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Black soybean improves the vascular function through an increase in nitric oxide and a decrease in oxidative stress in healthy women

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Abstract

Vascular dysfunction and injurious stimuli such as oxidative stress is closely related to the risk of cardiovascular diseases (CVD). Dietary polyphenols is reported to exert the beneficial effects on reducing the risk of CVD. Black soybean is rich in polyphenols, including isoflavones, anthocyanidins and flavan-3-ols, and its prevention effects on CVD risk were reported in the animal experiments. In this study, we investigated the effect of black soybean consumption on the vascular function and oxidative stress associating with the polyphenol concentrations in healthy women. Lowered vascular age was observed in 33 out of 44 volunteers who completed the 8-week trial. It was observed that improvement of the vascular stiffness, increasing in the urinary NO_2 and NO_3 level, and decreasing in the oxidative stress markers, 8-hydroxy-2'-deoxyguanosine, hexanoyl-lysine and myeloperoxidase. In addition, concentration of 12 polyphenols in black soybean increased in the plasma and urine. Increased concentration of polyphenols would be involved in the decreased oxidative stress. Thus, black soybean consumption improved the vascular function through an increase in nitric oxide and a decrease in oxidative stress accompanied by increasing the polyphenol concentrations in healthy women.

Key words:

vascular function; oxidative stress; nitric oxide; polyphenol; black soybean polyphenol

Abbreviations

APG; acceleration plethysmogram, BMI; body mass index, CVD; cardiovascular diseases, 2'-deoxyguanosine, HPLC; high-performance liquid chromatography, HRQOL; health-related quality of life, HEL; hexanoyl-lysine, 8-OHdG; 8-hydroxy-2'-deoxyguanosine, MPO; myeloperoxidase, NO; nitric oxide, SF-36; the 36-item Short-Form Health Survey,

1. Introduction

Vascular function is closely related to the risk of cardiovascular diseases (CVD) [1]. Aging process is related to the vascular dysfunction as the dominant risk factor [2, 3]. It causes functional and structural changes of the vascular wall. An increase in the vascular stiffness is the major symptom, and it compromises vascular adaption to blood flow and pressure changes. Oxidative stress is another important trigger of vascular dysfunction, because it is widely accepted that oxidative stress is closely related to the aging process [4], indicating that decline in the vascular function is an inevitable result

of aging. Alteration of nitric oxide (NO) is a key molecular event for the underlying mechanism of vascular function [5]: Releasing of NO from endothelial cells decreases the intracellular concentration of calcium, causes relaxation of vascular smooth muscle as a potential vasodilator. However, aging and oxidative stress quenches the production of NO and hampers the NO-mediated responses and eventually leads to the vascular dysfunction [6].

Black soybean (*Glycine max*) is originated from Asia and has been consumed as a health food and folk medicine for these centuries [7]. Grain of black soybean is rich in proteins, lipids, minerals and isoflavones as the same as that of yellow soybean. However, it has black pigments in the seed coat consisting of polyphenols such as anthocyanidins and flavan-3-ols [8-9]. These polyphenols contribute to the total antioxidant capacity of whole black soybean [10]. Polyphenols in the seed coat distinguish black soybean from the yellow one, and black soybean shows higher antioxidant capacity than other legumes including yellow soybean [11]. Previous study demonstrated that consumption of black soybean (35% of experiment diet) for 10 weeks in ovariectomized rats inhibited oxidative stress by increasing antioxidant activity and improving lipid profiles, resulting in the risk factors associated with CVD were greatly improved [12]. Results from another study demonstrated that an oral administration of

76 black soybean extract at 50 and 100 mg/kg body weight to rats for 14 days reduced the
77 risk of CVD by improving blood circulation through inhibiting platelet aggregation and
78 thrombus formation [13]. For human trial, it was reported that supplementation of black
79 soybean test extract (2.5 g/day) for 8 weeks improved visceral fat accumulation and
80 plasma lipid profiles in overweight Korean adults [14]. However, there is no human trial
81 on the beneficial effects of black soybean consumption on reducing the risk of CVD.
82 Moreover, these previous studies did not measure the physiological concentration of
83 each polyphenol after the consumption of test materials and analyze the correlation
84 between the observed effects and polyphenol concentrations in the body, although
85 polyphenols are considered to responsible for these beneficial effects. Therefore, in this
86 study, we investigated that the effect of black soybean consumption on the vascular
87 function and oxidative stress associating with polyphenol concentrations in healthy
88 women by an open-label study.

89

90 ## 2. Materials and Methods

91 ### *2.1 Design of human study*

92 This study was approved and conducted by the Institutional Review Board of
93 Fujicco Co., Ltd. (Trial registration: #5702) in accordance with the Declaration of
94 Helsinki. To perform the study, informed consent was obtained from all volunteers.

Volunteers were excluded if they fulfilled any of the following criteria: (1) having a clinical history of severe gastrointestinal disease, liver disease, kidney disease or heart disease; (2) currently undergoing treatment for metabolic syndrome or its associated diseases; (3) using medication for blood flow and/or pressure, such as Warfarin and Captopril; and (4) whose physical condition were considered inappropriate for this study by a physician. The main inclusion criterion was that the vascular age of volunteer was higher than her chronological age. Forty-seven female volunteers aged 20 to 70 were finally enrolled for this study. Volunteers ingested 30 g/day of roasted black soybeans for 8 weeks without strict restrictions on the intake patterns. Test material was produced from Fujicco Co., Ltd (Kobe, Japan) and composition of which was shown in Table 1. Dose of roasted black soybeans was decided by a one portion of commercially available soybean products with energy less than 150 cal. This dose is easily ingested with a meal or snack. During the 8-week trial, volunteers were asked to return to the facility at the 4^{th} and the 8^{th} week for the measurements of anthropometrics, accelerated plethysmogram (APG), blood pressure and assessments of health-related quality of life (HRQOL), as well as blood and urine collection under the fasting condition before breakfast. These measurements and collection were also conducted at starting day of the trial (0 week).

113

114 *2.2. Measurements of body composition*

115 Body weight, body mass index (BMI), body fat percentage, visceral fat percentage,
116 biological age, basal metabolic rate, estimated bone mass, and muscle mass was
117 measured using a body composition meter (BC-610-PB, TANITA. Co., Ltd. Tokyo,
118 Japan).

119

120 *2.3. Measurements of vascular function*

121 Vascular function was estimated by acceleration plethysmogram (APG) using a
122 "Pulse Analyzer" device (Pulse Analyzer Plus View, YKC Corporation, Tokyo, Japan).
123 The subject rested quietly and attached a device to the left middle finger to measure the
124 APG. APG is the second derivative wave of the photoplethysmogram. APG consisted of
125 *a*, *b*, *c* and *d* waves, namely, early systolic positive wave, early systolic negative wave,
126 late systolic re-increasing wave and late systolic re-decreasing wave, respectively. Their
127 magnitudes and the height ratios of *b/a*, *c/a* and *d/a* were measured. Vascular age,
128 vascular waveform, waveform score, and peripheral vascular health were calculated
129 from a wave pattern and the ratios of these 4 waves using a software for the Pulse
130 Analyzer. Systolic and diastolic blood pressure were measured in the right upper arm

using automated sphygmomanometer (HEM-9000AI, OMRON Corporation, Kyoto, Japan).

2.4. Assessments of HRQOL

HRQOL was assessed using the Japanese version of the 36-item Short-Form Health Survey (SF-36) questionnaire developed by iHope International Co., Ltd. (Kyoto, Japan) [15].

2.5. Measurements of biomarkers in the blood and urine

Plasma, which was prepared from the blood, and urine was used for the measurements of NO_2/NO_3, 8-hydroxy-2'-deoxyguanosine (8-OHdG), hexanoyl-lysine (HEL) and myeloperoxidase (MPO) by using corresponding commercial kit [NO: NO_2/NO_3 Assay Kit-C II (DOJINDO LABORATORIES, Kumamoto, Japan); 8-OHdG: New 8-OHdG Check ELISA (Japan Institute for the Control of Aging, NIKKEN SEIL Co., Ltd (JaICA) Shizuoka, Japan); HEL: HEL ELISA kits (JaICA); MPO: Human serum MPO and urine MPO ELISA kits (JaICA)]. Urinary NO was calculated by creatinine equivalent. Urinary creatinine level was measured by Creatinine (urinary) Colorimetric Assay Kit (Cayman CHEMICAL, Ann Arbor, MI, USA).

149 In addition, hematologic parameters including creatinine, total protein, blood urea
150 nitrogen, glucose, lactate dehydrogenase, alkaline phosphatase, γ-
151 glutamyltranspeptidase, aspartate aminotransferase, alanine aminotransferase,
152 triglycerides, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol,
153 Na^+, Cl^-, K^+, white blood cells, red blood cells, hemoglobin, hematocrit, mean
154 corpuscular volume, mean corpuscular hemoglobin, mean hemoglobin concentration,
155 and platelet were analyzed by LSI Medience Co. (Tokyo, Japan).
156
157 *2.6. Extraction of polyphenols from the plasma and urine.*
158 An aliquot of 10 ml urine was concentrated to 2 ml before analysis using a
159 centrifugal separator in vacuo. Concentrated urine or 500 μl of plasma were mixed with
160 2% (w/v) ascorbic acid (200 μl for urine and 50 μl for plasma) to prevent oxidation
161 during extraction and were transferred to a polypropylene centrifuge tube (15 ml, BD
162 Biosciences, San Jose, CA, USA) which were siliconized using Sigmacote® (Sigma-
163 Aldrich, St. Louis, MO, USA). These plasma and urine samples were hydrolyzed with
164 500 U of β-glucuronidase from *E. coli.,* (type IX-A, Sigma-Aldrich) and10 U of
165 sulfatase from *Abalone entrails* (type VIII, Sigma-Aldrich) for deconjugation according
166 to our previous reports [16].

A solid phase extraction method was used to extract polyphenols from the mixture: C18 Sep-Pak cartridge (50 mg resin, Waters Co., Milford, MA, USA) was conditioned with 5 ml of methanol and 5 ml of ultrapure water. Plasma and urine samples were centrifuged at 3000 × g for 15 min to remove precipitated protein and applied to the cartridge. After the cartridge was washed with 5 ml of 10% methanol, polyphenols were eluted by 2 ml of 95% methanol and evaporated to dryness using the centrifugal separator. Obtained precipitate was dissolved in 50 µl of 50% methanol for analysis of polyphenols using a high-performance liquid chromatography (HPLC).

2.7. HPLC analysis

Quantification of polyphenols was conducted using the HPLC system as described previously [16] with modifications. HPLC was performed using a system equipped with a DGU-20A 3R degassing unit, LC-20AD XR binary pump, SIL-20AC XR auto sampler, RF-20A XS fluorescence detector, SPD-M20A diode array detector, CTO-20AC column oven and CBM-20A communications bus module connected to an LC work station (Shimadzu Corporation, Kyoto, Japan). The analytical column was a Cadenza CL-C18 column (φ 250 mm × 4.6 mm, 3 µm, Imtakt, Kyoto, Japan), protected by a guard column (Cadenza CL-C18, φ 5 mm × 2 mm, 3 µm, Imtak).

