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Reduction of blood glucose and HbA1c levels by cyanidin 3,5-diglucoside in KKAy mice

Takuya Yamane[a,b,c,*], Momoko Imai[a,d,e], Satoshi Handa[a,b], Keiko Yamada[a], Tatsuji Sakamoto[a,b], Testuo Ishida[f], Hiroshi Inui[a,e], Yoshio Yamamoto[g], Takenori Nakagaki[c], Yoshihisa Nakano[a]

[a] Center for Research and Development Bioresources, Organization for Research Promotion, Osaka Prefecture University, Sakai, Osaka 599-8570, Japan
[b] Department of Applied Life Sciences, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai, Osaka 599-8531, Japan
[c] Institute of Food Sciences, Nakagaki Consulting Engineer and Co., Ltd, Nishi-ku, Sakai, Osaka 593-8328, Japan
[d] Faculty of Human Development, Department of Food and Nutrition Management Studies, Soai University, Osaka 559-0033, Japan
[e] Department of Nutrition, Osaka Prefecture University, Habikino, Osaka 583-8555, Japan
[f] Department of Chemistry, Biology and Marine Science, Faculty of Science, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan
[g] Iga Research Institute, Iga Regional Satellite Campus, Mie University, Iga, Mie 518-0131, Japan

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ABSTRACT

Aronia melanocarpa has beneficial effects on lifestyle-related diseases. Our previous studies showed that dipeptidyl peptidase IV (DPP IV) activity was inhibited by cyanidin 3,5-diglucoside from aronia juice and the weight of adipose tissues of mice given aronia juice was also reduced. The aim of this study is to investigate the effects of cyanidin 3,5-diglucoside, including aronia juice, on type-2 diabetes through inhibition of DPP IV activity *in vivo*. Type-2 diabetes and obesity model KKAy mice were divided into three groups containing five mice in each group: a control group that was given water, an aronia group that was given aronia juice, and a cy-dg group that was given cyanidin 3,5-diglucoside solution. Although increased blood glucose and hemoglobin A1c levels were reduced in KKAy mice administered cyanidin 3,5-diglucoside, weight of white adipose tissue was not reduced. These results suggest that cyanidin 3,5-diglucoside has an anti-diabetic effect but not an anti-obesity effect.

1. Introduction

Aronia berries (*Aronia melanocarpa*) have been used in traditional medicine in Russia and Eastern European countries (Kokotkiewicz, Jaremicz, & Luczkiewicz, 2010). Aronia berries have high contents of phenolic phytochemicals, and their concentration in aronia berries is over five-fold higher than that in cranberries (Wu et al., 2004, 2006).

Type-2 diabetes has become a public health challenge for many countries (International Diabetes Federation, 2017). The glucose-lowering medications such as DPP IV inhibitors, glucagon-like peptide 1 (GLP-1) receptor agonists and sodium-glucose cotransporter 2 inhibitors have been used in last 10 years (Sterrett, Bragg, & Weart, 2016).

Reduction of glycated hemoglobin (HbA1c) is accepted as a well-validated surrogate for glycemic control and prevention of microvascular complications (European Medicines Agency, 2012). HbA1c levels have become the standard outcome measure in many trials for various diabetes therapies (U.S. Department of Health and Human Services Food and Drug Administration, 2008).

Aronia berries and their derivatives have an anti-diabetic effect. In an animal model study, hyperglycemia was attenuated in diabetic rats given aronia juice (Valcheva-Kuzmanova, Kuzmanov, Tancheva, & Belcheva, 2007). Fasting blood glucose level was reduced in animals fed aronia extract (Qin & Anderson, 2012). Levels of glycemia were significantly decreased in rats with alloxan-induced diabetes that were given aronia juice (Oprea, Manolescu, Fărcăşanu, Mladin, & Mihele, 2014). Glucose tolerance was improved in rats fed a high-fat diet with aronia juice (Bhaswant, Shafie, Mathai, Mouatt, & Brown, 2017).

In a human intervention study, the fasting glucose level was reduced by aronia juice in patients with non-insulin-dependent diabetes (Simeonov et al., 2002). The postphedidetal blood glucose level was also decreased by drinking aronia juice before meals (Yamane et al., 2017).

