatant was separated. Further extraction from the resultant solid residue with 70% ethanol was repeated three times in the same manner. Four supernatants were collected and concentrated to about a 1 mL solution, which was diluted to constant volumes of 2.0 mL and was used as analytical samples of four-stage batchwise extraction for liquid chromatography-mass chromatography (LC/MS). Analytical samples from the single-stage extraction were obtained but without three repeated extractions similar to that described above.

2.4.2. Identification and quantitative analysis of free and bound ferulic acids

Polyphenols in the extracts were identified using LC-MS/MS. The LC-MS/MS system consisted of a Shimadzu LCMS-8030 quadrupole mass spectrometer and a Shimadzu Prominence® LC-30A HPLC system (Shimadzu, Kyoto, Japan). Chromatographic separation was achieved in a Kinetex EVO C18 column (150 mm \times 2.1 mm i. d., 2.6 μ m, Phenomenex). The mobile phase consisted of solvent A containing 0.1% (v/v) acetic acid in water and solvent B containing methanol. The separation conditions were a linear gradient from 2% of solvent B for 0–4 min, 2–10% of solvent B for 4–15 min, 10–50% of solvent B for 15–30 min, and 50% of solvent B for 30–35 min. The flow rate was 0.2 mL/min. The column temperature was set at 40 °C, and 5 μ L of a 5-fold diluted extract was injected. Ferulic acid derivatives were detected by UV absorbance at 320 nm. A Shimadzu LCMS-8030 quadrupole mass spectrometer equipped with an ESI source in the negative ion mode was operated under the following conditions: nebulizing gas and drying gas with nitrogen were set at flow rates of 3.0 and 15.0 L/min, respectively; the interface voltage was set to 4.5 kV; and the desolvation line (DL) and heat block temperature were set at 300 °C and 500 °C. The mass spectrometer was used for selected ion monitoring (SIM) or the multiple reaction monitoring (MRM) mode with argon as the collision-induced dissociation gas (CID) at a pressure of 230 kPa. The detector voltage was set at 1.78 kV. The m/z value of each ferulic acid derivative with the SIM or MRM mode was set as follows: feruloysucrose (1), 517; ferulic acid (2), 193 \rightarrow 134; and feruloysinapoylsucrose (3), 723.

Quantitation of free and bound ferulic acids was performed using an HPLC system equipped with a diode array detector (DAD-HPLC, Shimadzu, Japan). Analytical conditions were as follows: column, Kinetex EVO C18 (250 mm \times 4.6 mm i. d., 5 μ m) from Phenomenex; flow rate, 1.0 mL/min; and column temperature, elution condition, and injection volume the same as for the LC/MS analysis described above. The UV spectra were recorded from 250 to 600 nm. Free and bound ferulic acids were detected at 320 nm and quantified by an external standard method using a calibration curve prepared with authentic ferulic acid (Sigma-Aldrich Corp., St Louis, MO, USA).

2.5. Statistical analysis

Results are presented as mean values and their SD. Intergroup comparisons among FMD responses and glucose and insulin levels in the three groups, HMT-BR-C, BR-C, and WR-C, were analyzed using Dunnett's multiple comparison test vs. WR-C. Intragroup comparison of the FMD change from baseline was performed using the paired-*t* test. Contents of ferulic acids in three kinds of rice crackers were compared by Dunnett's multiple comparison test vs. HMT-BR-C. The difference between baseline FMD and 1 h post-intake was compared by effect size. Values of $P < 0.05$ were considered statistically significant. All statistical analyses were performed using EZR software version 1.27 (Saitama Medical Center, Jichi Medical University, Saitama, Japan).

3. Results

3.1. Rice crackers

Nutrient and non-nutrient compositions of cooked rice crackers (HMT-BR-C, BR-C, and WR-C) and native rice flours (HMT-BR-F, BR-F, and WR-F) are shown in Table 1. No difference was apparent between HMT-BR-C and BR-C but WR-C contained lower levels of dietary fiber, total polyphenols, bound ferulic acid, γ -oryzanol, free GABA, and α -tocopherol. These rice crackers had similar compositions to those of the corresponding native rice flours, HMT-BR-F, BR-F, and WR-F, except that free GABA was decreased through cooking.

3.2. Exp. 1. postprandial FMD measurement

3.2.1. Study participants

FMD values for all 12 participants (5 females and 7 males) were $<7.0\%$ at screening. All participants successfully completed this trial without dropping out. Their data were included in the final analysis, although baseline FMD values over 7.0% (exceeding the limit established by the criteria) were measured in three male participants (Nos. 9, 10, and 12) (Figure 1). Characteristics of the 12 participants at the screening visit are summarized in Table 2. Their FMD values were in the abnormal (<4.0 , $n = 6$) and borderline zones (≥ 4.0 and <7.0 , $n = 6$), as classified by previously published criteria [5].