185 For the analysis of cyanidin-3-*O*-glucoside, 10% (v/v) formic acid was mobile
186 phase A and formic acid: acetonitrile (10:90, v/v) was mobile phase B. Elution of
187 cyanidin-3-*O*-glucoside was achieved using these linear gradients: 15% B over 0–10
188 min; 80% B over 10-25 min; and 15% B, over 25-40 min. The flow rate was 0.8
189 ml/min, the injection volume was 10 µl and temperature of the column oven was set to
190 40°C. Absorbance of cyanidin-3-*O*-glucoside was measured at a wavelength of 513 nm.
191 For the analysis of flavan-3-ols and isoflavones, 0.1% (v/v) formic acid was mobile
192 phase A and acetonitrile was mobile phase B. Separation was achieved using these
193 linear gradients: 5-10% (v/v) B over 0-5 min; 10-85% (v/v) B over 5-65 min; 30-45%
194 (v/v) B, over 65-110 min; 80% (v/v) B, over 110–120 min; and 5% (v/v) B over 120-
195 140 min. The flow rate was 0.7 ml/min, the injection volume was 10 µl and temperature
196 of the column oven was set to 40°C. Fluorescence of the flavan-3-ols was measured
197 with the excitation and emission wavelengths at 276 and 316 nm, respectively.
198 Absorbance of isoflavones was measured with a wavelength at 254 nm and that of equol
199 was at 280 nm.
200
201 *2.8. Polyphenols and solvents used for HPLC analysis*
202 Following authentic compounds were used for HPLC analyses. (−)-Epicatechin

was purchased from Kurita Analysis Service Co. Ltd (Ibaraki, Japan). Procyanidin B2, procyanidin C1 and cinnamtannin A2 were prepared by Fujicco Co., Ltd. [17]. Cyandin-3-*O*-glucoside, daidzein, daidzin and genistein were from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan), glycitein, glycitin and genistin were from Extrasynthese (Genay, France), and *S*-equol was from Cayman Chemicals (MI, USA). Chemical structures of polyphenols analyzed in this study are shown in Fig. 1. As an internal standard used for the measurement of flavan-3-ols, procyanidin B3-OAc was kindly provided from Professor Akiko Saito (Osaka Electro-communication University, Osaka, Japan). Flavone (FUJIFILM Wako Pure Chemical Co.) was used as the internal standard for the measurement of isoflavones. HPLC grade methanol, acetonitrile and formic acid were obtained from FUJIFILM Wako Pure Chemical Co.

2.9. Statistical analysis

Data are expressed as the means ± standard deviation. Statistical analysis was performed with Dunnett`s test, Wilcoxon signed-rank test, or paired *t*-test using JMP statistical software version 11.2.0 (SAS Institute, Cary, NC, USA). Pearson correlation coefficient was applied to determine the association between the concentration of polyphenols and corresponding factors, including anthropometric measurements,

221 biomarkers of vascular function and oxidative stress, and hematologic parameters.
222 Statistical analysis of correlation efficient was performed using *t*-tests. The level of
223 significance was set as $p<0.05$.
224
225 ## *3.* **Results**
226 ### *3.1. General characteristics of volunteers*
227 Before the trial, an average vascular age of volunteers was 39.2 ± 10.2 years old
228 with the average BMI of 20.8 ± 0.42 kg/m^2. The vascular age was significantly higher
229 than the chronological age which was 33.5 ± 10.0 years old ($p<0.01$). At the end of the
230 trial, 3 volunteers dropped out for non-health related reasons and 44 volunteers
231 completed the 8-week trial with no report on poor health or any abnormality. No
232 significant changes in anthropometric and hematologic parameters were observed
233 throughout the trial (Supplementary Tables 1 and 2). HRQOL was assessed by SF-36,
234 but there is no significant change (data not shown).
235
236 ### *3.2. Vascular function*
237 During the 8-week trial, vascular function was significantly improved (Table 2).
238 The vascular age became about 2 years younger at the end of the trial compared to that

239 at the 0 week. Indicators of APG that reflect the vascular function were significantly
240 improved: *a*, *c*, *d* wave magnitude and the height ratios of *c*/*a* and *d*/*a* were increased; *b*
241 wave magnitude and height ratio of *b*/*a* were decreased. The significant change was
242 observed at both 4th and 8th week. In addition, significant improved vascular waveform,
243 waveform score and peripheral vascular health were also observed. Systolic and
244 diastolic blood pressure tended to decrease at the 4th and/or the 8th week without
245 statistical significance. In detail, 25 and 26 volunteers showed lowered vascular age at
246 the 4th week and 8th week, respectively (Fig. 2). In total, 33 volunteers showed lowered
247 vascular age during the trial. As to the remaining 11 volunteers, the vascular age of 8
248 volunteers did not change throughout the trial, and that of 3 volunteers was slightly
249 higher at the end of the trial compared to that at the 0 week. Based on the timing of
250 lowered vascular age being observed, the results of 25 volunteers whose lowered
251 vascular age was observed at the 4th week were extracted and denoted as an 'Improved
252 at the 4th week' group and the results of 26 volunteers whose lowered vascular age was
253 observed at the 8th week were extracted and denoted as an 'Improved at the 8th week'
254 group in this study. For following results, cluster analysis was performed in these two
255 groups ('Improved at the 4th week' and 'Improved at the 8th week' groups) in addition to
256 an 'All' group (the results of 44 volunteers).

257

258

259 **3.3. NO concentration in the plasma and urine**

260 Since NO is involved in the regulation of vascular function, including blood
261 pressure and blood flow [18], we measured the NO concentration in the urine and
262 plasma (Fig. 3). In the urine, NO_2/NO_3 of the 'All' group increased significantly at the
263 4^{th} and 8^{th} week compared to that of 0 week. More clear results were observed in the
264 urine of 'Improved at the 4^{th} week' and 'Improved at the 8^{th} week' groups compared to
265 that of the 'All' group. In the plasma, NO_2/NO_3 concentration did not changed, though
266 slight increasing trend was observed.

267

268 *3.4. Oxidative stress markers in the plasma and urine*

269 In this study, three oxidative stress markers, namely 8-OHdG, HEL and MPO, were
270 selected and measured (Fig. 4). In the plasma, 8-OHdG in the 'All' group significantly
271 decreased at the 4^{th} and 8^{th} week (Fig. 4A). After cluster analysis, significant decrease in
272 8-OHdG was observed in both 'Improved at the 4^{th} week' and 'Improved at the 8^{th}
273 week' groups. In the urine, 8-OHdG also decreased without significant difference,
274 because the results vary widely in 0-week. HEL in the plasma also significantly

decreased at both the 4th and the 8th week, and after cluster analysis, significant decrease was observed in both 'Improved at the 4th week' and 'Improved at the 8th week' groups (Fig. 4B). In the urine, HEL did not alter after consumption of black soybeans. In the case of MPO, this marker also showed decreasing tendency in both plasma and urine in all groups, but significant difference was not observed (Fig. 4C).

3.5. Polyphenol concentrations in the plasma and urine

Polyphenol concentrations were measured in the plasma and urine after consumption of black soybeans. Results of the 'All' group in the plasma and urine were shown in Table 3A and 3B, respectively. Polyphenols detected with and without enzymatic hydrolysis were denoted as 'Total' and 'Free', respectively. Although there is no significant difference was observed in the concentration of each polyphenol, but the sum of four flavan-3-ols, the sum of seven isoflavones and the sum of 12 polyphenols significantly increased in both plasma and urine. Cyanidin-3-*O*-glucoside was only detected in the urine.

After cluster analysis, besides the sum of polyphenols, the significant increases were also observed in certain polyphenols (Tables 4 and 5). In the 'Improved at the 4th week' group, procyanidin B2, daidzin, daidzein, glycitin and glycitein significantly

293 increased in the plasma (Table 4A), and cyandin-3-O-glucoside, (−)-epicatechin,
294 procyanidin C1, cinnamtannin A2, daidzin, daidzein and genistein significantly
295 increased in the urine (Table 4B) after consumption of black soybeans. In the 'Improved
296 at the 8th week' group, procyandin B2, procyanidin C1, cinnamtannin A2 significantly
297 increased in the plasma (Table 5A), and cinnamtannin A2, daidzein, genistin and
298 genistein significantly increased in the urine (Table 5B). Overall, procyanidin B2,
299 procyanidin C1, cinnamtannin A2, daidzein and genistein were typically increased
300 polyphenols (to 2- and 3-fold) after consumption of black soybeans.
301
302 ***3.6. Correlation between each polyphenol concentration and biomarker***
303 The correlation between each polyphenol concentration and measured biomarker
304 was analyzed using Pearson correlation coefficient and heatmap data was shown in Fig.
305 5. All 12 polyphenols were found significantly correlated to at least one biomarker that
306 related to vascular function. Among these polyphenols, total procyanidin B2 in the
307 plasma and total cinnamtannin A2 in the urine of the 'Improved at the 4th week' group
308 were correlated to the vascular function markers (Fig 5). Negative correlation was
309 observed between total procyanidin B2 and the vascular age (r=−0.323, p=0.032), *b*
310 wave (r=−0.448, p=0.002), height ratio of *b/a* (r=−0.424, p=0.004), systolic blood

311 pressure (r=−0.418, p=0.005) and diastolic blood pressure (r=−0.379, p=0.011). On the
312 contrary, positive correlation was observed between total cinnamtannin A2 and the
313 biological age (r=0.570, p<0.001), vascular age (r=0.348, p=0.021), *b* wave (r=0.311,
314 p=0.040), height ratio of *b/a* (r=0.292, p=0.040) and systolic blood pressure (r=0.317,
315 p=0.036).

316 Negative correlation between each polyphenol concentration and oxidative stress
317 marker was also observed (Fig. 5). 8-OHdG was the most negatively correlated to free
318 daidzin (r=−0.310, p=0.041), free glycitin (r=−0.329, p=0.029), total glycitein
319 (r=−0.302, p=0.046), free equol (r=−0.325, p=0.031) and total procyanidin
320 C1(r=−0.333, p=0.027). Significant negative correlation was also found between HEL
321 or MPO and other polyphenols, but strong positive correlation was observed between
322 HEL and free (−)-epicatechin (r=0.596, p<0.001) as well as total (−)-epicatechin
323 (r=0.697, p<0.001).