Although those studies showed that aronia is very useful for

* Corresponding author at: Center for Research and Development Bioresources, Research Organization for Research Promotion, Osaka Prefecture University, Sakai, Osaka 599-8570, Japan.
E-mail address: tyt29194@osakafu-u.ac.jp (T. Yamane).

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lowering blood glucose level and improving diabetes, the majority of the mechanisms are have not been clarified.

In our previous study, cyanidin 3,5-diglucoside, one of the components of aronia juice, inhibits dipeptidyl peptidase IV (DPP IV) activity (Kozuka et al., 2015). DPP IV (EC 3.4.14.5) is an exopeptidase (Ohkubo, Huang, Ochiai, Takagaki, & Kani, 1994) and degrades incretins as do glucagon-like peptide 1 (GLP-1) and insulinotropic peptide (GIP). Incretins stimulate insulin secretion from the pancreas to reduce the blood glucose level (Baggio & Drucker, 2007; Kieffer, McIntosh, & Pederson, 1995; Mentlein, 1999; Pridal et al., 1996). Therefore, blood glucose level is reduced by DPP IV inhibitors.

Our previous study showed that blood glucose level and weight of white adipose tissues were reduced in KKAy diabetes and obesity model mice that were given aronia juice (Yamane et al., 2016). However, the effect of cyanidin 3,5-diglucoside in diabetes *in vivo* is not clear.

In this study, to clarify the effect of cyanidin 3,5-diglucoside identified as a DPP IV inhibitor present in aronia juice in our previous study on reduction of blood glucose level, cyanidin 3,5-diglucoside was administered to KKAy mice, and their blood glucose and HbA1c levels and weights of white adipose tissues were measured. Furthermore, we discuss the effect of cyanidin 3,5-diglucoside on type-2 diabetes and obesity.

2. Materials and methods

2.1. Materials

Aronia juice was provided by Nakagaki Consulting and Engineer Co., Ltd. (Osaka, Japan). Cyanidin 3,5-diglucoside, and cyanidin 3-O-glucoside were purchased from EXTRASYNTHESE (Lyon, France). Cyanidin 3-O-garactoside and cyanidin 3-O-arabinoside were purchased from TOKIWA (Chiba, Japan). HbA1c cartridges and strips for blood glucose levels were provided by SIEMENS (Tokyo, Japan) and Nova Biomedical (Massachusetts, USA), respectively. Gly-Pro-MCA and GLP-1 ELISA kits were purchased from Peptide Institute (Osaka, Japan) and IBL (Gunma, Japan), respectively. All other chemicals were of analytical grade and were purchased from Wako Pure Chemicals (Osaka, Japan).

2.2. Animals

KKAy male mice were obtained at 4 weeks of age from CLEA Japan (Tokyo, Japan). All mice were fed a normal diet (CE2 diet, CLEA Japan, Tokyo, Japan). After 4 days, KKAy mice were divided into three groups of five mice in each group: a control group that was given water, an aronia group that was given aronia juice, and a cy-dg group that was given 10 μg/mL solution of cyanidin 3,5-diglucoside (99.7% purity) prepared with water. Aronia juice and cyanidin 3,5-diglucoside solution were given by free intake. At 49 days after starting the diets, the mice were sacrificed by isoflurane anesthesia. Serum, liver, small intestine, kidney and adipose tissues were isolated and weighed.

2.3. Blood glucose and HbA1c levels

Blood glucose levels were measured using a small blood glucose measurement apparatus, Xpress 900 (Nova Biomedical, Massachusetts, USA). HbA1c levels were measured using DCA Vantage Analyzer (SIEMENS, Tokyo, Japan).

2.4. DPP IV activity

Enzyme activity was measured by fluorometrical determination (excitation, 380 nm; emission, 440 nm) of the liberation of 7-amino 4-methylcoumarin at 37 °C in a mixture containing 10 μl of 10 mM substrate Gly-Pro-4-methyl-coumarinamide, 100 μL of 0.5 M Tris–HCl (pH 9.0), 5 μL of enzyme solution and Milli Q water (18 mΩ) in a total volume of 1 ml. After incubation for 30 min, 2 ml of 0.2 M acetic acid was added to the mixture to terminate the reaction.