3.2.2. FMD measurement

Mean pre-intake baseline FMD values for HMT-BR-C, BR-C, and WR-C were 4.9%, 5.1%, and 4.9%, respectively, and those for 1 h post-intake were 6.3%, 5.1%, and 4.8%, respectively. Changes in FMD values from baseline (delta FMD) for HMT-BR-C, BR-C, and WR-C at 1 h post-intake were 1.4%, 0.0%, and -0.1%, respectively. Intergroup comparisons among FMD values for the three groups (HMT-BR-C, BR-C, and WR-C) as analyzed using Dunnett's multiple comparison test vs. WR-C revealed no significant difference in observed values ($P = 0.152$, HMT-BR-C vs. WR-C) or delta ($P = 0.173$, HMT-BR-C vs. WR-C). However, intragroup comparisons of the change 1 h after intake from the corresponding baseline level using the paired-*t* test found a significant increase in FMD in the HMT-BR-C group ($P = 0.042$) and the delta ($P = 0.042$). The effect size was 0.63. Figure 1).

3.3. Exp. 2. measurement of postprandial glucose and insulin levels

3.3.1. Study participants

All 12 participants (5 females and 7 males) successfully completed this trial, and their data were included in the final analysis.

3.3.2. Glucose and insulin levels

Glucose levels at 0 min (baseline), 30 min, 60 min, 90 min, and 120 min, respectively, after intake of the three kinds of rice crackers were as follows: HMT-BR-C, 5.16, 7.89, 8.43, 7.03 and 5.71 mmol/L; BR-C, 5.03, 7.84, 8.49, 6.97, and 6.07 mmol/L; and WR-C, 5.14, 8.36, 8.62, 7.34, and 5.92 mmol/L (Table 3). Insulin levels at 0 min (baseline), 30 min, 60 min, 90 min, and 120 min, respectively, were as follows: HMT-BR-C, 44.4, 327.1, 454.9, 344.4, and 258.3 pmol/L; BR-C, 49.9, 320.1, 428.3, 315.1, and 258.5 pmol/L; and WR-C, 56.2, 408.5, 475.0, 399.4, and 265.0 pmol/L (Table 3). From these data, profiles of changes from baseline were obtained as shown in Figure 2. No significant differences in either changes in glucose or insulin among the three groups were found using repeated measures analysis of variance.

3.3.3. Incremental areas under the curve of glucose (AUCg) and insulin (AUCi)

AUCg at 0 min (baseline), 30 min, 60 min, 90 min, and 120 min, respectively, after intake of the three kinds of rice crackers were as

Table 1. Nutrient and non-nutrient compositions of cooked rice crackers (HMT-BR-C, BR-C, and WR-C) and native rice flours (HMT-BR-F, BR-F, and WR-F).

Component	Cooked rice cracker (/60 g)			Native rice flour (/60 g)		
	HMT-BR-C	BR-C	WR-C	HMT-BR-F	BR-F	WR-F
Energy (Kcal)	241	241	240	218	218	207
Protein (g)	4.4	4.5	4.2	3.8	3.8	3.4
Fat (g)	2.1	2.1	1.1	1.9	1.8	0.8
Ash (g)	0.72	0.78	0.36	0.66	0.66	0.3
Carbohydrate (g)	50.9	51.1	53.3	46.5	46.7	46.4
Available (g)	49	49.1	52.5	44.9	45.2	46.0
Dietary fiber (g)	2	2	0.8	1.6	1.5	0.4
Moisture (g)	1.8	1.5	1.0	7.1	7	9.0
Salt equivalent (g)	0.01	0.00	0.00	0.01	0.01	0.00
Total polyphenols (g)	0.05	0.05	0.02	0.04	0.04	0.01
Bound ferulic acid (mg)	28	25	8	24	23	7
Free ferulic acid (mg)	nd	nd	nd	0.3	nd	nd
γ -Oryzanol (mg)	14.8	14.3	2.6	16.2	15.0	3.2
Free GABA (mg)	3	3	0.6	4.8	5.4	2.4
α -Tocopherol (mg)	0.48	0.60	0.30	0.48	0.54	0.30

HMT-BR-C, heat-moisture treated brown rice cracker; HMT-BR-F, heat-moisture treated brown rice flour; BR-C, brown rice cracker; BR-F, brown rice flour; WR-C, white rice cracker; WR-CF white rice flour; GABA, γ -aminobutyric acid; nd, not detected.