324

325 **4. Discussion**

326 Vascular function is closely related to the high risk of CVD [1-4]. Recently, many
327 researchers are focusing on the effect of dietary intake of polyphenols, especially
328 flavonoids on the prevention of CVD [19, 20]. Black soybeans are rich in flavonoids

329 and its amelioration and preventive effects on CVD was reported in the animal
330 experiments [12, 13, 21, 22]. In our best knowledge, this is the first report showing the
331 improvement of vascular function after consumption of black soybeans in human trial,
332 though the design is the open-label study. In this study, the significant improvement of
333 vascular function, including lowered vascular age and improved APG, were observed in
334 women during the 8-week trial (Table 2). The vascular age is one of the common
335 indicators that predict the risk of CVD by age, and it performs better than the
336 chronological age [23]. In this study, lowered vascular age was observed in 33
337 volunteers who had higher vascular age than their chronological age before the trial.
338 The intake of black soybean for 4 weeks improved vascular function and further slight
339 improvement of the function was observed after the intake for 8 weeks (Table 2).
340 However, the vascular age of volunteers was still higher than their chronological age at
341 the end of the trial. Therefore, further continuous consumption of black soybeans might
342 reduce the vascular age lower than their chronological age. Compared to the vascular
343 age, APG provides more detailed information about the health condition of blood
344 vessels, including cardiac output intensity (*b* wave), residual blood volume (*c* wave) and
345 vascular compliance (*d* wave). In addition, the height ratios of each wave to *a* wave
346 (*b/a*, *c/a* and *d/a*) are the typical indicators used in APG for assessing the arterial

347 stiffness [24, 25]. In this study, significantly increased cardiac output intensity, reduced
348 residual blood volume and improved vascular compliance were observed during the trial
349 (Table 2). Improvement in the vascular stiffness was also reported in healthy adults after
350 6-week supplementation of isoflavones (genistein and daidzein) [26], in patients with
351 coronary artery disease after 4-week consumption of cranberry juice [27], and in healthy
352 males after 1-week consumption of black tea [28]. These results indicated that
353 consumption of polyphenol-rich food materials are possible to improve the vascular
354 stiffness.

355 NO is another indicator to evaluate the vascular function: It contributes to the
356 relaxation of blood vessels and eventually leads to improvement in the vascular stiffness
357 [29]. In this study, we found that urinary-NO_2/NO_3 concentration significantly increased
358 after consumption of black soybeans in both 'Improved at the 4^{th} week' and 'Improved
359 at the 8^{th} week' groups (Fig. 3), though the NO level in the plasma remained unchanged.
360 These findings indicated that consumption of black soybeans improved vascular
361 function through increasing NO concentration in human. It was presumed that produced
362 NO in the plasma was excreted to urine during the fasting period, indicating that plasma
363 NO was a basal level, while urinary NO (sum of NO2 + NO3) was an accumulated
364 level. This is a reason why NO_2/NO_3 increased only in urine but not in the plasma.

Coincide results were reported by a previous study showing that NO concentration increased in the plasma and urine of healthy human after the ingestion of a cocoa drink accompanied by improvement of the vascular stiffness [30]. Therefore, increased NO concentration is involved in the underlying mechanism by which consumption of polyphenol-rich food materials improve the vascular function.

Oxidative stress is closely related to the underlying mechanism of vascular dysfunction, because it quenches the production of NO, hampers the NO-mediated responses and eventually leads to the vascular dysfunction [31]. In this study, biomarkers for oxidative stress, 8-OHdG, HEL and MPO, decreased in the plasma and/or urine (Fig. 4), suggesting that oxidative stress was ameliorated after consumption of black soybeans. These biomarkers reflected the different aspects of the oxidative stress. Decreased 8-OHdG (Fig. 4A) reflects the amelioration of DNA damage which is a trigger of the development of CVD and type II diabetes [32]. Decreased 8-OHdG was also reported in human after a drinking of red wine with a high-fat diet during 3 months [33]. Decreased HEL (Fig. 4B) suggested the reduction of lipid peroxidation [34], and similar result was reported in human ingested cocoa powder for 12 weeks [35]. MPO is another biomarker for lipid peroxidation and inflammation [36]. Although decreasing of MPO (Fig. 4C) was not significant, decreasing tendency was observed in this study.

383 Observed decreasing tendency of MPO may suggest to reduce lipid peroxidation and
384 inflammation in volunteers. Therefore, consumption of black soybeans improved
385 vascular function through ameliorating oxidative stress in human and polyphenols
386 would be contribute to this effect.

387 Polyphenols are usually considered responsible for the beneficial effects exerted by
388 food materials [26-28, 30, 33, 35], but it is not fully understood yet that the correlation
389 between physiological concentration of polyphenols after the consumption of test
390 material and observed beneficial functions in human. In this study, increased plasma and
391 urinary concentrations of 11 black soybean polyphenols, and 1 intestinal bacterial
392 metabolite of daidzein, namely equol, were observed after consumption of roasted black
393 soybeans (Table 3). Although significant increase was only observed in their sum
394 amounts, these results strongly suggested that consumption of black soybeans increased
395 physiological concentration of total polyphenols in human, resulting in improved
396 vascular function. From our results, volunteers with higher physiological concentration
397 of polyphenols revealed more improved vascular function and more ameliorated
398 oxidative stress after consumption of black soybeans. Schroeter *et al.* reported that (−)-
399 epicatechin, (+)-catechin, their related metabolites and the sum flavanols in the plasma
400 increased after ingestion of flavanol-rich cocoa [30]; Urquiaga *et al.* reported that total

401 plasma and urinary polyphenols increased during the supplementation with red wine
402 [33]. These results indicate that dietary intake of polyphenol-rich food materials is an
403 effective way to increase the physiological concentration of polyphenols, but to prove
404 the direct relation between consumed polyphenols and observed beneficial effects, more
405 detailed evidence is needed. In this study, after the cluster analysis, significant increases
406 were observed not only in the sum amounts of polypehnols but also in several
407 polyphenol compounds (Tables 4 and 5), i.e., procyanidin B2, procyanidin C1,
408 cinnamtannin A2, daidzein and genistein increased to 2- and 3-fold after consumption of
409 black soybeans. Moreover, heatmap data indicated that plasma procyanidin B2 was well
410 correlated to the improvement of vascular function (Fig. 5). This result suggest that
411 procyanidin B2 is one of the candidates for the active compound in black soybean to
412 improve the vascular function. On the other hand, urinary cinnamtannin A2 revealed
413 negative effect on the vascular function. Although absolute amount of urinary
414 cinnamtannin A2 was the lowest among the polyphenols measured in this study, further
415 careful experiment is needed to clarify this phenomenon. Although concentration of
416 some isoflavones related to the suppression of oxidative stress markers, concentration of
417 isoflavones in neither plasma nor urine correlated to the vascular function (Fig. 5).
418 These result suggested that contribution of isoflavones to the improvement in vascular

function might be low.

It is usually considered more accurate to assess the beneficial effects of food consumption with restrictions on the diet, but some of the restrictions in the previous studies were not usually achieved in daily life, such as abstaining from coffee and green tea before and during 7-week trial in the study of coffee consumption [37], and forbidden consumption of berries, wine and all related products in the study of strawberry and cranberry polyphenols [38]. Volunteers in this study were asked to keep their usual diet before and during the trial period, and consume the 30 g/day of roasted black soybeans at any time. This instruction is easy for consumption of the test material in daily life. Indeed, the consumption of the roasted black soybeans leached around 90%. Even with the possible interference from the daily diet, beneficial effects of the intake of black soybeans on the vascular function and oxidative stress correlated to the increased physiological concentration of polyphenols.

Other components in the black soybeans may also contribute to the observed effects. Soy protein, accounting for approximately 36% of dry soybeans by weight, was reported to lower the risk of CVD through regulating the lipid profile, including lowering total cholesterol, low-density lipoprotein and triglycerides without affecting high-density lipoprotein [39]. Another previous study also demonstrated that soy protein

437 itself reduced oxidative stress in rats [40]. Therefore, black soybean is a nutritious and
438 functional food that should be recommended for daily consumption. The intake of black
439 soybeans in this study was 30 g/day. According to the Annual Health, Labor and
440 Welfare Report 2016 by the Japanese Government, the average dietary intake of soy
441 products of Japanese female reached 57.7 g/day [41], which was approximately 2-fold
442 higher than the intake of this study, indicating that 30 g/day is a dietary intake that can
443 be achieved by daily diet. It is an important finding that the dietary intake, but not
444 supplementary intake, of black soybeans can exert beneficial effects to human.
445

446 **5. Conclusions**

447 The intake of 30 g/day of roasted black soybeans for 8 weeks significantly
448 improved the vascular function and reduced oxidative stress. The intake of black
449 soybeans also increased the physiological concentration of polyphenols in the plasma
450 and urine, which contributed to the improvement of vascular function and reduction of
451 oxidative stress leading to lowering the risk of CVD in human.
452

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Conflict of interest

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References

[1] K. Jensen-Urstad, J. Johansson, M. Jensen-Urstad, Vascular function correlates with risk factors for cardiovascular disease in a healthy population of 35-year-old subjects, J. Intern. Med. 241 (1997) 507-513.

[2] L. Rodríguez-Mañas, M. El-Assar, S. Vallejo, P. López-Dóriga, J. Solís, R. Petidier, M. Montes, J. Nevado, M. Castro, C. Gómez-Guerrero, C. Peiró, C.F. Sánchez-Ferrer, Endothelial dysfunction in aged humans is related with oxidative stress and vascular inflammation, Aging. Cell. 8 (2009) 226-238.

[3] J. Angulo, S. Vallejo, M. El Assar, J. García-Septiem, Sánchez-Ferrer CF and Rodríguez-Mañas L. Age-related differences in the effects of α and γ peroxisome

475 proliferator-activated receptor subtype agonists on endothelial vasodilation in
476 human microvessels. Exp. Gerontol. 2012, 47, 734-740.
477 [4] D. Harman, Aging: a theory based on free radical and radiation chemistry, J.
478 Gerontol. 11 (1956) 298-300.
479 [5] Y. Zhao, P.M. Vanhoutte, S.W. Leung, Vascular nitric oxide: Beyond eNOS, J.
480 Pharmacol. Sci. 129 (2015) 83-94.
481 [6] P. Pacher, J.S. Beckman, L. Liaudet, Nitric oxide and peroxynitrite in health and
482 disease, Physiol. Rev. 87 (2007) 315-424.
483 [7] I.R.A.P. Jati, V. Vadivel, H.K. Biesalski. Bioactive food as Dietary Interventions for
484 Liver and Gastrointestinal Disease, ed. Watson RR and Preedy VR, Elsevier Inc.,
485 San Diego 2013, Antioxidant Activity of Anthocyanidins in Common Legume
486 Grains, 485-495.
487 [8] Y. Takahata, M. Ohnishi-Kameyama, S. Furuta, M. Takahashi I. Suda, Highly
488 polymerized procyanidins in brown soybean seed coat with a high radical-
489 scavenging activity, J. Agric. Food. Chem. 49 (2001) 5843-5837.
490 [9] J.H. Lee, N.S. Kang, S.O. Shin, S. H. Shin, S. G. Lim, D. Y. S, I.Y. Baek, K.P. Tae,
491 J. Ha, Characterizations of anthocyanins in the black soybean (Glycine max L.) by
492 HPLC-DAD-ESI/MS analysis, Food Chem. 112 (2009) 226-231.
493 [10] B. Xu, S.K. Chang, Antioxidant capacity of seed coat, dehulled bean, and whole
494 black soybeans in relation to their distributions of total phenolics, phenolic acids,
495 anthocyanins, and isoflavones, J. Agric. Food. Chem. 56 (2008) 8365-8373.
496 [11] B.J. Xu, S.H. Yuan, S.K. Chang, Comparative analyses of phenolic composition,
497 antioxidant capacity, and color of cool season legumes and other selected food
498 legumes, J. Food. Sci. 72 (2007) 167-177.