2.5. Enzyme immunoassay for GLP-1 and insulin

The blood level of GLP-1 was measured using a mammalian active-GLP-1 measurement kit (IBL, Gunma, Japan).

2.6. Analysis of liver triglyceride content

Liver triglyceride content was measured as described previously (Blige & Dyer, 1958). Briefly, the liver tissue (50 mg) was homogenized with 0.1 M acetic acid and 1.8 ml of methanol-chloroform (2:1, v/v) was added. After centrifugation, the lower chloroform layer was collected, and then the residues were washed with 0.5 ml of chloroform. The combined chloroform layer was dried in air. The pellet was dissolved in 2-propanol containing 10% Triton X-100. Triglyceride concentrations were measured using Wako Triglyceride E-test (Wako, Osaka, Japan).

2.7. Extraction of anthocyanin from aronia juice

Extraction of anthocyanin was carried out according to the method described by Vieira et al (Vieira, Marques, Machado, Silva, & Hubinger, 2017) with some modifications. One g of Wakogel 50C18 (Fujifilm Wako Chemicals) was put into a small glass column and washed in the order with ethyl acetate, methanol and water. Five mL of aronia juice was loaded into this pre-conditioned column, and the column was washed with about 5 ml of water and then with about 10 ml of ethyl acetate. The bound anthocyanins were eluted with 90% methanol in water containing 0.1% formic acid. The fraction with 300 μL of aliquote in 1.5 ml tubes was concentrated to near-dryness under vacuum using a centrifugal evaporator. The sample in the tube was redissolved in 300 μL of the equilibration solvent used for HPLC analysis (see below).

2.8. HPLC analysis of anthocyanin

HPLC analysis of anthocyanins was carried out according to the method described by Gouvêa et al (Gouvêa et al., 2015) with some modifications. A Shimadzu HPLC system was used to perform the chromatographic analysis, and separation was performed on a Cosmosil 3C18-EB column (2.0 mm ID × 150 mm, Nacalai tesque). Solvent A contained 5% formic acid in water, and solvent B contained 5% formic acid, 90% methanol and 5% water. The column was equilibrated with 10% B. A fifty-μL aliquot of the sample and calibration standard solutions (1.25–10 μmol/L in 10% B) were loaded onto a 40-μL sample loop. Anthocyanins were eluted using the following time program: 0–10 min, 10% B; 10–50 min, linear increase in methanol from 10% B to 50% B; and 51–59 min, 90% B. The flow rate was 100 μL/min, and the column temperature was at 20 °C. The elution of anthocyanins was monitored by measuring absorbance at 520 nm. The anthocyanin contents of aronia juice was shown in Table 1.

Table 1
Anthocyanins content in aronia juice.

Anthocyanin	nmol/mL juice
Cyanidin 3,5-diglucoside	0.234 ± 0.013
Cyanidin 3- O-galactoside	2.70 ± 0.05
Cyanidin 3- O-glucoside	0.262 ± 0.039
Cyanidin 3- O-arabinoside	2.79 ± 0.16

All data is presented as mean ± standard deviation of four replicates.

2.9. Statistical analysis

Data are expressed as means ± standard error. Statistical analyses were performed using one-way analysis of variance followed by unpaired Student's *t* test. For comparison of multiple samples, the Tukey–Kramer test was used.

2.10. Ethics statement

All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the Committee for Animal Research at Osaka Prefecture University (permit number: 30–89).

3. Results

3.1. Reduction of blood glucose and HbA1c levels by cyanidin 3,5-diglucoside

To examine the effects of cyanidin 3,5-diglucoside on blood glucose and HbA1c levels, a solution containing water, aronia juice and cyanidin 3,5-diglucoside was administered orally to mice in the control group, aronia group and cy-dg group. As shown in Fig. 1A, blood

