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Apple juice relieves loperamide-induced constipation in rats by downregulating intestinal apical sodium-dependent bile acid transporter ASBT

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Abstract

Apples are known to exhibit various beneficial effects on human health. In the present study, we investigated the effect of continuous intake of apple juice (AJ) on constipation status. A single dose of loperamide in rats as the constipation model markedly decreased the weight and number of fecal pellets compared to saline-administered rats as a control. After the administration of AJ twice a day for seven days, recovery of defecation close to that of the control was observed in loperamide-treated rats. In addition, the total bile acid content in the feces increased from day 4 after the administration of AJ. Among hepatic and intestinal transporters and enzymes that regulate bile acids, the mRNA and protein expression of apical sodium-dependent bile acid transporter (asbt, *slc10a2*) was decreased by AJ in rats. Furthermore, the asbt-mediated bile acid transport activity in the rat ileum decreased after AJ administration. Moreover, in human colonic cancer-derived Caco-2 cells, AJ exposure for 24 and 48 h decreased the expression of ASBT mRNA, protein, and uptake activity of taurocholic acid in both 7- and 21-d cultures. Several components of AJ, such as procyanidins, decreased the expression of ASBT in Caco-2 cells. In conclusion, ASBT downregulation is a possible mechanism responsible for the constipation-relieving effect of apples, and procyanidins may play a role in downregulating ASBT, which leads to beneficial effects of apples against constipation. Although it is generally agreed that the common dietary compositions play a role in constipation relief, the novel specific mechanism of apples found in this study would facilitate understanding food functions.

1. Introduction

Apple is a globally popular fruit that contains polyphenols, vitamins, minerals, and dietary fibers. Apples exhibit various biological functions: intestinal transporter OATP2B1, which facilitates drug absorption such as fexofenadine is susceptible to apple juice (AJ), causing drug-food interaction[1,2]; apples also change the levels of various biomarkers in plasma: lowering low-density lipoprotein cholesterol[3], lowering plasma triglyceride[4], and improving insulin[5], showing health benefits, including lowering the risk of stroke[6], relieving constipation[7], and others[8,9]. Among the various benefits, a relieving constipation has long been known.

Besides foods such as apples, laxatives, including stool softeners, osmotic laxatives, and stimulant laxatives, have been used for treating constipation. In 2018, GOOFICE® tablet was successfully approved as a new drug for chronic idiopathic constipation in Japan[10]. The active pharmaceutical ingredient (API) of GOOFICE® is elobixibat, which was developed as a selective intestinal bile acid transporter inhibitor that promotes spontaneous bowel movement and secretion of water into the gut lumen by increasing bile acid (BAs) content in the gut lumen.

BAs are important biological detergents produced from cholesterol in hepatocytes and are secreted into the lumen of the small intestine to facilitate the dissolution of lipids to be absorbed. The BAs are then reabsorbed from the intestine and back to the liver, while less than 10% of BAs are excreted into feces, escaping enterohepatic circulation. A series of transporters and enzymes are involved in enterohepatic circulation, among which the intestinal apical sodium-dependent bile acid transporter ASBT (*SLC10A2*, also known as iBAT, ileal bile acid transporter) is responsible for the reabsorption of BAs in the small intestine and plays a pivotal role in enterohepatic circulation to maintain homeostasis of BAs. Owing to the multiple physiological functions of BAs, including solubilizing dietary lipids[11] and regulating cholesterol and glucose by combining several hepatic and intestinal receptors, such as TGR5 and FXR[12], ASBT dysfunction results in multiple diseases. An increase in ASBT activity may lead to progressive familial intrahepatic cholestasis[13], necrotizing enterocolitis[14], and diabetes mellitus[15]. A decrease in ASBT activity results in colonic bile acid accumulation and diarrhea[16]. Thus, ASBT has been receiving increasing attention as a potential drug target in recent years.

Depression of ASBT shows similar alterations in these biomarkers in apples: decreased low-density lipoprotein cholesterol[17], decreased plasma triglyceride[18], and increased insulin[18]. Considering the high consistency between apple intake and ASBT depression and our previous finding of decreased expression of ASBT mRNA in Caco-2 cells after exposure to apple-derived small extracellular vesicles[19], we hypothesized that apples relieve constipation by altering the bile acid disposition caused by downregulation of ASBT. The present study aimed to clarify the mechanism underlying the beneficial effects of apples on constipation. We demonstrated that AJ could relieve loperamide-induced constipation by increasing fecal BAs. We then investigated the alteration of ASBT expression and ASBT-mediated transport activity in rats and Caco-2 cells. Finally, we studied the components of AJ that can depress ASBT expression.