[12] J.S. Byun, Y.S. Han, S.S. Lee, The effects of yellow soybean, black soybean, and sword bean on lipid levels and oxidative stress in ovariectomized rats, Int. J. Vitam. Nutr. Res. 80 (2010) 97-106.

[13] K.Y. Kim, K.M. Lim, C.W. Kim, H.J. Shin, D.B. Seo, S.J. Lee, J.Y. Noh, O.N. Bae, S. Shin, J.H. Chung, Black soybean extract can attenuate thrombosis through inhibition of collagen-induced platelet activation, J. Nutr. Biochem., 22 (2011) 964-970.

[14] M. Lee, S.R. Sorn, Y. Park, H.K. Park, Anthocyanin Rich-Black Soybean Testa Improved Visceral Fat and Plasma Lipid Profiles in Overweight/Obese Korean Adults: A Randomized Controlled Trial, J. Med. Food. 19 (2016) 995-1003.

[15] J.E. Ware C.D. Sherbourne, The MOS 36-item Shoro-Form Health Survey (SF-36): I. Conceptual framework and item selection, Med. Care. 30 (1992) 473-489.

[16] L.Q. Wang, Y. Yamashita, A. Saito, H. Ashida, An analysis method for flavan-3-ol using high performance liquid chromatography coupled with a fluorescence detector, J. Food. Drug. Anal. 25 (2017) 478-487.

[17] C. Ito, T. Oki, T. Yoshida, F. Nanba, K. Yamada, T. Toda, Characterisation of proanthocyanidins from black soybeans: Isolation and characterisation of proanthocyanidin oligomers from black soybean seed coats, Food Chem. 141 (2013) 2507–2512.

[18] J.G. Umans, R. Levi, Nitric oxide in the regulation of blood flow and arterial pressure, Annu. Rev. Physiol. 57 (1995) 771-790.

[19] F. Fusi, A. Trezza, M. Tramaglino, G. Sgaragli, S. Saponara, O. Spiga, The beneficial health effects of flavonoids on the cardiovascular system: Focus on K+ channels, Pharmacol. Res. 152 (2020)104625.

523 [20] K. Yamagata, Polyphenols Regulate Endothelial Functions and Reduce the Risk of
524 Cardiovascular Disease, Curr. Pharm. 25 (2019) 2443-2458.
525 [21] H.J. Kim, I. Tsoy, J.M. Park, J.I. Chung, S.C. Shin, K.C. Chang, Anthocyanins from
526 soybean seed coat inhibit the expression of TNF-alpha-induced genes associated
527 with ischemia/reperfusion in endothelial cell by NF-kappaB-dependent pathway
528 and reduce rat myocardial damages incurred by ischemia and reperfusion in vivo,
529 FEBS. Lett. 580 (2006) 1391-1397.
530 [22] K. Ganesan, B. Xu, A Critical Review on Polyphenols and Health Benefits of Black
531 Soybeans. Nutrients. 9 (2017) doi: 10.3390/nu9050455.
532 [23] K.A. Groenewegen, H.M. den Ruijter, G. Pasterkamp, J.F. Polak, M.L. Bots, S.A.
533 Peters, Vascular age to determine cardiovascular disease risk: A systematic review
534 of its concepts, definitions, and clinical applications, Eur. J. Prev. Cardiol. 23
535 (2016) 264-274.
536 [24] M. Elgendi On the analysis of fingertip photoplethysmogram signals, Curr. Cardiol.
537 Rev. 8 (2012) 14-25.
538 [25] J.M. Ahn, New Aging Index Using Signal Features of Both Photoplethysmograms
539 and Acceleration Plethysmograms, Healthc. Inform. Res. 23 (2017) 53-59.
540 [26] H.J. Teede, B.P. McGrath, L. DeSilva, M. A. Cehun, Fassoulakis, P.J. Nestel,
541 Isoflavones reduce arterial stiffness: a placebo-controlled study in men and
542 postmenopausal women, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 1066-1071.
543 [27] M.M. Dohadwala, M. Holbrook, N.M. Hamburg, S.M. Shenouda, W.B .Chung, M.
544 Titas, M.A. Kluge, N. Wang, J. Palmisano, P.E. Milbury, J.B. Blumberg, J.A.Vita,
545 Effects of cranberry juice consumption on vascular function in patients with
546 coronary artery disease, Am. J. Clin. Nutr., 93 (2011) 934-940.

547 [28] D. Grassi, T.P. Mulder, R. Draijer, G. Desideri, H.O. Molhuizen, C. Ferri, Black
548 testa consumption dose-dependently improves flow-mediated dilation in healthy
549 males, J. Hypertens. 27 (2009) 774-781.
550 [29] R.M. Palmer, A.G. Ferrige, S. Moncada, Nitric oxide release accounts for the
551 biological activity of endothelium-derived relaxing factor, Nature. 327 (1987) 524-
552 526.
553 [30] H. Schroeter, C. Heiss, J. Balzer, P. Kleinbongard, C.L. Keen, N.K. Hollenberg, H.
554 Sies, C. Kwik-Uribe, H.H. Schmitz, M. Kelm, (−)-Epicatechin mediates beneficial
555 effects of flavanol-rich cocoa on vascular function in humans. Proc. Natl. Acad. Sci.
556 U S A. 103 (2006) 1024-1029.
557 [31] P. Pacher, J.S. Beckman, L. Liaudet, Nitric oxide and peroxynitrite in health and
558 disease, Physiol. Rev. 87 (2007) 315-424.
559 [32] C.Q. Lai, K.L. Tucker, L.D. Parnell, et al. PPARGC1A Variation Associated With
560 DNA Damage, Diabetes, and Cardiovascular Diseases: The Boston Puerto Rican
561 Health Study. Diabetes. 2008, 57, 809-816.
562 [33] I. Urquiaga, P. Strobel, D. Perez, X. Adiconis, B. García-Bailo, J. Griffith, M.
563 Meydani, J.M. Ordovás, Mediterranean diet and red wine protect against oxidative
564 damage in young volunteers, Atherosclerosis. 211 (2010) 694-699.
565 [34] Y. Kato, Y. Mori, Y. Makino, Morimitsu Y, Hiroi S, Ishikawa T, Osawa T.
566 Formation of Nepsilon-(hexanonyl)lysine in protein exposed to lipid
567 hydroperoxide. A plausible marker for lipid hydroperoxide-derived protein
568 modification. J Biol Chem. 1999, 274, 20406-20414.
569 [35] S. Baba, N. Osakabe, Y. Kato, M. Natsume, A. Yasuda, T. Kido, K. Fukuda, Y.
570 Muto, K. Kondo, Continuous intake of polyphenolic compounds containing cocoa

571 powder reduces LDL oxidative susceptibility and has beneficial effects on plasma
572 HDL-cholesterol concentrations in humans, Am J Clin Nutr. 85 (2007) 709-717.
573 [36] N. Anatoliotakis, S. Deftereos, G. Bouras, G. Giannopoulos, D. Tsounis, C.
574 Angelidis, A. Kaoukis, C. Stefanadis, Myeloperoxidase: expressing inflammation
575 and oxidative stress in cardiovascular disease, Curr. Top. Med. Chem. 13 (2013)
576 115-138.
577 [37] N. Ota, S. Soga, T. Murase, A. Shimotoyodome, T. Hase, Consumption of Coffee
578 Polyphenols Increases Fat Utilization in Humans, J. Health. Sci. 56 (2010) 745-751.
579 [38] M. Paquette, A.S. Medina Larqué, S.J. Weisnagel, Y. Desjardins, J. Marois, G.
580 Pilon, S. Dudonné, A. Marette, H. Jacques, Strawberry and cranberry polyphenols
581 improve insulin sensitivity in insulin-resistant, non-diabetic adults: a parallel,
582 double-blind, controlled and randomised clinical trial, Br. J. Nutr. 117 (2017) 519-
583 531.
584 [39] J.W. Anderson, B.M. Johnstone, M.E. Cook-Newell, Meta-analysis of the effects of
585 soy protein intake on serum lipids, N. Engl. J. Med. 333 (1995) 276-282.
586 [40] H. Aoki, Y. Otaka, K. Igarashi, A. Takenaka, Soy protein reduces paraquat-induced
587 oxidative stress in rats, J. Nutr. 132 (2002) 2258-2262.
588 [41] Ministery of Health, Labour and Welfare, Annual Health, Labour and Welfare
589 Report 2016, p77-76.

590 **Figure legends**

591 Figure 1. Chemical structures of 11 major polyphenols in black soybeans and 1
592 intestinal bacterial metabolite equol.

593

594 Figure 2. The effect of black soybean consumption on the vascular age. The results are
595 represented as the means ± standard deviation. *p<0.05 *vs.* 0 week by paired *t*-test. The
596 results of volunteers who showed lowered vascular age at the 4th or 8th week are denoted
597 as 'Improved at the 4th week' and 'Improved at the 8th week', respectively.

598

599 Figure 3. The effect of black soybean consumption on NO concentration in the plasma
600 and urine. The results are represented as the means ± standard deviation. *p<0.05 *vs.* 0
601 week by Dunnett′s test. $^{\#}p$<0.05 *vs.* 0 week by Wilcoxon signed-ranked test. The results
602 of 44 volunteers are denoted as 'All', and the results of volunteers who showed lowered
603 vascular age at the 4th or 8th week are denoted as 'Improved at the 4th week' and 'Improved
604 at the 8th week', respectively.

605

606 Figure 4. The effect of black soybean consumption on biomarkers related to the
607 oxidative stress. (A) 8-OHdG, (B) HEL and (C) MPO were measured using
608 corresponding ELISA kit. The results are represented as the means ± standard deviation.
609 *p<0.05 *vs.* 0 week by Dunnett′s test. $^{\#}p$<0.05 *vs.* 0 week by Wilcoxon signed-ranked
610 test. The results of 44 volunteers are denoted as 'All', and the results of volunteers who
611 showed lowered vascular age at the 4th or 8th week are denoted as 'Improved at the 4th
612 week' and 'Improved at the 8th week', respectively.

613

614 Figure 5. Correlation between concentrations of polyphenols and the vascular function
615 and oxidative stress by heatmap. Each square indicates the Pearson correlation
616 coefficient vales of corresponding factors. Positive correlations are shown in blue,
617 negative ones are shown in red and significant results are marked with '+' and '−',
618 respectively, $p<0.05$ by Student′s *t*-test.
619
620

Table 1. Composition of roasted black soybean

Nutrition component	**(per 30 g)**
Calories	132 kcal
Protein	10.6 g
Fat	6.5 g
Carbohydrate	5.0 g
Dietary fiber	5.6 g
Sodium	1.8 mg
NaCl	4.75 mg
Calcium	50.7 mg
Polyphenol content	**(per 30 g)**
Anthocyanidin	
Cyanidin-3-*O*-glucoside	12.4 mg
Flavan-3-ols	
(−)-Epicatechin	0.3 mg
Procyanidin B2	0.5 mg
Procyanidin C1	1.2 mg
Cinnamtannin A2	1.6 mg
Isoflavones	
Daizein	0.5 mg
Daidzin	78.1 mg
Glycitein	0.1 mg
Glycitin	0.5 mg
Genistein	48.4 mg

Table 2. The effect of black soybean consumption on the vascular function.