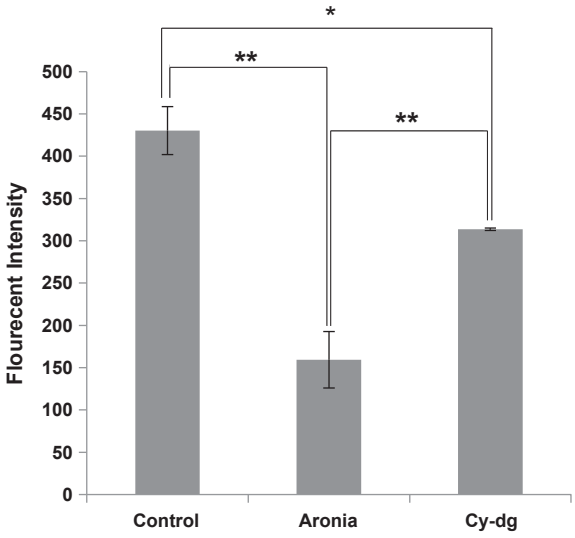

Fig. 2. DPP IV activities in the serum. Water or aronia juice or cyanidin-3,5-diglucoside was administered orally to mice. After 49 days, serum was obtained, and their DPP IV activities were measured. *$p < 0.05$, **$p < 0.01$, n = 5.

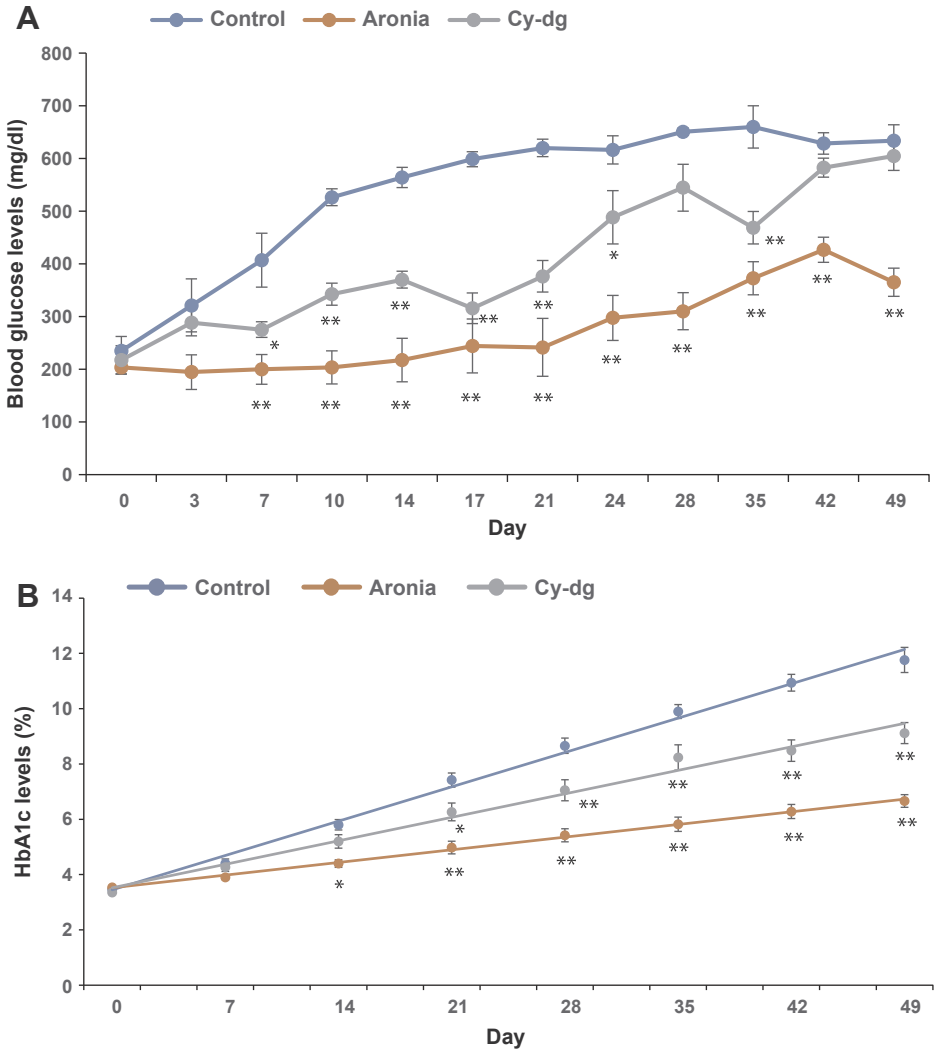

Fig. 1. Differences in blood glucose and HbA1c levels between control and cyanidin-3,5-diglucoside or aronia juice-administered mice. Aronia juice was used as a positive control against cyanidin-3,5-diglucoside. Blood glucose (A) and HbA1c (B) levels in mice were measured every 3 or 4 days for 49 days. There were significant differences between control mice and mice fed cyanidin-3,5-diglucoside or aronia juice. *$p < 0.05$, **$p < 0.01$, n = 5.