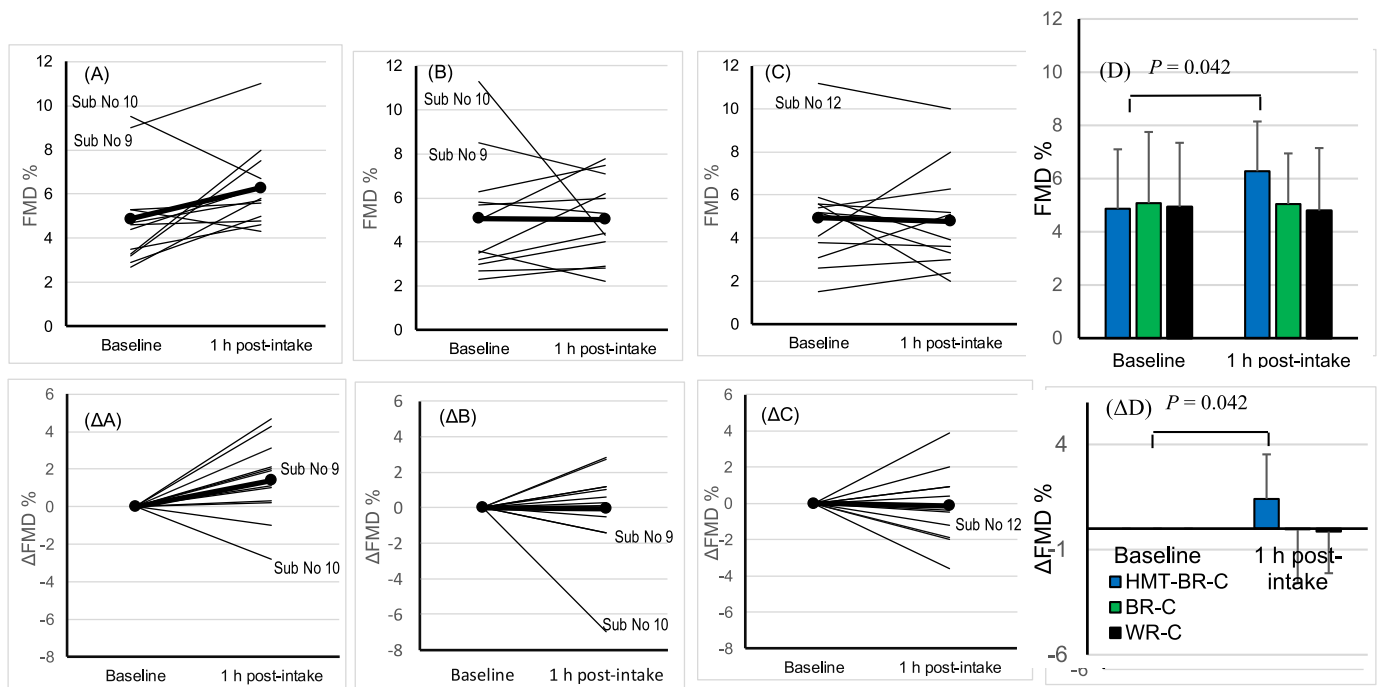


Figure 1. Individual and average flow-mediated dilation values. Individual (thin line) and average (thick line) flow-mediated dilation (FMD) values (A–C) and the delta ($\Delta A - \Delta C$) at baseline and 1 h post-intake and their statistical comparisons (D, ΔD). A and ΔA , heat-moisture treated brown rice cracker (HMT-BR-C); B and ΔB , brown rice cracker (BR-C); and C and ΔC , white rice cracker (WR-C). In D and ΔD , values are means ($n = 12$) with their standard deviations represented by vertical bars and P values are for intragroup comparisons using the unpaired-t test. Participants No. 9, 10, and 12 refer to participants with baseline FMD values over the 7.0% inclusion criteria limit. **Figure 1-D** and **1- ΔD** . Heat-moisture treated brown rice crackers (HMT-BR-C, blue), brown rice crackers (BR-C, green), and white rice crackers (WR-C, black).

follows: HMT-BR-C, 0, 41.1, 131.3, 208.4 and 244.7 min mmol/L; BR-C, 0, 42.2, 136.2, 217.1 and 261.8 min mmol/L; and WR-C, 0, 48.3, 148.9, 234.1 and 278.8 min mmol/L (**Table 3**).