Vascular function	**0 week**	**4th week**	**8th week**
APG			
Vascular age	39.27±10.2	37.36±9.69*	37.07±9.61*
a wave	107.07±9.27	107.39±11.8	110.80±9.15*
b wave	−59.07±14.6	−65.32±15.9*	−67.98±13.9*
c wave	−23.48±10.8	−18.52±10.6*	−17.89±11.1*
d wave	−35.43±13.4	−29.91±12.2*	−30.02±13.5*
b/a	−0.55±0.13	−0.61±0.14*	−0.61±0.11*
c/a	−0.22±0.10	−0.17±0.10*	−0.16±0.10*
d/a	−0.33±0.13	−0.28±0.12*	−0.27±0.12*
Vascular waveform	2.84±1.26	2.43±0.94*	2.27±0.84*
Waveform score	53.34±11.9	58.41±10.8*	59.95±10.0*
Peripheral vascular health	64.93±12.8	70.59±10.1*	72.41±8.69*
Blood pressure			
Systolic blood pressure	112.09±14.4	109.59±14.0	111.73±10.9
Diastolic blood pressure	67.66±10.9	66.30±11.5	67.39±8.32

Means ± standard deviation are shown. * $p<0.05$ *vs.* 0 week, *Dunnett`s* Test.

Table 3A. The effect of black soybean consumption on polyphenol concentrations in the plasma of the 'All' group.

Polyphenols	Plasma					
	0 week		**4th week**		**8th week**	
	Free	Total	Free	Total	Free	Total
Cyanidin-3-*O*-glucoside	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
(−)-Epicatechin	0.048±0.033	0.066±0.059	0.049±0.033	0.072±0.060	0.061±0.053	0.085±0.087
Procyanidin B2	0.013±0.009	0.015±0.009	0.023±0.015	0.022±0.016	0.027±0.016	0.028±0.017
Procyanidin C1	0.001±0.001	0.001±0.001	0.001±0.002	0.002±0.002	0.002±0.002	0.003±0.003
Cinnamtannin A2	0.002±0.002	0.002±0.002	0.003±0.003	0.004±0.003	0.004±0.004	0.004±0.003
Sum of flavan-3-ols	0.063±0.038	0.079±0.052	0.073±0.040	0.099±0.064	0.091±0.062*	0.127±0.089††
Daidzin	0.012±0.006	0.013±0.011	0.018±0.010	0.020±0.009	0.018±0.012	0.018±0.007
Daidzein	0.010±0.005	0.011±0.007	0.013±0.007	0.016±0.010	0.012±0.008	0.013±0.010
Glycitin	0.014±0.007	0.016±0.010	0.021±0.014	0.029±0.022	0.020±0.015	0.021±0.017
Glycitein	0.024±0.007	0.027±0.008	0.026±0.009	0.029±0.009	0.026±0.009	0.028±0.009
Genistin	0.007±0.010	0.008±0.011	0.008±0.010	0.010±0.010	0.008±0.010	0.012±0.013
Genistein	0.034±0.021	0.038±0.025	0.041±0.019	0.047±0.025	0.039±0.017	0.040±0.026
Equol	0.008±0.006	0.011±0.012	0.010±0.009	0.016±0.012	0.008±0.008	0.013±0.014
Sum of isoflavones	0.101±0.035	0.116±0.042	0.136±0.041**	0.162±0.054††	0.119±0.042	0.142±0.051†
Sum of 12 polyphenols	0.164±0.059	0.195±0.064	0.209±0.066**	0.261±0.092††	0.210±0.073**	0.268±0.104††

Means ± standard deviation are shown, μmol/L. *, †p<0.05, **, ††p<0.01. * and ** represent for significant differences from free compound of 0 week. † and †† represent for that from total compound of 0 week. *Dunnett`s* test.

Table 3B. The effect of black soybean consumption on polyphenol concentrations in the urine of the 'All' group.

Polyphenols	Urine					
	0 week		**4th week**		**8th week**	
	Free	Total	Free	Total	Free	Total
Cyanidin-3-*O*-glucoside	0.009±0.009	0.011±0.010	0.016±0.012	0.020±0.014	0.013±0.010	0.015±0.011
(−)-Epicatechin	0.038±0.059	0.062±0.049	0.088±0.084	0.165±0.144	0.067±0.063	0.127±0.120
Procyanidin B2	0.118±0.137	0.144±0.124	0.234±0.166	0.262±0.194	0.219±0.154	0.264±0.231
Procyanidin C1	0.008±0.009	0.008±0.010	0.014±0.012	0.019±0.016	0.012±0.011	0.016±0.015
Cinnamtannin A2	0.002±0.003	0.004±0.004	0.009±0.009	0.010±0.008	0.008±0.008	0.008±0.009
Sum of flavan-3-ols	0.161±0.201	0.267±0.243	0.477±0.632	0.495±0.576††	0.425±0.697**	0.458±0.679††
Daidzin	0.055±0.062	0.071±0.125	0.107±0.122	0.147±0.163	0.122±0.323	0.175±0.243
Daidzein	0.596±0.770	4.741±3.595	1.718±1.994	8.387±4.574	1.992±2.180	9.961±6.744
Glycitin	0.041±0.041	0.057±0.123	0.048±0.041	0.083±0.086	0.061±0.055	0.093±0.125
Glycitein	0.263±0.391	1.034±1.380	0.394±0.507	2.152±1.703	0.487±0.463	2.468±2.624
Genistin	0.078±0.112	0.172±0.231	0.124±0.169	0.327±0.396	0.180±0.252	0.450±0.460
Genistein	0.397±0.451	5.319±6.086	1.100±1.172	11.891±9.831	1.443±2.066	14.949±11.34
Equol	0.146±0.116	0.443±0.905	0.374±1.433	3.439±6.942	1.439±7.390	5.418±14.54
Sum of isoflavones	2.279±2.552	10.638±9.989	3.893±3.942	29.898±24.25††	5.083±5.544**	28.579±29.82††
Sum of 12 polyphenols	2.597±2.579	10.978±9.954	4.470±3.897	30.770±24.57††	5.685±5.618**	29.237±26.73††

Means ± standard deviation are shown, μmol/L. *, †p<0.05, **, ††p<0.01. * and ** represent for significant differences from free compound of 0 week. † and †† represent for that from total compound of 0 week. *Dunnett`s* test.

Table 4A. The effect of black soybean consumption on polyphenol concentrations in the plasma of the 'Improved at the 4th week' group.

Polyphenols	Plasma			
	0 week		4th week	
	Free	Total	Free	Total
Cyanidin-3-*O*-glucoside	N.D.	N.D.	N.D.	N.D.
(−)-Epicatechin	0.048±0.032	0.077±0.070	0.052±0.037	0.082±0.062
Procyanidin B2	0.013±0.010	0.014±0.008	0.021±0.016	0.023±0.019‡‡
Procyanidin C1	0.001±0.001	0.001±0.001	0.001±0.001	0.002±0.002
Cinnamtannin A2	0.001±0.002	0.002±0.002	0.001±0.002	0.003±0.003
Sum of flavan-3-ols	0.063±0.037	0.083±0.059	0.073±0.044	0.109±0.065
Daidzin	0.012±0.006	0.013±0.011	0.017±0.009	0.021±0.009‡
Daidzein	0.009±0.005	0.010±0.007	0.014±0.007#	0.017±0.011‡‡
Glycitin	0.014±0.006	0.014±0.009	0.021±0.014#	0.026±0.021‡
Glycitein	0.024±0.007	0.024±0.009	0.025±0.009	0.030±0.009‡‡
Genistin	0.004±0.006	0.008±0.012	0.006±0.006	0.011±0.012
Genistein	0.037±0.023	0.044±0.031	0.046±0.016	0.054±0.027
Equol	0.007±0.006	0.011±0.011	0.010±0.009	0.016±0.013
Sum of isoflavones	0.105±0.038	0.119±0.049	0.139±0.034##	0.170±0.064‡‡
Sum of 12 polyphenols	0.168±0.060	0.203±0.070	0.211±0.064	0.279±0.102‡

Means ± standard deviation are shown, μmol/L. #, ‡$p<0.05$, ##, ‡‡$p < 0.01$. # and ## represent for significant differences from free compound of 0 week. ‡ and ‡‡ represent for that from total compound of 0 week. Wilcoxon signed-rank test.

Table 4B. The effect of black soybean consumption on polyphenol concentrations in the urine of the 'Improved at the 4th week' group.

Polyphenols	Urine			
	0 week		4th week	
	Free	Total	Free	Total
Cyanidin-3-*O*-glucoside	0.009±0.007	0.012±0.009	0.015±0.010#	0.019±0.013‡
(−)-Epicatechin	0.024±0.042	0.136±0.242	0.175±0.253#	0.460±0.472‡
Procyanidin B2	0.217±0.294	0.191±0.229	0.505±0.576	0.587±1.120
Procyanidin C1	0.008±0.009	0.010±0.010	0.019±0.017#	0.029±0.027‡‡
Cinnamtannin A2	0.005±0.009	0.005±0.009	0.012±0.013#	0.018±0.017‡‡
Sum of flavan-3-ols	0.318±0.594	0.313±0.357	0.698±0.688	1.099±1.218‡‡
Daidzin	0.043±0.030	0.052±0.065	0.105±0.120#	0.142±0.190
Daidzein	1.396±2.373	3.741±3.360	3.033±6.957	11.375±8.408‡‡
Glycitin	0.041±0.035	0.070±0.058	0.045±0.030	0.083±0.092
Glycitein	0.329±0.505	1.124±1.479	0.446±0.612	3.006±3.226
Genistin	0.047±0.053	0.127±0.153	0.119±0.176	0.267±0.186
Genistein	0.522±0.516	5.202±4.765	1.104±1.109	16.973±15.99‡‡
Equol	0.163±0.120	0.590±1.128	0.160±0.217	2.386±4.763
Sum of isoflavaones	2.165±2.239	10.613±8.538	3.391±3.401	30.774±20.97‡‡
Sum of 12 polyphenols	2.482±2.225	10.926±8.558	4.080±3.400	31.761±20.98‡‡

Means ± standard deviation are shown, μmol/L. #, ‡$p<0.05$, ##, ‡‡$p<0.01$. # and ## represent for significant differences from free compound of 0 week. ‡ and ‡‡ represent for that from total compound of 0 week. Wilcoxon signed-rank test.

Table 5A. The effect of black soybean consumption on polyphenol concentrations in the plasma of the 'Improved at the 8th week' group.