2. Materials and Methods

2.1 Materials

The apples (Sun Fuji) were harvested from Sawaguchi Farm (Iwate, Japan). Caco-2 cells were purchased from the RIKEN Cell Bank (Tsukuba, Japan). Fluorescent bile acid, tauro-nor-THCA-24-DBD(N-(24-[7-(4-N,N-dimethylaminosulfonyl-2,1,3-benzoxadiazole)]amino-3α,7α,12α-trihydroxy-27-nor-5β-cholestan-26-oyl)-2'-aminoethanesulfonate), was purchased from GenoMembrane Co., Ltd. (Yokohama, Japan).

[^{3}H]Taurocholic acid ([^{3}H]TCA, specific activity 20 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Elobixibat was extracted using dimethylformamide from GOOFICE® tablets from EA Pharma Co., Ltd. (Tokyo, Japan). RNAiso Plus®, M-MLV reverse transcriptase, and SYBR® green qPCR master mix were obtained from Takara Bio Inc. (Shiga, Japan), Promega Corporation (Tokyo, Japan), and Agilent Technologies Japan Ltd. (Tokyo, Japan), respectively. Anti-*SLC10A2* (GTX03115) and anti-GAPDH (60004-1-Ig) antibodies were purchased from GeneTex (Irvine, CA, USA) and Cell Signaling Technology (Danvers, MA, USA), respectively. All other chemicals and reagents of the highest commercially available purity or reagent grade were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), Nacalai Tesque Inc. (Kyoto, Japan), Cayman Chemical (Ann Arbor, MI, USA), and Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan).

2.2 Preparation of apple juice

Whole apples (containing skin and core) were ground with a plastic grater, and the obtained juice was centrifuged at 2,000 x *g* for 20 min at 4 °C to exclude debris. The supernatant was further centrifuged at 13,000 x *g* for 70 min at 4 °C and the supernatant was collected as AJ.

2.3 Cell culture

Caco-2 cells were used at passage numbers between 15 and 35, and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.1 mM nonessential amino acids, antibiotics benzylpenicillin (100 U/mL) and streptomycin (100 μg/mL). The cells were seeded on collagen-coated plates and cultured for 7 or 21 d in a humidified incubator (5% CO_2, 37 °C).

2.4 AJ treatment and preparation of constipation model in rats

Male Wistar rats (6–8 weeks) were purchased from Sankyo Labo Service Corp., Inc. (Toyama, Japan). All animals were allowed free access to food and water under a standard 12 h light/12 h dark cycle in a temperature-controlled (23 ± 1 °C) and humidity-controlled (55 ± 5%) room until use. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Kanazawa University and experiments were approved by the Animal Ethics Committee of Kanazawa University (Permit No. AP-204199).

AJ (or saline as control) was administrated by oral gavaging at a dose of 10 mL/kg every 12 h for seven consecutive days, and loperamide or saline as control was administered intraperitoneally at a concentration of 0.3 mg/kg once at 1 h after the last administration of AJ. Feces were collected daily during AJ administration and 12 h after loperamide injection. The wet weight was measured, and water content was calculated by subtracting the dry weight measured after vacuum drying. Ethanol was then added to extract BAs. The supernatant obtained after centrifugation of the feces at 21,600 x *g* for 15 min at 4 °C was dried under reduced pressure. The concentration of BAs in the supernatant was measured using the total bile acid assay kit following the manufacturer's instructions (Diazyme Laboratories Inc., Poway, CA, USA).

For tissue collection of intestine and liver, rats were anesthetized by intraperitoneal injection of a triple anesthetic combination (medetomidine, midazolam and butorphanol) after continuous AJ administration for 7 d. The liver and intestine were washed with ice-cold phosphate-buffered saline (PBS) to remove blood and intestinal contents and collected for further gene and protein expression analyses.