glucose levels in the aronia group and cy-dg group were about 39% and 60%, respectively, of that in the control group at 21 days after the start of administration. On the other hand, blood glucose levels in the aronia group and cy-dg group were about 58% and 95%, respectively, of that in control group at 49 days after the start of administration. The blood glucose levels were increased in the cy-dg group at 24 days and in the aronia group at 35 days after the start of administration. Furthermore, HbA1c levels in the aronia group and cy-dg group were about 67% and 84%, respectively, of that in control group at 21 days after the start of administration. On the other hand, HbA1c levels in the aronia group and cy-dg group were about 56% and 77%, respectively, of that in the control group at 49 days after the start of administration (Fig. 1B).

3.2. Inhibition of DPP IV activity in KKAy mice fed cyanidin 3,5-diglucoside

Forty-nine days after the start of administration, serum was extracted from mice, and their DPP IV activities were measured. As shown in Fig. 2, serum DPP IV activity in the control group was significantly higher than that in the cy-dg and aronia groups. Inhibition of DPP IV activity was observed in serum of KKAy mice given aronia juice and cyanidin 3,5-diglucoside, and their activities were about 38% and 74%, respectively, of those in the control group.

3.3. Active GLP-level in serum

Forty-nine days after the start of administration, serum was extracted from the mice, and active GLP-1 levels were measured. As shown in Fig. 3, serum GLP-1 levels in the cy-dg and aronia groups were significantly increased compared to that in the control group. Increased active GLP-1 levels in the serum from KKAy mice given aronia juice and cyanidin 3,5-diglucoside were about 10-fold and 6.4-fold of those in the control group, respectively.

3.4. Weights of livers and white adipose tissues from KKAy mice administered cyanidin 3,5-diglucoside

Forty-nine days after the start of administration, livers and white adipose tissues were extracted from the mice and weighed. As shown in Fig. 4, weights of livers and white adipose tissues from KKAy mice given aronia juice but not cyanidin 3,5-diglucoside were significantly decreased compared to that in the control group.

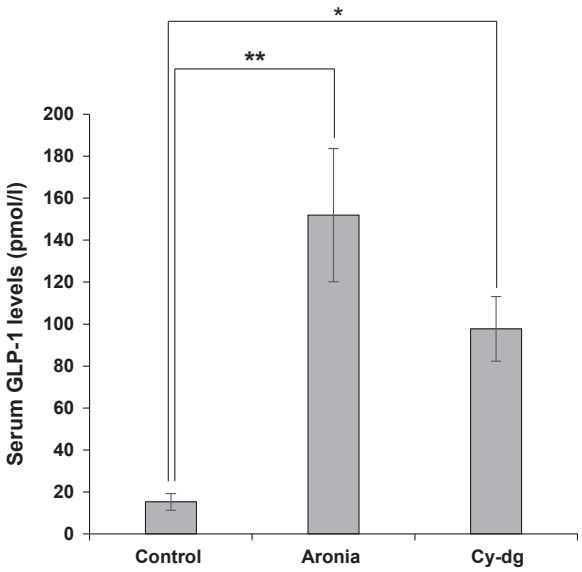

Fig. 3. Active GLP-1 levels in the serum. Protein level of active GLP-1 was examined by ELISA in the serum. *$p < 0.05$, **$p < 0.01$, n = 5.