Polyphenols	Plasma			
	0 week		8th week	
	Free	Total	Free	Total
Cyanidin-3-*O*-glucoside	N.D.	N.D.	N.D.	N.D.
(−)-Epicatechin	0.042±0.022	0.063±0.053	0.058±0.048	0.104±0.097
Procyanidin B2	0.014±0.008	0.013±0.008	0.028±0.013$^{\#\#}$	0.029±0.015‡‡
Procyanidin C1	0.001±0.001	0.001±0.001	0.002±0.002$^{\#}$	0.003±0.003‡
Cinnamtannin A2	0.002±0.002	0.002±0.002	0.004±0.005	0.005±0.003‡‡
Sum of flavan-3-ols	0.059±0.026	0.080±0.051	0.100±0.067$^{\#\#}$	0.151±0.099‡‡
Daidzin	0.012±0.006	0.015±0.013	0.020±0.014	0.020±0.007
Daidzein	0.010±0.005	0.011±0.007	0.012±0.006	0.013±0.008
Glycitin	0.016±0.006	0.018±0.010	0.018±0.007	0.019±0.017
Glycitein	0.026±0.006	0.026±0.007	0.027±0.009	0.030±0.008
Genistin	0.006±0.007	0.006±0.007	0.005±0.006	0.010±0.014
Genistein	0.037±0.023	0.039±0.030	0.038±0.018	0.039±0.025
Equol	0.009±0.006	0.013±0.012	0.010±0.004	0.014±0.013
Sum of isoflavones	0.111±0.032	0.127±0.043	0.124±0.045	0.142±0.048
Sum of 12 polyphneols	0.170±0.050	0.207±0.068	0.225±0.070$^{\#\#}$	0.293±0.103‡‡

Means ± standard deviation are shown, μmol/L. $^{\#}$, $^{‡}p<0.05$, $^{\#\#}$, $^{‡‡}p<0.01$. $^{\#}$ and $^{\#\#}$ represent for significant differences from free compound of 0 week. ‡ and ‡‡ represent for that from total compound of 0 week. Wilcoxon signed-rank test.

Table 5B. The effect of black soybean consumption on polyphenol concentrations in the urine of the 'Improved at the 8th week' group.

Polyphenols	Urine			
	0 week		8th week	
	Free	Total	Free	Total
Cyanidin-3-*O*-glucoside	0.010±0.008	0.012±0.009	0.012±0.008	0.015±0.009
(−)-Epicatechin	0.076±0.151	0.110±0.158	0.096±0.129	0.125±0.120
Procyanidin B2	0.197±0.269	0.179±0.194	0.473±0.408	0.494±0.466
Procyanidin C1	0.010±0.015	0.012±0.016	0.014±0.010	0.016±0.014
Cinnamtannin A2	0.002±0.003	0.003±0.004	0.008±0.007#	0.008±0.008‡
Sum of flavan-3-ols	0.287±0.556	0.260±0.283	0.522±0.448	0.623±0.555
Daidzin	0.044±0.023	0.046±0.055	0.057±0.056	0.107±0.122
Daidzein	1.410±2.396	3.344±3.499	3.627±4.832	8.360±7.339‡
Glycitin	0.035±0.034	0.065±0.059	0.058±0.064	0.092±0.144
Glycitein	0.371±0.517	1.034±1.486	0.787±1.261	2.573±3.003
Genistin	0.057±0.083	0.103±0.150	0.252±0.359#	0.419±0.427‡‡
Genistein	0.419±0.444	5.268±6.528	1.600±2.564#	11.414±10.37
Equol	0.157±0.116	0.481±1.113	2.411±9.691	6.597±17.07
Sum of isoflavones	1.565±1.511	8.950±9.434	5.030±5.848#	27.629±29.58‡‡
Sum of 12 polyphenols	1.852±1.510	9.210±9.467	5.552±5.804##	28.273±29.46‡‡

Means ± standard deviation are shown, μmol/L. #, ‡$p<0.05$, ##, ‡‡$p<0.01$. # and ## represent for significant differences from free compound of 0 week. ‡ and ‡‡ represent for that from total compound of 0 week. Wilcoxon signed-rank test.

Supplementary Table 1. Anthropometric parameters of 44 volunteers during the 8-week trial.

Anthropometric parameters	0 week	4th week	8th week
Body weight (kg)	52.6±1.03	52.71±1.04	52.78±1.02
BMI	20.82±0.42	20.87±0.42	20.89±0.42
Body fat(%)	26.80±0.82	27.46±0.79	27.64±0.82
Visceral fat (%)	3.45±0.30	3.50±0.31	3.55±0.31
Biological age	27.93±1.66	29.02±1.67	29.32±1.72
Basal metabolic rate (kcal/day)	1140±15	1134±14	1133±14
Estimated bone mass (kg)	2.20±0.04	2.17±0.04	2.17±0.04
Muscle mass (%)	36.03±0.42	35.78±0.43	35.50±0.49

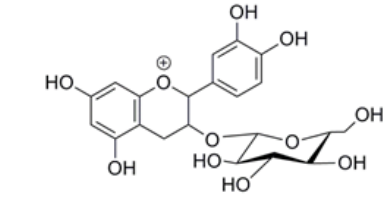
Supplementary Table 2. Hematologic parameters of 44 volunteers during the 8-week trial.

Hematologic parameters	0 week	4th week	8th week
TP	7.37±0.35	7.38±0.31	7.33±0.32
BUN	11.35±2.88	12.98±3.07	12.04±2.42
CRE	0.69±0.08	0.67±0.08	0.66±0.07
GLU	88.21±5.86	89.74±6.01	86.63±6.36
LDH	164.74±19.34	165.37±21.28	163.04±21.10
ALP	157.49±38.93	164.72±42.78	165.84±45.81
γ-GTP	17.05±11.03	18.26±11.94	17.88±13.20
AST	17.63±5.37	19.02±4.29	18.28±5.05
ALT	13.53±6.87	14.60±6.21	14.28±6.49
TG	61.37±34.32	63.28±35.00	60.30±29.58
LDL-C	102.72±30.02	101.51±28.71	101.02±29.03
HDL-C	68.74±11.41	68.60±13.47	71.16±12.48
Na	138.53±1.69	137.65±1.40	138.21±1.42
Cl	104.60±1.59	103.63±1.49	103.23±1.91
K	4.32±0.29	4.39±0.24	4.48±0.32
WBC	5582±1633	5441±1477	5698±1457
RBC	440±29	452±26	449±27
Hb	13.04±0.91	13.19±0.87	13.28±0.98
Ht	39.78±2.16	40.59±2.06	41.06±2.42
MCV	90.77±4.65	90.59±4.55	91.63±4.63
MCH	29.72±1.95	29.21±1.82	19.64±1.92
MCHC	32.75±0.93	32.47±0.80	32.31±0.82
PLT	25.21±6.24	25.93±7.10	25.89±6.21

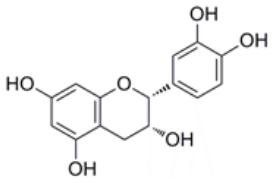
TP, total protein; BUN, blood urea nitrogen; CRE, creatinine; GLU, glucose; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; γ-

852 GTP, γ- glutamyltranspeptidase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TG, triglycerides; HDL-C, high-
853 density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol, WBC, white blood cells; RBC, red blood cells; Hb,
854 hemoglobin; Ht, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean hemoglobin
855 concentration; PLT, platelet.
856
857

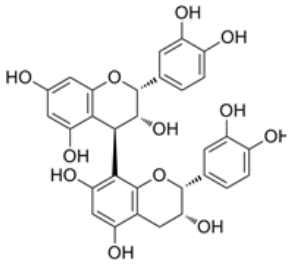
858



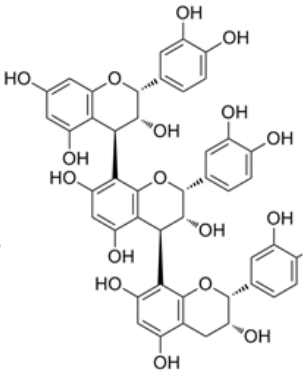
Cyanidin-3-*O*-glucoside



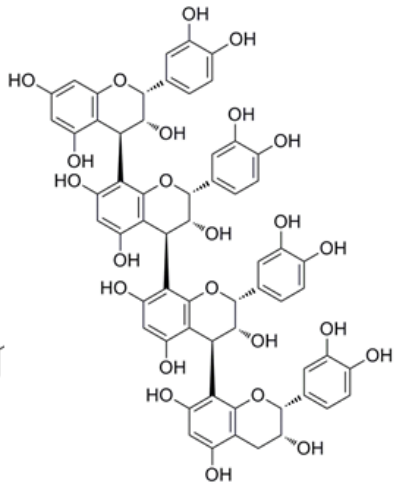
(−)-Epicatechin



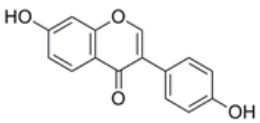
Procyanidin B2



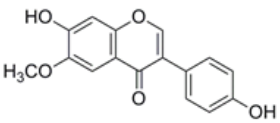
Procyanidin C1



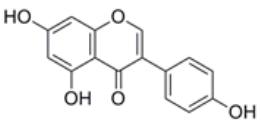
Cinnamtannin A2



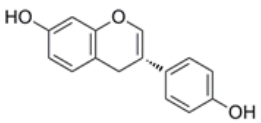
Daidzein



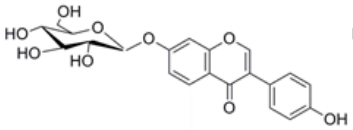
Glycitein



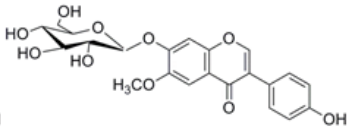
Genistein



Equol



Daidzin



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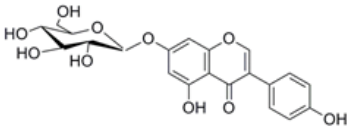