AUC_i at 0 min (baseline), 30 min, 60 min, 90 min, and 120 min, respectively, were as follows: HMT-BR-C, 0, 4.24, 14.63, 25.29 and 33.00

min nmol/L; BR-C, 0, 4.05, 13.78, 23.43 and 30.54 min nmol/L; and WR-C, 0, 5.28, 16.85, 28.28 and 36.56 min nmol/L (**Table 3**).

Although AUC_g and AUC_i of HMT-BR-C and BR-C were lower than those of WR-C, there were no significant differences among the three groups using repeated measures analysis of variance.

Table 2. Characteristics and laboratory values of participants in FMD studies (Experiment-1), blood glucose and blood insulin measurements (Experiment-2).

Variables	FMD measurement (Experiment-1) N = 12		BG and BI measurement. (Experiment-2) N = 12	
	Mean	SD	Mean	SD
Male/female	7/5		5/7	
Age (years)	49.0	18.2	37.1	12.8
BMI (kg/m ²)	24.8	4.2	21.2	2.7
Height (cm)	163.3	7.8	164.1	10.6
Body weight (kg)	66.9	15.5	57.4	10.9
SBP (mmHg)	122.8	9.5	113.5	10.4
DBP (mmHg)	76.1	7.0	66.5	9.8
Triglycerides (mg/dL)	98.2	49.2	70.7	18.8
LDL-cholesterol (mg/dL)	119.3	17.1	119.3	38.0
HDL-cholesterol (mg/dL)	58.8	16.8	66.3	12.1
Fasting blood glucose (mmol/L)	5.26	0.73	5.13	0.83
Creatinine (mg/dL)	0.72	0.14	0.65	0.22
AST (U/L)	25.1	7.0	20.4	7.1
ALT (U/L)	32.8	22.6	16.0	11.4
γ -GTP (U/L)	39	25	25.3	9.9
Hemoglobin (g/dL)	14.3	1.4	14.9	1.5
FMD (%)	3.68	1.58	nm	
Abnormal (<4.0 %, $n = 6$) [†]	2.42	1.03	nm	
Borderline (4.0 % ≤ and <7.0 %, $n = 6$) [†]	4.9	0.78	nm	

ALT, alanine aminotransferase; AST, aspartate aminotransferase; DBP, diastolic blood pressure; FMD, flow-mediated dilation; γ -GTP, γ -glutamyl transpeptidase; nm, not measured; SBP, systolic blood pressure.

[†] Classified by the criteria of ref. (5).

Table 3. Exp. 2. Postprandial glucose (A, mmol/L) and insulin (B, pmol/L), the incremental areas under the curve of glucose (C, AUC_G, min mmol/L) and insulin (D, AUC_I, min nmol/L).

(A) Glucose	Baseline	30 min	60 min	90 min	120 min
HMT-BR-C	5.16 ± 0.33	7.89 ± 0.84	8.43 ± 1.04	7.03 ± 1.29	5.71 ± 1.31
BR-C	5.03 ± 0.36	7.84 ± 0.83	8.49 ± 1.64	6.97 ± 1.39	6.07 ± 1.43
WR-C	5.14 ± 0.28	8.36 ± 0.81	8.62 ± 1.64	7.34 ± 1.77	5.92 ± 1.34
(B) Insulin	Baseline	30 min	60 min	90 min	120 min
HMT-BR-C	44.4 ± 12.5	327.1 ± 100.0	454.9 ± 185.4	344.4 ± 165.2	258.3 ± 206.9
BR-C	49.9 ± 23.2	320.1 ± 154.6	428.3 ± 153.5	315.1 ± 103.1	258.5 ± 101.2
WR-C	56.2 ± 23.7	408.5 ± 147.8	475.0 ± 193.4	399.4 ± 183.2	265.0 ± 138.7
(C) AUC _G	Baseline	30 min	60 min	90 min	120 min
HMT-BR-C	0 ± 0	41.1 ± 12.9	131.3 ± 36.3	208.4 ± 48.3	244.7 ± 59.9
BR-C	0 ± 0	42.2 ± 14.0	136.2 ± 49.7	217.1 ± 87.3	261.8 ± 110.0
WR-C	0 ± 0	48.3 ± 10.7	148.9 ± 38.0	234.1 ± 76.2	278.8 ± 108.2
(D) AUC _I	Baseline	30 min	60 min	90 min	120 min
HMT-BR-C	0 ± 0	4.24 ± 1.46	14.63 ± 5.19	25.29 ± 9.39	33.00 ± 13.63
BR-C	0 ± 0	4.05 ± 2.18	13.78 ± 6.14	23.43 ± 8.84	30.54 ± 10.25
WR-C	0 ± 0	5.28 ± 1.97	16.85 ± 6.25	28.28 ± 10.69	36.56 ± 13.70

HMT-BR-C; heat-moisture treated brown rice cracker. BR-C; brown rice cracker. WR-C; white rice cracker. mean values ±SD.