2.5 *In situ* intestinal closed-loop method

The asbt activity in the intestine was quantified using an *in situ* closed loop of the ileum in rats, as described previously[20]. After free access to AJ for four days, the 24-h fasted rats were

anesthetized, and the intestine was exposed. Care was taken to avoid affecting the intestinal blood supply. A 10-cm loop was made at the end of the ileum segment by cannulating the tube into incisions at both ends. Warmed saline and air were flushed alternately to remove the intestinal contents. PBS (pH 6.5, 1 mL) containing tauro-nor-THCA-24-DBD (2 μM) in the presence and absence of elobixibat (10 nM) were pushed into the loop and followed by tightening the ends of loop immediately. The intestinal solution was collected after 20 min by washing the intestine with a warmed mobile phase used for high-performance liquid chromatography (HPLC) analysis. Finally, intestinal tissues were isolated immediately to evaluate ASBT mRNA expression.

The entire luminal solution was collected and centrifuged at 3,000 x *g* for 15 min at 4 °C, and the resultant supernatant was used to quantify remaining tauro-nor-THCA-24-DBD using HPLC analysis. The quantification was performed as described previously[21]. To estimate the ASBT activity of the ileum, the apparent permeability coefficient (P*app*, cm/s) was calculated using the following equation:

$$P_{app} = (k_a \times V_d)/2\pi rl$$

where k_a is the first-order absorption rate constant of tauro-THCA-24-DBD estimated from its disappearance rate during 20 min, V_d is the volume of tauro-THCA-24-DBD solution added to the closed loop (1 mL), and r and l are the radius (0.178 cm, reported by Fagerholm et al.[22]) and length (10 cm) of the ileum segment, respectively. Permeability was measured in the presence and absence of elobixibat, and elobixibat-sensitive permeation was regarded as ASBT-mediated permeability.

2.6 Gene expression analysis using quantitative real-time reverse transcription-PCR (qRT-PCR)

For mRNA determination, total RNA was extracted from the rat intestine, rat liver, and Caco-2 cells using RNAiso Plus® and reverse-transcribed to complementary DNA using random primers and M-MLV reverse transcriptase. The obtained cDNA was used to perform quantitative real-time PCR to detect the BA-related gene expression using SYBR® Green qPCR Master Mix and the corresponding primers listed in Table 1 on an AriaMx Real-Time PCR system (Agilent Technologies, Inc.), using HPRT as an endogenous control.

2.7 Western blotting

Total protein was extracted from Caco-2 cells using M-PER™ mammalian protein extraction reagent (Thermo Fisher Scientific Inc., Rockford, IL, USA) containing a 1% protease inhibitor cocktail. After determining the protein concentration using a bicinchoninic acid assay kit (FUJIFILM Wako Pure Chemical Corporation), equal amounts of protein were loaded into the wells of a 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis gel, along with a marker for electrophoresis, and then transferred to a polyvinylidene fluoride membrane on a Mini Trans-Blot® Cell (Bio-Rad Laboratories Inc., Hercules, CA, USA). The membrane was blocked with 2% skimmed milk and then was incubated with anti-*SLC10A2* antibody at 4 °C overnight following incubation with goat anti-rabbit IgG antibody at room temperature for 2 h. Protein bands were detected using Immunostaining Zeta on LAS-4000 (FujiFilm Co., Ltd., Tokyo, Japan). Similarly, anti-GAPDH and goat anti-mouse IgG antibodies were used as endogenous controls for GAPDH detection.

2.8 Uptake studies in Caco-2 cells

The uptake study was performed as described previously[21]. The 7- or 21-d cultured Caco-2 cells were incubated at 37 °C for 5 min in an uptake buffer (110 mM NaCl, 4 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM HEPES, and 50 mM D-mannitol, pH 7.4, adjusted with NaOH) containing 1 μM [^{3}H]TCA with or without elobixibat after being washed twice with 250 μL/well of prewarmed uptake buffer, and the uptake reaction was terminated by washing the cells with ice-cold uptake buffer. Protein content was measured to calculate transport activity using Bio-Rad protein assay reagent (Bio-Rad Laboratories Inc.).

2.9 Statistical analysis

Data are expressed as the mean values obtained from at least three experiments with the S.E.M. Statistical analyses were performed using Student's *t*-test and results were considered statistically significant when *p* value was less than 0.05 ($p < 0.05$).