Genistin

Fig. 1

859

860

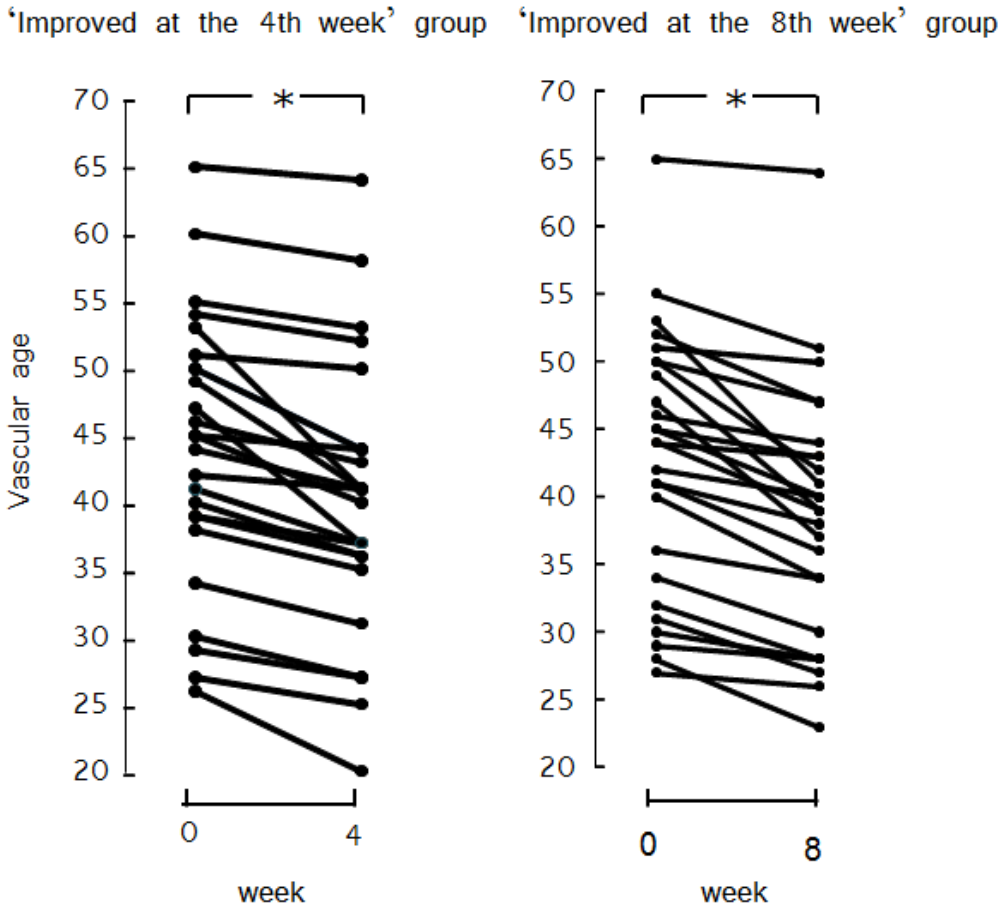
'Improved at the 4th week' group
'Improved at the 8th week' group
Vascular age
70
65
60
55
50
45
40
35
30
25
20
*
0
4
week
0
8
week

Fig. 2

861
862

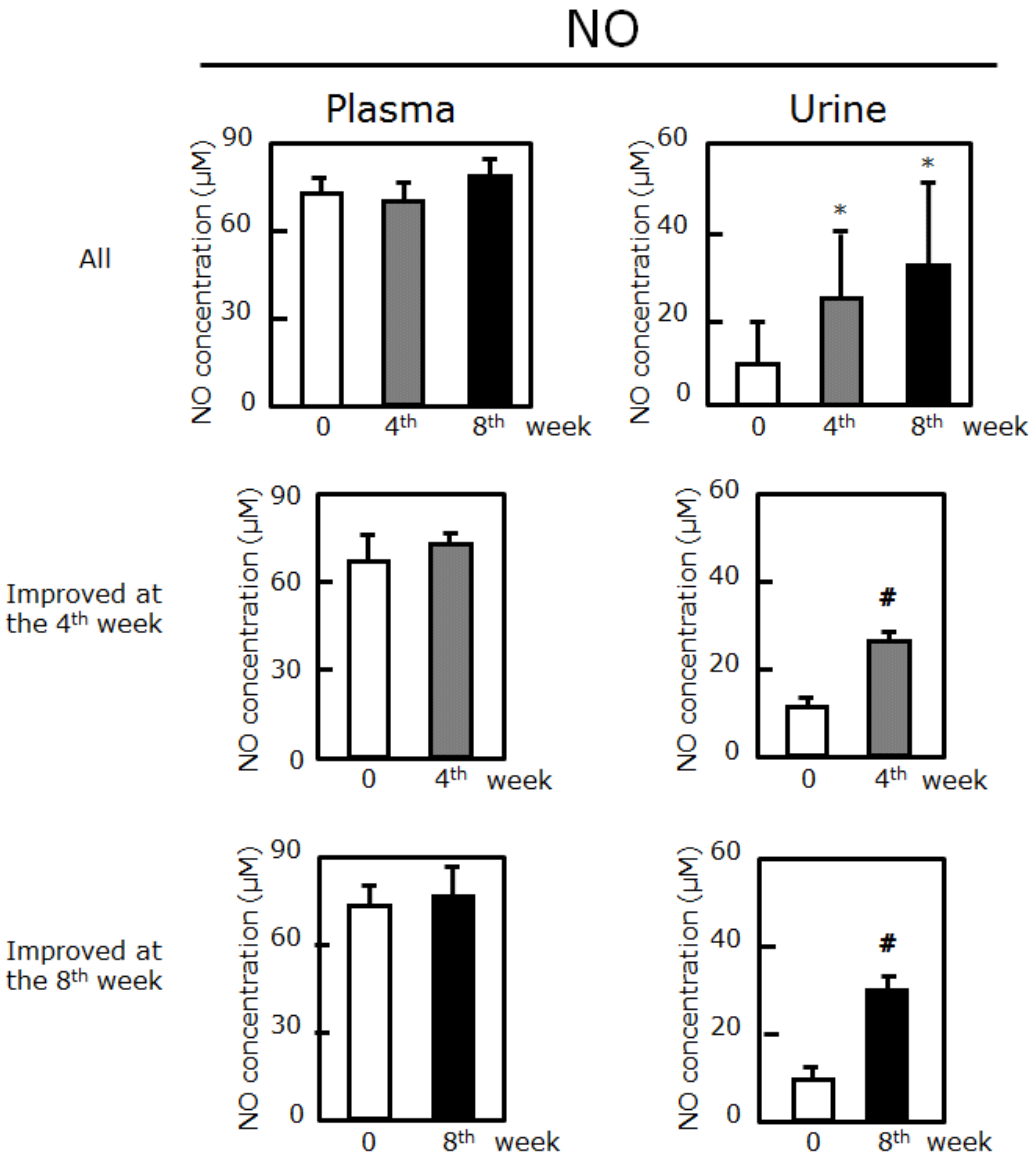
NO
Plasma
Urine
All
Improved at the 4th week
Improved at the 8th week
NO concentration (μM)
90
60
30
0
60
40
20
0
0
4th
8th
week
*
#