3.4. Exp 3. extraction efficiencies of free and bound ferulic acids from test rice crackers by 70% ethanol

3.4.1. Ferulic acid analysis in extracts

Each type of rice cracker was treated with four-stage batchwise extraction by 70% ethanol, and the resultant extract concentrates were analyzed by HPLC. By LC/MS analysis, three peaks in HPLC were identified as free ferulic acid and bound ferulates corresponding to feruloylsucrose and feruloylsinapoylsucrose (Figures 3 and 4).

3.4.2. Comparison of efficiency of extraction of free and bound ferulic acids by 70% ethanol

Quantitative determination of free and bound ferulic acids using an external standard for ferulic acid revealed three ferulic acid derivatives in all three kinds of rice crackers. In four-stage 70% ethanol extraction, these ferulic acid derivatives and their contents (mg FA equiv/100 g of rice cracker) in HMT-BR-C, BR-C, and WR-C were as follows: feruloylsucrose, 0.316 ± 0.005, 0.332 ± 0.006, and 0.113 ± 0.007; ferulic acid, 0.365 ± 0.016, 0.311 ± 0.008, and 0.159 ± 0.008; and feruloylsinapoylsucrose, 0.374 ± 0.003, 0.350 ± 0.001, and 0.143 ± 0.000, respectively (Figure 4-A). Percentage differences in all three ferulic acids

extracted from WR-C without rice bran were less than half of those in HMT-BR-C (Figure 4-A). On the other hand, the feruloylsucrose ratio (105.3%) in BR-C without HMT was higher than that of HMT-BR-C but ferulic acid (85.3%) and feruloylsinapoylsucrose (93.4%) ratios were lower, indicating that extractable ferulic acid contents with HMT were different from those without HMT, depending on the structure of ferulic acids. With single-stage 70% ethanol extraction, the percent changes in extractable ferulic acid with and without HMT were large based on the following ferulic acid contents (mg FA equiv/100 g of rice cracker) in HMT-BR-C and BR-C: feruloylsucrose, 0.213 ± 0.010 and 0.214 ± 0.005; ferulic acid, 0.280 ± 0.003 and 0.217 ± 0.006; and feruloylsinapoylsucrose, 0.252 ± 0.003 and 0.216 ± 0.002, respectively (Figure 4-B). The percent of feruloylsucrose (100.8%) in BR-C without HMT was almost the same as that in HMT-BR-C, and the ratios of ferulic acid (77.7%) and feruloylsinapoylsucrose (85.9%) were lower than in HMT-BR-C.

4. Discussion

Effect of HMT-BR-C on postprandial FMD in adults with mild endothelial dysfunction was compared with that of BR-C and WR-C in 12

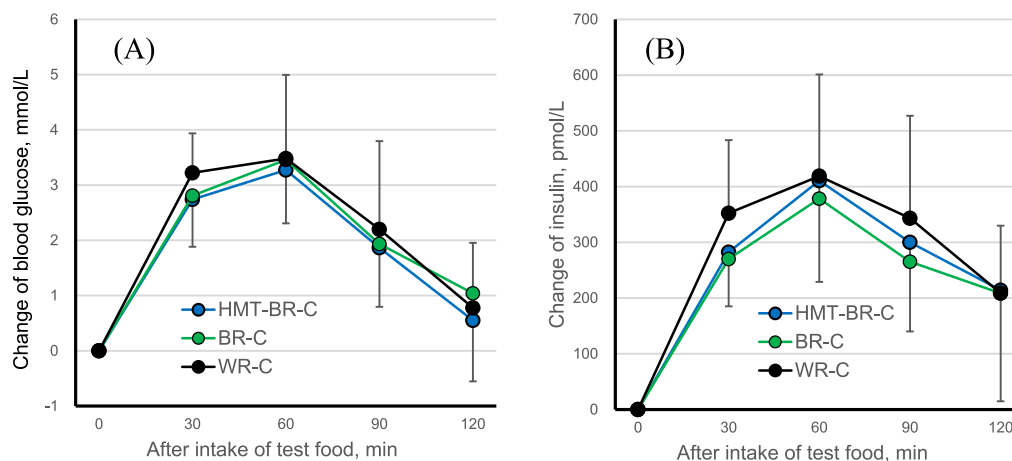


Figure 2. Time courses of changes in blood glucose (A) and insulin (B) levels. Time-courses of changes in blood glucose (A) and insulin (B) levels after intake of heat-moisture treated brown rice crackers (HMT-BR-C, blue circle), brown rice crackers (BR-C, green circle), and white rice crackers (WR-C, black circle). Values are means (n = 12) with their standard deviations represented by vertical bars.