3. Results

3.1 Effect of apple juice administration on defecation and fecal bile acids in rats

To study the effects of AJ on constipation, loperamide was used to induce constipation. Figure 1 shows the effects of loperamide on the number of pellets (A), wet weight (B) and water content (C) of feces, respectively. Loperamide markedly decreased the number of pellets, wet weight and water content of feces compared to the control, demonstrating the establishment of a constipation model. Under physiological conditions (in the absence of loperamide), AJ did not affect defecation. However, under constipation conditions (with loperamide), AJ caused recovery of the loperamide-induced decrease in all the pellet number, wet weight and water content of feces, suggesting that AJ relieves constipation in rats.

As increased fecal BAs can strengthen intestinal peristalsis, we monitored the alteration of fecal BAs after AJ administration. AJ was administered every 12 h for a week, and feces were collected every 24 h. Figure 2 shows the BAs contained in feces over 7 d. AJ administration increased the fecal BAs and the difference between AJ administration and control groups became greater with time and significant difference was observed after day 4 (control group: 0.74 ± 0.04 μmol/d; AJ group: 2.34 ± 0.56 μmol/d, 3.2 folds of control group). These results suggest that AJ facilitates fecal excretion by increasing the amount of fecal BAs.

3.2 Fecal bile acids promoting effect of apple juice by downregulating asbt expression in rats

Since the effect of AJ administration on fecal BAs was observed gradually, the change of expression of any factors that affect intestinal disposition of BAs was considered. We quantified mRNA expression of genes involved in BA homeostasis. As shown in Fig. 3A, AJ administration did not significantly change the expression of genes in the liver, indicating that AJ does not affect the hepatic transporters and enzymes responsible for BA handling. As shown in Fig. 3B, the expression of the genes in the ileum were not significantly changed by AJ administration, except for asbt. AJ downregulated the expression of asbt to 40% of that of the control, suggesting that an increase in fecal BAs by AJ resulted from decreased reabsorption of BAs by decreased expression of asbt.

3.3 Effect of apple juice on asbt expression and function in rats

The asbt-mediated ileal transport capability of BAs was measured to confirm that the reduction of asbt mRNA expression caused by AJ is associated with BA reabsorption activity. Figure 4 shows the change in asbt mRNA expression and intestinal membrane permeability of BAs after 4-d AJ feeding. When AJ was administered, the asbt mRNA expression (Fig. 4A) decreased to 51% of that of the control in a manner similar to that observed in Fig. 3B. The ileal membrane permeability of BAs was evaluated by *in situ* closed-loop method using tauro-nor-THCA-24-DBD, a fluorescence analog of taurocholic acid, and the ASBT selective inhibitor elobixibat[21]. As shown in Fig. 4B, AJ administration significantly decreased the asbt-mediated transport of tauro-nor-THCA-24-DBD to 73% of that in the control. These results suggest that increased fecal BA is explained by the impaired reabsorption of BAs by downregulation of asbt by AJ. Accordingly, the constipation-

relieving effect of apples could be explained by the increased BAs in the intestinal lumen due to the reduction of asbt expression.

3.4 Effect of apple juice on ASBT expression and transport activity in Caco-2 cells

To investigate whether AJ can relieve constipation by downregulating ASBT in humans, Caco-2 cells were used[15, 23, 24]. Seven-d cultured Caco-2 cells, a model for developing intestinal cells, and 21-d cultured Caco-2 cells, a model for human intestinal epithelium[25-28], were used.

Figures 5A-C show the results for 7-d cultured Caco-2 cells. AJ exposure for 24 and 48 h reduced ASBT mRNA expression to 51.2% and 28.3%, respectively (Fig. 5A) and protein levels to 47.8% and 45.1%, respectively (Fig. 5B). Correspondingly, the ASBT-mediated transport activity of [^{3}H]TCA decreased to 62.9% and 37.6% after 24- and 48-h, respectively (Fig. 5C). The results for the 21-d cultured Caco-2 cells are shown in Fig. 5D–F. AJ exposure for 24 and 48 h significantly reduced ASBT mRNA expression to 61.8% and 40.0%, respectively (Fig. 5D) and protein to 81.1% and 57.6%, respectively (Fig. 5E). Correspondingly, the ASBT-mediated transport activity of [^{3}H]TCA decreased to 76.7% and 63.5% of the control after 24- or 48-h AJ exposure, respectively (Fig. 5F). Similar results in a human intestinal epithelium model and developing intestine cell model with rats indicate that AJ could relieve constipation by downregulating ASBT expression in humans.