Fig. 3

863

864

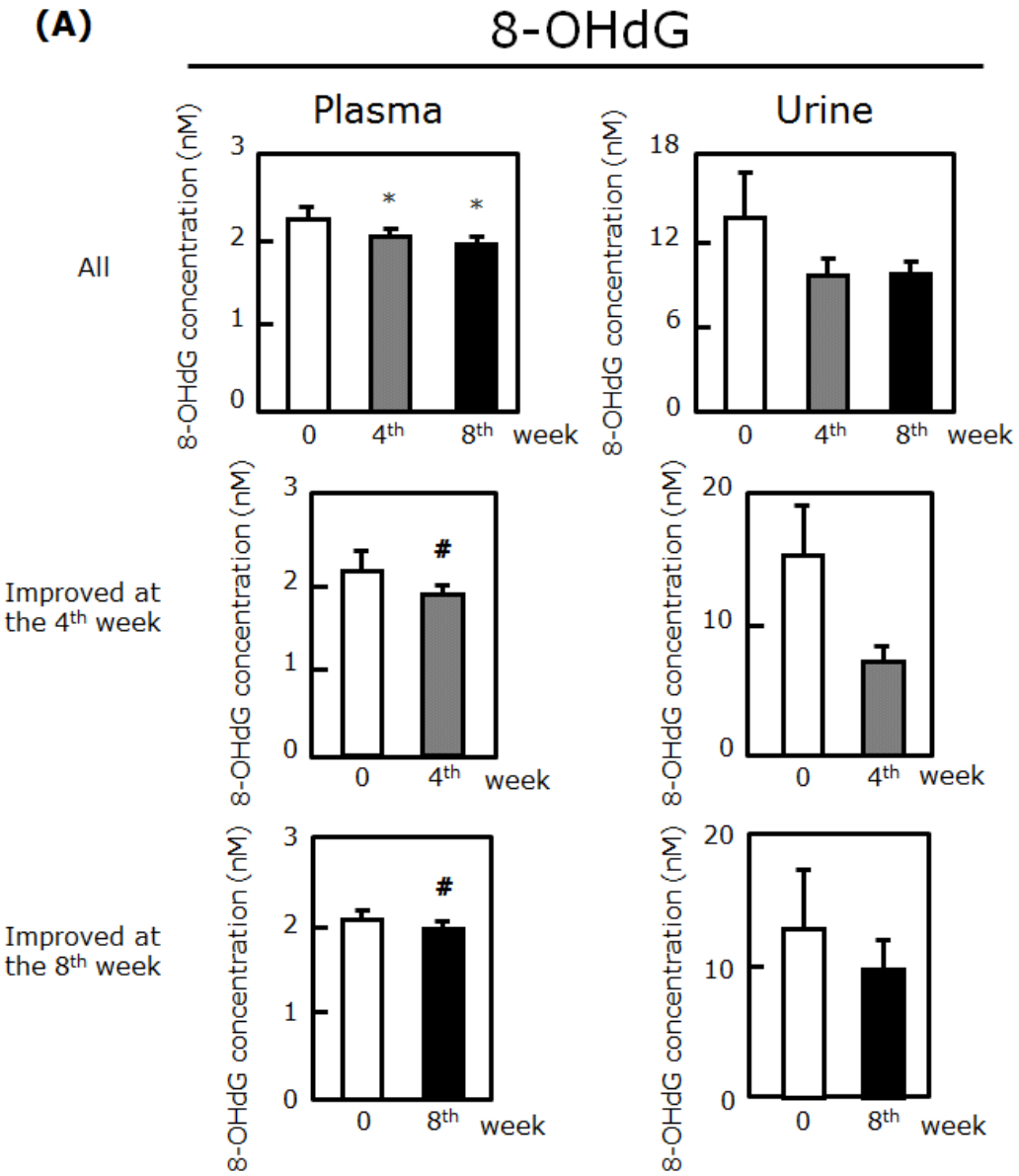

Fig. 4A

865
866

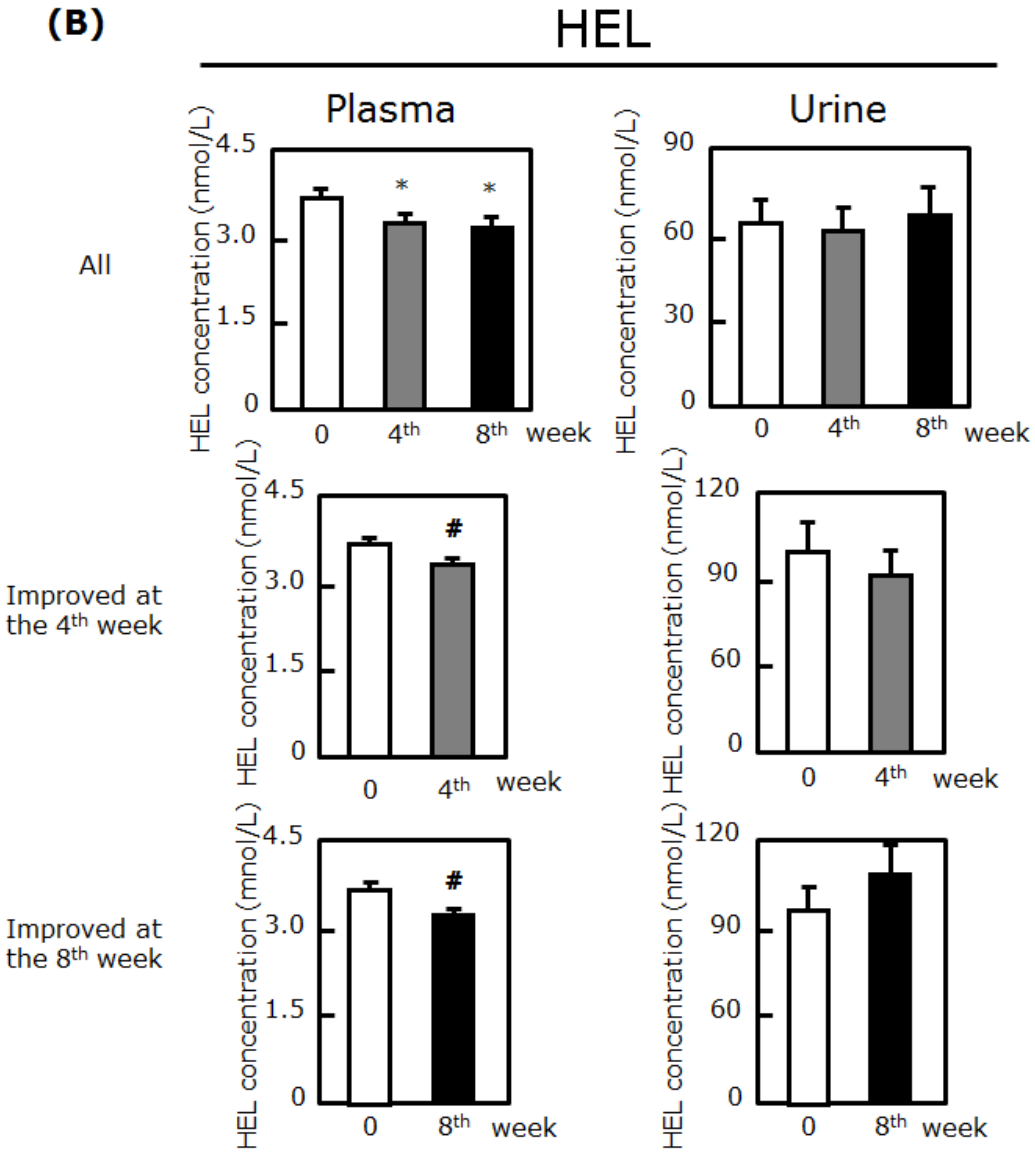

Fig. 4B

867

868

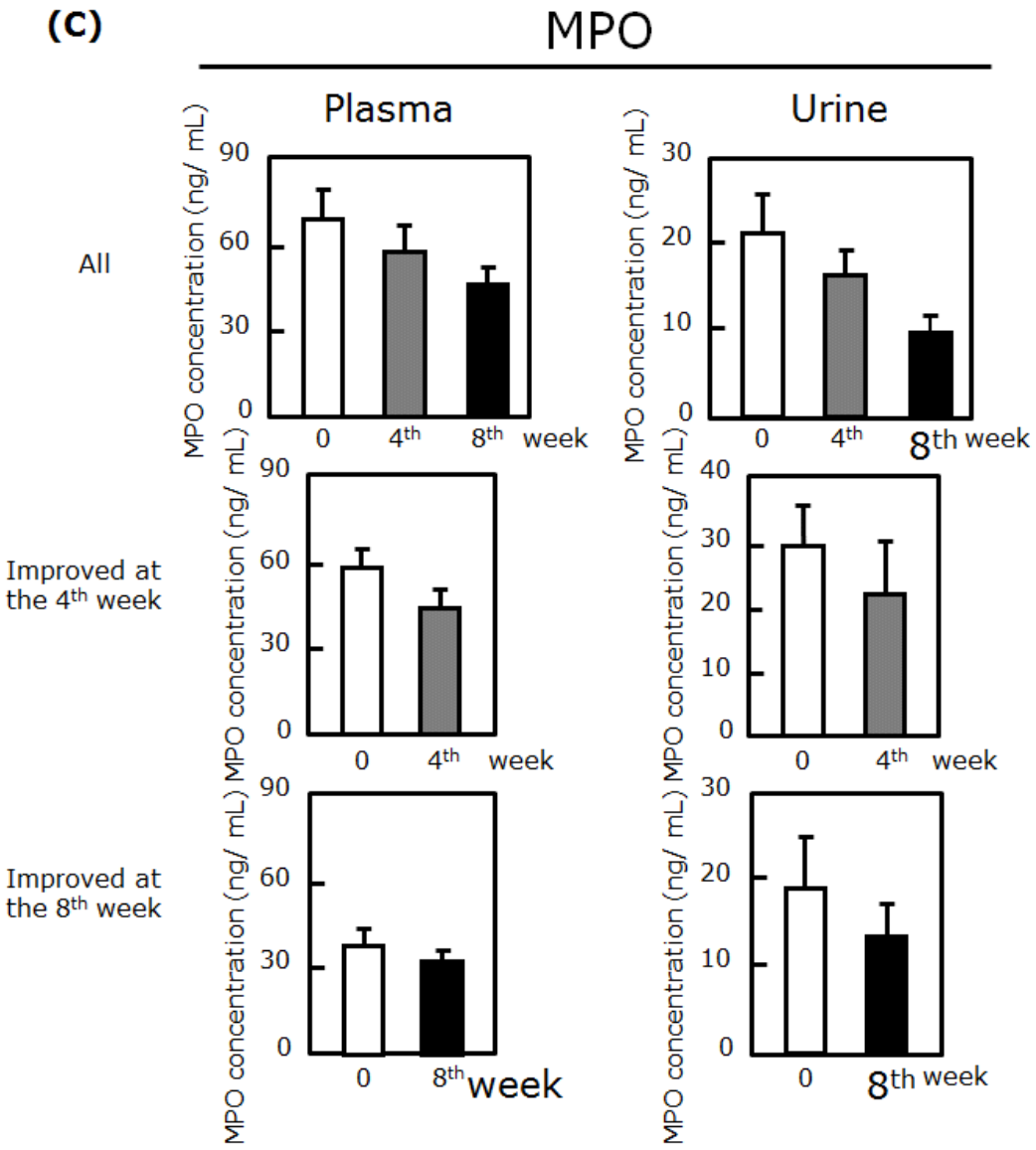

Fig. 4C

869

870

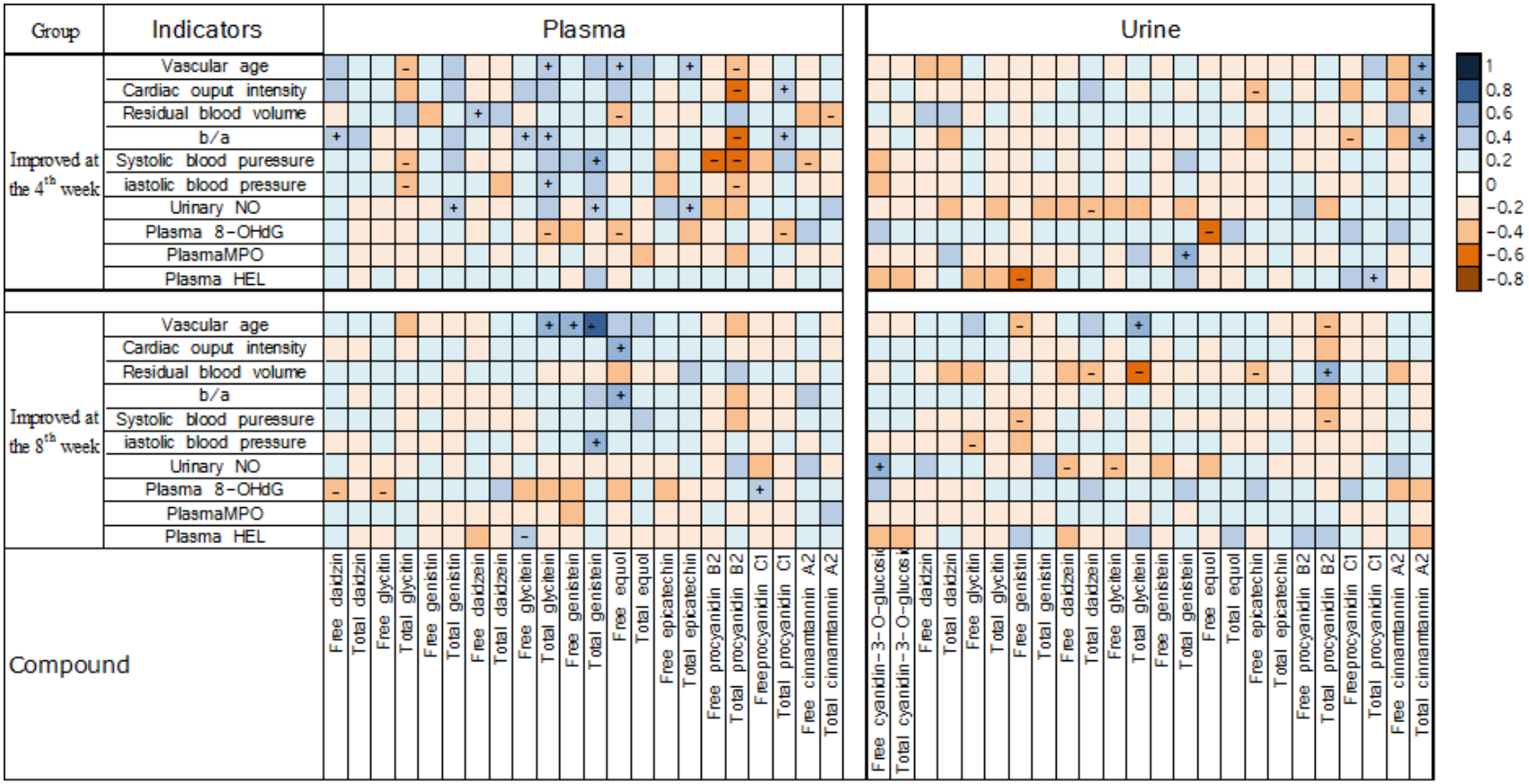
Group
Indicators
Plasma
Urine
Improved at the 4th week
Vascular age
Cardiac ouput intensity
Residual blood volume
b/a
Systolic blood puressure
iastolic blood pressure
Urinary NO
Plasma 8-OHdG
PlasmaMPO
Plasma HEL
Improved at the 8th week
Compound
Free daidzin
Total daidzin
Free glycitin
Total glycitin
Free genistin
Total genistin
Free daidzein
Total daidzein
Free glycitein
Total glycitein
Free genistein
Total genistein
Free equol
Total equol
Free epicatechin
Total epicatechin
Free procyanidin B2
Total procyanidin B2
Free procyanidin C1
Total procyanidin C1
Free cinnamtannin A2
Total cinnamtannin A2
Free cyanidin-3-O-glucosi
Total cyanidin-3-O-glucosi
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8

Fig. 5

871