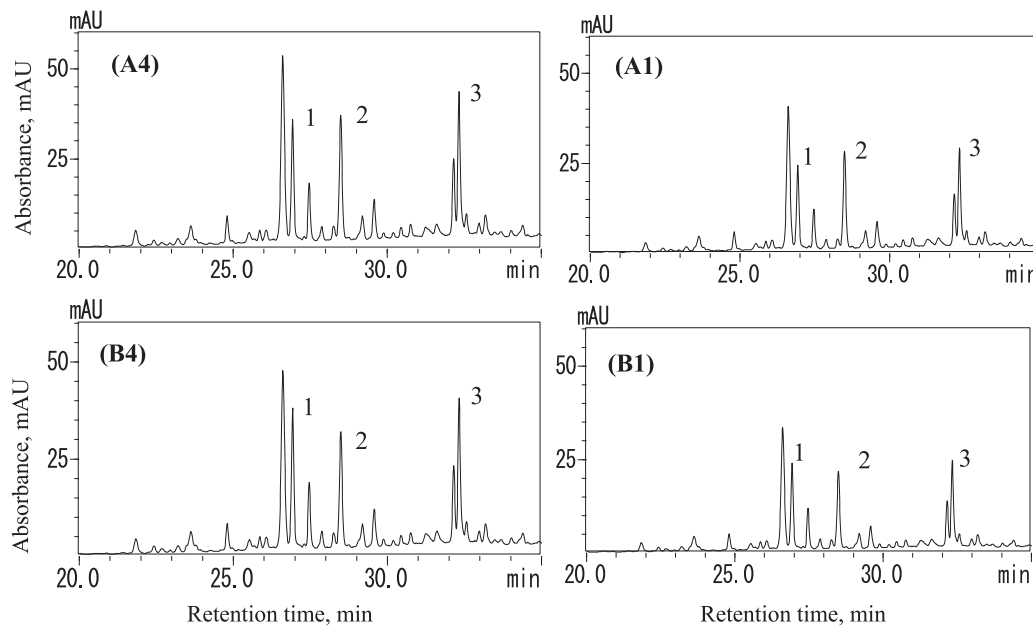


Figure 3. High performance liquid chromatography (HPLC). High performance liquid chromatography (HPLC) profiles detected at UV 320 nm and identified as free and bound ferulic acids (1–3) in four-stage (A4 and B4) and single-stage extract (A1 and B1) of heat-moisture treated brown rice crackers (HMT-BR-C) and brown rice crackers (BR-C) with 70% ethanol, respectively. 1, Feruloylsucrose; 2, Ferulic acid; 3, Feruloylsinapoylsucrose

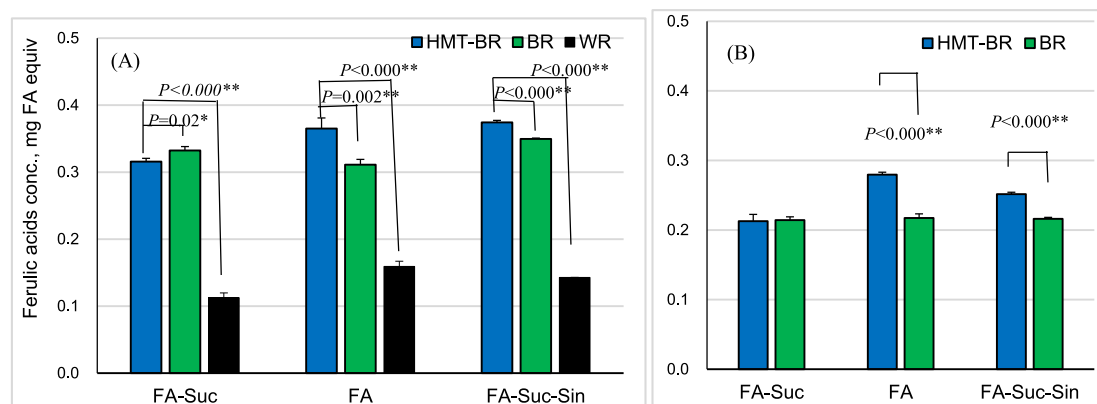


Figure 4. Contents of feruloylsucrose, ferulic acid and feruloylsinapoylsucrose. Contents of feruloylsucrose (FA-Suc), ferulic acid (FA), and feruloylsinapoylsucrose (FA-Suc-Sin) in four-stage (A) and single-stage extracts (B) of heat-moisture treated brown rice crackers (HMT-BR-C, blue), brown rice crackers (BR-C, green), and white rice crackers (WR-C, black) with 70% ethanol. Values are means (Number = 3) with their standard deviations represented by vertical bars. *P* values are by a Dunnett's multiple comparison test among the three groups (vs. HMT-BR-C) (A) and an unpaired-*t* test between the two groups (B).