3.5 Contribution of procyanidins to the downregulation of ASBT in Caco-2 cells

Furthermore, we assessed if the apple components can decrease ASBT expression in 21-d cultured Caco-2 cells. Eight polyphenols, chlorogenic acid, phloridzin, quercetin, kaempferol, catechin, (-)-epicatechin, procyanidin B1, and procyanidin B2, were used. Exposure to 50 μM of each compound for 48 h resulted in ASBT mRNA expression of 101.8%, 116.2%, 90.2%, 96.4%, 72.1%, 70.6%, 73.7%, and 72.3% of the control, respectively (Fig. 6). Procyanidins (catechin, (-)-epicatechin, procyanidin B1, and procyanidin B2) tended to decrease ASBT expression, with (-)-epicatechin showing a statistically significant decrease. When we further examined the effect of a mixture of four procyanidins (12.5 μM each as low, 50 μM each as high), they repressed ASBT mRNA expression to 66.3% and 54.1% of control, respectively. In addition, IC_{50} values of AJ, (-)-epicatechin and procyanidin B2 on ASBT mRNA expression were evaluated in 21-d cultured Caco-2 cells. Concentration dependent inhibitory curves are showed in Supplemental Fig. 1, and IC_{50} values were estimated to 67.3 ± 29.7 μM, 67.0 ± 35.2 μM and 5.9 ± 1.9 %, respectively. These results suggest that procyanidins in apples contribute to decreased ASBT expression.

4. Discussion

Constipation is one of common gastrointestinal disorders with the number of patients more than 15% of population worldwide[29]. In constipation, the quality of life of patients declines due to symptoms such as sensation of incomplete evacuation and hard stools, in addition to reduction of bowel movement frequency. Compared with medical treatment, lifestyle modifications, such as exercise, increasing fluid intake, and increasing helpful food intake, are more acceptable, especially for the elderly and children. Apple is typically regarded as food good for alleviating constipation. In this study, ASBT downregulation was identified as a novel mechanism responsible for the constipation-relieving effects of apples.

In this study, the constipation-relieving effects of apples were first confirmed in rats (Fig. 1). As defecation is influenced by factors other than the intestinal condition, the body weight and amount of food intake were monitored, and no significant differences were observed between the AJ and control groups, indicating that AJ relieved constipation mainly by improving the intestinal condition. Many mechanisms reported to be involved in this

process, such as effect of fibers, are not specific to AJ[7]. Therefore, we attempted to identify a specific mechanism of AJ for the defecation effect. Recently, the regulation of luminal BAs is becoming a novel treatment strategy for chronic constipation[30] since BAs regulate colonic motility[31] and water secretion[32], and elobixibat was recently approved as a novel drug for treating chronic constipation by inhibiting the intestinal BA reabsorption transporter ASBT[33]. Accordingly, in the present study, we focused on altered bile acid enterohepatic circulation and investigated whether continuous AJ administration increases fecal BAs levels (Fig. 2). As intestinal BAs are regulated by various transporters and enzymes in the liver and small intestine, we analyzed the expression of BA-related genes (Fig. 3). BA homeostasis is regulated by enterohepatic circulation, where BAs circulate between the liver and the small intestine. In the liver, BAs are synthesized by cytochrome P450 family 7 subfamily A member 1 (Cyp7a1, encoded by *Cyp7a1*) and effluxed into bile by a bile salt export pump (Bsep, encoded by *Abcb11*) and multidrug resistance-associated protein 2 (Mrp2, encoded by *Abcc2*). In the ileum, BAs are reabsorbed into epithelial cells by an apical sodium-dependent bile acid transporter (Asbt, encoded by *Slc10a2*), binding to ileal bile acid-binding protein (Ibabp, encoded by *Fabp6*) to be transported to the basal side of intestinal cells. In rats, BAs are effluxed out by the organic solute transporter alpha/beta (Ostα/β, encoded by *Slc51a/Slc51b*) and multidrug resistance-associated protein 3 (Mrp3, encoded by *Abcc3*) into the portal vein and taken up by hepatocytes mainly by sodium taurocholate cotransporting polypeptide (Ntcp, encoded by *Slc10a1*), and by sodium-independent organic anion transporting polypeptides (Oatps), including Oatp1a1, encoded by *Slco1a1*, Oatp1a4, encoded by *Slco1a4*, and Oatp1b2, encoded by *Slco1b2*. A significant decrease in the mRNA expression of asbt by AJ in the rat ileum was observed (Fig. 3B). ASBT is responsible for the ileal reabsorption of BAs, and intestinal BA permeability was evaluated using the *in situ* closed-loop method (Fig. 4B). Since administration of AJ for 4 d was sufficient to increase the fecal BAs amount (Fig. 2), ileal BAs transport was measured after 4 d of AJ administration. Here, tauro-nor-THCA-24-DBD and elobixibat were used. Tauro-nor-THCA-24-DBD, has been previously confirmed as a useful fluorescent BA analog for ASBT evaluation[21] and was used to avoid contamination of endogenous BAs for evaluation; elobixibat was used to measure asbt-specific permeation in order to avoid any nonspecific permeation that could be observed as an artifact of experimental method used. To minimize the technical influence of repeated administration of AJ by gastric tubes, AJ was fed by free access. Using this procedure, AJ administration decreased asbt mRNA expression to 51% of the control (Fig. 4A), a little weaker than 7-d administration (40% of control, Fig. 3B), and ileal BAs transport activity decreased to 73% of the control, indicating that AJ decreased luminal BAs absorption by decreasing asbt expression. These results show that repeated doses of AJ suppress asbt expression, resulting in decreased reabsorption of luminal BAs and promoting defecation in a luminal BAs-dependent manner.