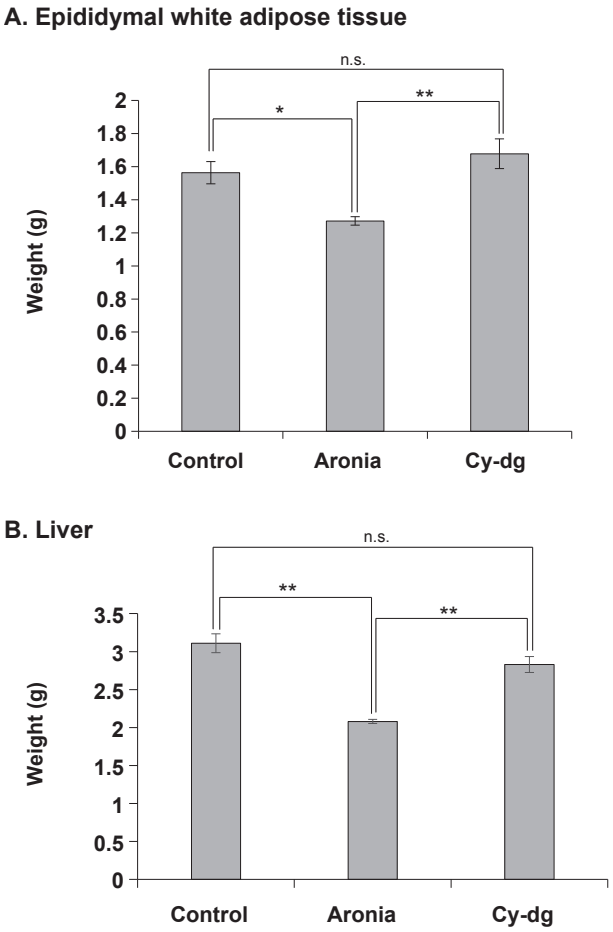

Fig. 4. Reduction of tissue weighs in KKAy mice administered aronia juice but not cyanidin-3,5-diglucoside. Water, cyanidin-3,5-diglucoside or aronia juice was administered orally to mice. After 49 days, epididymal white adipose tissue (A) and the liver were obtained and weighed. *$p < 0.05$, n.s.: not significant. n = 5.

3.5. Lipid accumulation in the livers from KKAy mice fed cyanidin 3,5-diglucoside

Forty-nine days after the start of administration, livers were extracted from the mice, and triglyceride levels were measured. As shown in Fig. 5, triglyceride levels in the livers from KKAy mice given aronia juice and cyanidin 3,5-diglucoside were about 50% and 95% of those in the control group, respectively.

4. Discussion

The present study revealed that blood glucose and HbA1c levels were reduced by giving aronia juice or cyanidin 3,5-diglucoside. However, the magnitudes of reduction in blood glucose and HbA1c by administration of cyanidin 3,5-diglucoside were about 50% of those by giving aronia juice in KKAy mice. In our previous study, blood glucose levels in KKAy mice given aronia juice were reduced by inhibiting DPP IV and α-glucosidase activities in the small intestine but not in serum (Yamane et al., 2016).

Interestingly, serum DPP IV activity was inhibited in KKAy mice given aronia juice and cyanidin 3,5-diglucoside for 49 days, and the rate of inhibition of DPP IV activity by giving cyanidin 3,5-diglucoside was about 50% of that by giving aronia juice. The increase in the level of active GLP-1 in serum from KKAy mice by giving cyanidin 3,5-diglucoside was also about 64% of that by giving aronia juice.

Although the reduced blood glucose and HbA1c levels in KKAy mice

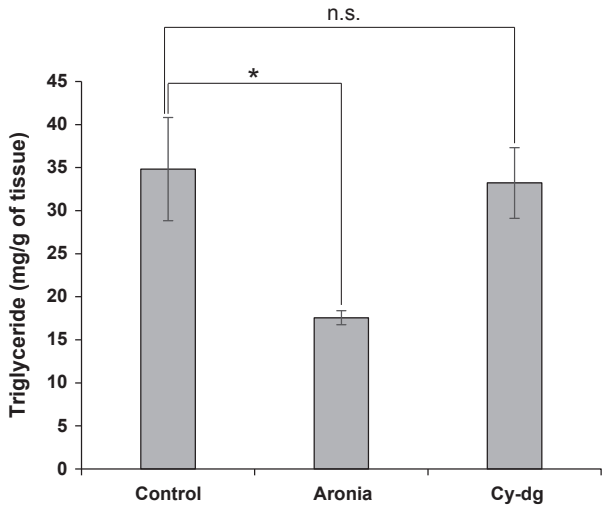

Fig. 5. Changes in the liver triglyceride levels in KKAy mice administered aronia juice but not cyanidin-3,5-diglucoside. Triglyceride levels of the liver in control and aronia juice or cyanidin-3,5-diglucoside-administered mice were measured. *$p < 0.05$, n.s.: not significant. n = 5.