adults with mild endothelial dysfunction (<7.0% FMD) by a randomized, single-blind, three-treatment three-period crossover trial. The primary outcome was FMD responses. We assessed FMD at baseline and 1 h post-intake of three kinds of rice crackers and only observed a significant increase in FMD of $1.4 \pm 2.1\%$ at 1 h post-intake for HMT-BR-C (Figure 1.). Unfortunately, five baseline values >7.0% were measured in three male participants (Nos. 9, 10, and 12) (Figure 1.). The relevant persons in the trial period were two in the first FMD phase, two in the second, and one in the third, but no clear reason for these increases was obtained from medical inquiries and daily health records. However, we did consider these increases to be the result of a period-effect in that fasting FMD values of three participants changed from values in the screening phase after 5 weeks. In the 9 participants (5 females and 4 males) the baseline FMD values <7.0% in the HMT-BR-C, BR-C, and WR-C groups at 1 h post-intake were $5.5 \pm 1.0\%$, $4.6 \pm 1.9\%$, and $4.0 \pm 1.8\%$, respectively, and deltas from baseline were $1.4 \pm 1.6\%$, $0.8 \pm 1.4\%$, and $-0.4 \pm 2.1\%$, respectively. Statistical analyses showed a significant difference only in intragroup comparisons of measured and delta FMD

values for HMT-BR-C (both $P = 0.027$) and no difference in intergroup comparisons of either measured or delta FMD. The results for the 9 people were similar to those of all 12 participants.

Of interest were FMD results showing that only HMT-BR-C intake resulted in an increase in postprandial FMD while intake of BR-C without HMT did not. We speculated that HMT of BR might cause a constitutional change in nutrients to increase the beneficial effects FMD. Table 1 shows the nutritional components of the three kinds of rice crackers (HMT-BR-C, BR-C, and WR-C) and pre-cooked native rice flours (HMT-BR-F, BR-F, and WR-F). As expected, WR-C and WR-F contained lower amounts of bran components, such as dietary fiber, polyphenols, and GABA, than the corresponding values for BR-C and BR-F because of removal of the majority of bran [22, 23]. The contents of WR-F and BR-F were not inconsistent with reported values. However, bound ferulic acid in HMT-BR-C was found to be the major polyphenol component, and the increase in bound ferulic acid from 25 mg to 28 mg/60 g was assumed to be due to HMT processing. This increase along with the decrease of GABA was similar to that by high pressure treatment reported by Yu et al. [18]

Because the GABA contents in HMT-BR-C and BR-C were 5 times higher than that in WR-C (Table 1), we considered that FMD might be affected by GABA. However, a difference in the effects on FMD was observed between HMT-BR-C and BR-C (Figure 1-D), with a significant increase in the former and no increase in the latter, suggesting that GABA was not a primary active component responsible for the improvement in FMD.

As elevations in glucose levels were reported to decrease the FMD response [7, 8], we expected that the HMT process would change starch characteristics to affect carbohydrate contents to increase bioavailability, including digestion and absorption profiles of test meals. However, carbohydrate contents were not only nearly similar among the cooked crackers analyzed (Table 1) but no significant differences were observed among postprandial blood glucose levels (Figure 2.). The three types of starch (rapidly digestible, slowly digestible, and resistant starch fractions) described by Englyst were not measured, but dietary fiber contents by the modified Prosky method were <2 g/60 g in each cracker (Table 1). The dietary fiber contents of HMT-BR-C, BR-C, and WR-C were assumed to be too small to show statistical differences among the corresponding postprandial blood glucose levels. This result indicated that the difference in FMD responses between HMT-BR-C and BR-C did not primarily depend on postprandial glucose elevations although the glucose elevation affected the FMD response.