Reduced ASBT expression and BA transport activity caused by AJ were examined in human-derived intestinal Caco-2 cells (Fig. 5). Considering the effect of cell status on the regulation of transporter expression, we examined both pre- and post-differentiated Caco-2 cells. Although the effect of AJ on ASBT expression in pre-differentiated cells was more noticeable than in post-differentiated cells, both exhibited essentially similar responses to AJ in downregulating ASBT expression. Differences in sensitivity to AJ may be due to lower absorption of active AJ components caused by the difference in the development of tight junctions, transporter/enzyme expression, and others[34]. Since the self-renewal of the intestinal epithelium takes approximately 5 d[35], continuous AJ drinking for over 5 d is recommended to relieve constipation. The downregulation of ASBT by AJ is expected to be more stable and potent with time, and long-term AJ administration should be better in preventing constipation clinically.

Finally, we investigated the active components of AJ that downregulate ASBT. Since polyphenols are well-known active ingredients in apples, concentration of eight typical polyphenols reported in previous studies[36, 37] are summarized (Supplemental Tab. 1) and the effect of eight typical polyphenols in AJ on ASBT expression was examined (Fig. 6). Among them, catechin, (-)-epicatechin, procyanidin B1, and procyanidin B2 tended to decrease ASBT expression, whereas (-)-epicatechin changed it significantly. These four are

procyanidins, and grape seed procyanidins extract was reported to suppress the expression of ASBT in human Caco-2 cells and in mice[38]. Furthermore, the mixture of these four procyanidins decreased ASBT expression in a concentration-dependent manner, and procyanidins in apples are thought to be responsible for suppressing ASBT mRNA expression. Moreover, IC_{50} was estimated to 5.9 ± 1.9 % (Supplemental Fig. 1). To understand further about the inhibitory effect of procyanidins, (-)-epicatechin, which showed significantly strong inhibition on ASBT among the four, and procyanidin B2, which is contained most abundantly in AJ (Supplemental Tab. 1) were selected and their IC_{50} values were 67.3 ± 29.7 μM, 67.0 ± 35.2 μM, respectively (Supplemental Fig. 1). All these results suggest that AJ components downregulate the expression of ASBT, leading to increased intestinal luminal BAs and promoting defecation by the action of BAs through mechanisms of facilitation of gut motility and an increase in the water content in the lumen.

The results of the present study suggest that the downregulation of ASBT by AJ is a mechanism by which apples ameliorate constipation. The nonspecific effects of sugar alcohols (represented by sorbitol) and soluble fibers (represented by pectin) can relieve constipation. Sorbitol retains water in the large intestine through osmotic pressure to stimulate intestinal peristalsis and exert its laxative effect[39]. Changed intestinal osmotic pressure by sugar alcohols decrease concentration of luminal sodium that drives ASBT transport activity. It may impress ASBT activity, but not ASBT expression. Pectin escapes degradation by gastric acid and intestinal enzymes and is fermented by gut microbiota into short-chain fatty acids to modify peristalsis movement in the colon[40]. However, pectin does not change the mRNA expression of ileal ASBT expression in mice[41]. Therefore, the downregulation of ASBT is considered a distinctly specific novel mechanism of the constipation-relieving effect of apples.