Table 2
Intake contents of cyanidin 3,5-diglucoside.

Groups	Total intake (mL)	Cyanidin 3,5-diglucoside (mg)
Aronia	470 ± 21	0.07 ± 0.01
Cy-dg	578 ± 9	5.78 ± 0.01

All data is presented as mean ± standard error of five animals.

given aronia juice began to increase slightly from the 21st day, those in KKAy mice given cyanidin 3,5-diglucoside sharply increased from the same day. These different elevation curves of blood glucose and HbA1c levels suggest that other compounds such as α-glucosidase inhibitors exist in aronia juice. These results also indicate that aronia juice and cyanidin 3,5-diglucoside are highly effective in preventing type-2 diabetes.

Furthermore, DPP IV inhibitory activity and increased active GLP-1 level in serum from KKAy mice given cyanidin 3,5-diglucoside were about 50% of those in serum from KKAy mice given aronia juice. Intake of cyanidin 3,5-diglucoside was also low in aronia group in comparison with cy-dg group (Table 2). These results indicate that other DPP IV inhibitors exist in aronia juice. Since aronia juice contained at least cyanidin 3-O-glucoside, cyanidin 3-O-galactoside and cyanidin 3-O-arabinoside, these anthocyanins inhibited DPP IV activity with cyanidin 3,5-diglucoside.

It has been reported that many polyphenols are included in aronia berries (Xie et al., 2016). Activities of proteases such as DPP IV and angiotensin-converting enzyme were inhibited by these polyphenols from plants including berries (Yamane, 2018).

DPP IV activity was inhibited by several polyphenols such as cyanidin 3-O-glucoside, quercetin and cyanidin (Yamane, 2018). DPP IV inhibitory activity of cyanidin 3-O-glucoside was higher than that of cyanidin (Kalhotra, Chittepu, Osorio-Revilla, & Gallardo-Velázquez, 2018). DPP IV activity was inhibited by cyanidin 3,5-diglucoside more than by cyanidin 3-O-glucoside (Kozuka et al., 2015). On the other hand, α-glucosidase activity was also inhibited by giving aronia juice (Yamane et al., 2016), and polyphenols such as anthocyanins have an inhibitory effect on α-glucosidase activity (McDougall & Stewart, 2005). Since these polyphenols are included in aronia juice, reduction of blood glucose and HbA1c levels by giving aronia juice may occur through inhibition of DPP IV and α-glucosidase activities by the combination of cyanidin 3,5-diglucoside and these polyphenols.

On the other hand, weights of the livers and white adipose tissues from KKAy mice given aronia juice, but not those from KKAy mice given cyanidin 3,5-diglucoside, were reduced. Furthermore, the triglyceride level in the liver was reduced by giving aronia juice but not by giving cyanidin 3,5-diglucoside. Since a reduction of white adipose tissue by giving aronia juice has been reported (Yamane et al., 2016), another compound(s) including aronia juice may have an effect on white adipose tissue. These results suggest that cyanidin 3,5-diglucoside, included in aronia juice, has a beneficial effect on diabetes but not an obesity.

In a further study, it is necessary to identify anti-diabetic compounds other than cyanidin 3,5-diglucoside in aronia juice and to examine therapeutic effects of aronia juice on type-2 diabetes, including association with islet cells and insulin resistance. Identification of anti-obesity compounds in aronia juice is also important.

Conflict of interest

None.

Author contributions

Conceived and designed the experiments: T.Y., T.S., T.I., H.I., T.N., and Y.N. Performed the experiments: T.Y., M.I., S.H., K.Y. and T.I. Analyzed the data: T.Y. Wrote the paper: T.Y.

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