Polyphenols in HMT-BR-C were also evaluated as candidates for increases in the FMD response. The polyphenol content in BR was higher than that in WR, but total polyphenol content did not change with and without HMT processing (HMT-BR-C and BR-C in Table 1). In rice, ferulic acid is found mainly as a bound form in cell walls of bran, and the average contents of 21 varieties of Japonica were reported to be about 200 and 100 times higher than those of free forms in BR (0.069 ± 0.048 mg/60g) and WR (0.039 ± 0.037 mg/60g), respectively, and our data on bound and free ferulic acid in Table 1 were not inconsistent with the previous report [24]. Therefore, we focused on bound ferulic acid, a major polyphenol component in HMT-WR-C, because an increase in bound ferulic acid from 25 mg to 28 mg/60 g was shown after HMT processing and ferulic acid was reported to cause a dose-dependent increase in postprandial FMD values [27]. Thermal treatment of endogenous polyphenols in foods was reported to become a double-edged sword with contradictory effects, which were increased release of polyphenol through softening or disruption of cell walls, and decreased release through degradation and oxidation of polyphenols [16]. There were some differences between the data in Table 1 and Figure 4. In Table 1 the values for bound ferulic acid indicated the total amount of ferulic acid generated from all bound ferulate compounds by alkaline hydrolysis (refluxing in 2N NaOH). In Figure 4, individual ferulate compounds, FA-Suc, FA, and FA-Suc-Sin, in HMT-BR-C, BR-C and WR-C extracts were quantitatively measured by LC/MS analysis, respectively, and the contents of each compound were statistically compared. That there was no change in total polyphenol content might be explained by our method of HMT (12% moisture content and 3-min time period), which was too mild to degrade polyphenols in comparison with the usual process of HMT of BR at a higher moisture content (typically 35%) for a longer time period (15 min–16 h) [14]. It was suggested that polyphenol release from the food matrix could be enhanced by our method of processing and provides support that high-pressure cooking increases the amount of phenolic compounds [18]. The release of polyphenols is very important to polyphenol bioavailability, as the plasma response depended on the released amount of polyphenols followed by absorption [25]. To investigate the amounts of ferulic acids released in test rice crackers, we prepared two types of 70% ethanol extracts, four-stage and single-stage extracts, supposing that almost all releasable ferulic acids would be extracted with the former but that ferulic acids might be easily releasable with the latter. Ferulic acid extraction percentages based on the amount of ferulic acid in HMT-BR-C extracts were determined by quantitative analysis. In both four-stage and single-stage extracts, the extraction percentages of free and bound ferulic acid from HMT-BR-C were significantly higher than those from BR-C (Figure 4). From these data, we speculated that there

was a relationship between the polyphenol release enhanced by HMT processing and the increased postprandial FMD response despite the lack of analytical data from blood studies. The reason was that the effect of ferulic acid on increasing FMD was clarified by recent human studies describing that the intake of cranberry juice polyphenols containing ferulic acids caused a dose-dependent increase in postprandial FMD values and that the increase significantly correlated with the amounts of ferulic acid sulfate as metabolites in blood [26, 27, 28].

4.1. Study limitations

This study has several limitations. First, the number of participants was small and the study was conducted as a crossover trial with an unexpected period-effect. FMD data in this study suggested that a large-scale study ($n = 32$ –, based on 80% power, alpha 0.05) would reveal significant differences between the HMT-BR-C and WR-C groups. Second, FMD was calculated as the percentage change in the brachial artery diameter from resting baseline to peak using a traditional method according to guidelines of the Japanese Circulation Society [29], without applying new techniques to adjust for the baseline artery diameter and normalization to shear stimulus [30, 31]. Third, we measured extraction efficiency as a marker of release of polyphenols from the food matrix, but the actual bioavailability of polyphenols should be evaluated by measuring absorbed polyphenols and the metabolites in blood in a future study. Although the exact mechanism is still unclear, HMT-BR-C intake increased the postprandial FMD response.

In conclusion, our data provided support that HMT processing would enhance the release of polyphenols in HMT-BR-C to increase the postprandial FMD response.

Declarations

Author contribution statement

Kenichi WATANABE: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Masao HIRAYAMA: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Somasundaram ARUMUGAM: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Masayoshi SUGAWARA: Conceived and designed the experiments.

Hisanori KATO, Sumiko NAKAMURA, Ken'ichi OHTSUBO, Noriyuki HOMMA, Yoshifumi FUJII, Naoto MUROHASHI, Hiroshi SUZUKI: Performed the experiments.

Hitoshi MATSUMOTO, Yuri NOMI: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Rajarajan A THANDAVARAYAN, Satoru KODAMA: Analyzed and interpreted the data.

Kazuya FUJIHARA, Hirohito SONE: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

The authors do not have permission to share data.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

The clinical trial described in this paper was registered at UMIN-CTR Clinical Trial under the registration numbers UMIN 000034898 and UMIN 000035168.

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