Moreover, four typical procyanidins in apples were found to downregulate ASBT expression. As for the effective composition of apples on constipation relief, fiber is well-known. However, its side effect has been also reported that fibers do not improve the treatment success[42] and FODMAPS (fermentable oligosaccharides, disaccharides, monosaccharides and polyols, a kind of high-fiber food) worsen constipation-type irritable bowel syndrome[43]. The finding that apple-derived procyanidins can downregulate ASBT shows that using these natural products is potential to relieve constipation, giving the possibility to improve the constipation patients' quality of life, especially for constipation-type IBS. On the other hand, the IC_{50} values of (-)-epicatechin, procyanidin B2 and AJ were 67.3 ± 29.7 μ M, 67.0 ± 35.2 μM and 5.9 ± 1.9 %, respectively (Supplemental fig. 1), while 7.4 μM (-)-epicatechin and 4.3 μM procyanidin B2 in 5.9% juice are contained (Supplemental Tab. 1), indicating that contribution of these components to constipation relieving effect of apples is possible. In addition, contribution of other components cannot be excluded. Besides small molecules, large molecules such as apple-derived microRNAs may also contribute to decreased expression of ASBT, since small extracellular vesicles from apples were recently reported to downregulate several intestinal transporters including ASBT in Caco-2 cells[19] and one of intestinal transporters, OATP2B1, is downregulated by specific apple microRNAs contained in apple-derived small extracellular vesicles[44]. We are now investigating the effect of apple-derived small extracellular vesicles on ASBT expression.

About the alteration of intestinal luminal BAs, in addition to these gene-regulating effects in apples, the direct effect of AJ components on ASBT transport activity may also be involved because AJ inhibited the uptake of taurocholic acid by Caco-2 cells in an AJ-concentration-dependent manner with an IC_{50} value of 61.9% ± 16.9% AJ (data not shown). As well, changes in the expression of other hepatic and intestinal BA-related genes cannot be completely excluded (Fig. 3). Apart from asbt, ibabp showed a high tendency to decline by AJ with p value of 0.11, followed by mrp3, ostα, and ostβ, whereas genes in the liver showed no differences. Changes in these intestinal transporters might be involved in the decreased reabsorption of BAs. Accordingly, AJ exposure longer than 7 d conducted in the present study may promote synthesis of BAs in the liver and increase hepatic uptake of cholesterol, thereby alleviating hyperlipidemia. In addition, more interesting modifications might occur if the time of AJ intake could be extended further.

Conclusions

The present study demonstrated that the beneficial effect of apples on constipation was due to reduced expression of intestinal BA reabsorption transporter ASBT, which increases intestinal luminal content of BAs, thereby promoting motility of the gut and water content, resulting in the relief of constipation. Several procyanidins contributed to ASBT downregulation. Downregulation of ASBT may explain other beneficial effect of apple intake for health. However, other mechanisms could also be considered to contribute to this effect in parallel, and further studies are needed to completely understand the complicated effects of food on intestinal function.

Author Contributions

Data curation: Zhu, Iwai, Okaguchi; Formal analysis: Zhu, Iwai; Visualization: Zhu; Funding acquisition: Tamai, Zhu; Supervision: Tamai, Shirasaka; Writing (original draft): Zhu; Writing (review and editing): Tamai, Shirasaka.

Conflicts of interest

There are no conflicts to declare.

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519 **Table 1. Primer list for qRT-PCR.**

Gene		Primer Sequence	
		Forward (5'→3')	Reverse (5'→3')
Rat			
Abcb11	Bsep	GCCATTGTGCGAGATCCTAAA	TGCAGGTCCGACCCTCTCT
Abcc2	Mrp2	TTTTGACACAACTCCCACAGG	CAGCGATGCCAAAGAAACAC
Abcc3	Mrp3	GCATTTGTGAGCAGCCAGCC	TCGTCTAAAACCAGGACACGGC
Cyp7a1	Cyp7a1	GAATTGCCGTGTTGGTGAGC	CCCAGGTACGGAATCAACCC
Fabp6	Ibabp	TCAGTTGCCTCTCTGCTGC	CTGCTGGACCTCTGTGATGA
Slc10a1	Ntcp	GGAGACCTTAAGGACAAGGTG	ATGCTGATGGTGCGTCTG
Slc10a2	Asbt	TGGCTACAGCCTTGGTTTCT	GCAAAGACGAGCTGGAAAAC
Slco1a1	Oatp1a1	AACCCTGAAATGTGGTCAGC	TCCTTCTCTCCGAGCATCAT
Slco1a4	Oatp1a4	GCCCTTTGATTGGACTTCTG	AAGGGAAAGCTGGTCAGGAT
Slco1b2	Oatp1b2	TGGACCAATCCTTGGCTTTA	TCCTCCTGTGACCTCTTTGG
Slc51a	Ostα	ATTGGGCTCAGTGGAAATTG	GACCAAAGCAGCAGAACACA
Slc51b	Ostβ	GTTCTGGCAGTCCTGGTGAT	GCCAAGTCTGCCTTCTCTGA
Hprt	Hprt	GACTTTGCTTTCCTTGGTCA	GTCTGGCCTGTATCCAACAC
Human			
SLC10A2	ASBT	TCGACTCTGGGAGCATCGTA	CTTTTTGGGGCCATTTGTGA
HPRT	HPRT	TTCTTTGCTGACCTGCTGGA	CCCCTGTTGACTGGTCATTACA

520

Figure legend

Figure 1. Effect of AJ administration on loperamide-induced constipation in rats.

Rats were orally administered saline as vehicle or apple juice (AJ) at 10 mL/kg every 12 h for 7 d. Loperamide or saline was given intraperitoneally at a dose of 0.3 mg/kg at 1 h after the last administration of saline or AJ. Stool samples were collected at 12 h after loperamide dosing. Pellet number (A),wet weight (B) and water content (C) were measured. Median and quartiles are indicated (n=4–6). *: Significantly different from "AJ - Loperamide -" group by Student t-test (*p<0.05), †: Significantly different from "AJ - Loperamide +" group by Student t-test (†p<0.05)

Figure 2. Effect of AJ administration on fecal bile acids excretion in rats.

Rats were orally administered saline (hollow circle) as vehicle or AJ (dark circle) at 10 mL/kg every 12 h for 7 d. Feces were collected every 24 h and fecal total bile acids concentration was measured. Each result represents the mean ± S.E.M. (n=4–6). *: Significantly different from control group by Student *t*-test (*p<0.05).

Figure 3. Effect of AJ administration on bile acids-related gene expression in liver (A) and ileum (B) of rats.

Rats were orally administered saline as vehicle (slash-filled column presents the average, hollow circle presents each point) or AJ (black column presents the average, dark circle presents each point) at 10 mL/kg every 12 h for 7 d. Ileum and liver were collected 24 h after the last administration. mRNA expression of BA-related genes in liver (A) and ileum (B) was detected. Hprt was used as a housekeeping gene. Each result represents the mean ± S.E.M. (n=10). *: Significantly different from control group by Student *t*-test (*p<0.05).

Figure 4. Effect of AJ administration on the expression and transport activity of ileal asbt in rats.

Rats were allowed free access to water (white column) as vehicle or AJ (black column) for 4 d. (A) Asbt mRNA expression in ileum was detected. (B) Transport activity of asbt was studied by evaluating asbt-mediated permeability of tauro-nor-THCA-24-DBD by in situ closed ileal loop method. Each result represents the mean ± S.E.M. (n=4). *: Significantly different from control group by Student *t*-test (*p<0.05).

Figure 5. Effect of AJ exposure on the expression and transport activity of ASBT in Caco-2 cells.

The mRNA (A, D) and protein (B, E) expression and transport activity (C, F) of ASBT was evaluated in 7- (A–C) and 21-d cultured Caco-2 cells (D–F) after being exposed to water (white column) as vehicle or AJ (black column) for designed time, respectively. Transport activity of ASBT was described as ASBT-mediated uptake of [^{3}H]TCA. Each result represents the mean ± S.E.M. (n=3–4). *: Significantly different from control group by Student *t*-test (*p<0.05).

Figure 6. Effect of apple-contained polyphenols on ASBT expression in Caco-2 cells.

ASBT mRNA expression was evaluated in 21-d cultured Caco-2 cells after being exposed to apple-contained polyphenols for 48 h. Each result represents the mean ± S.E.M. (n=3-4). *: Significantly different from control group by Student *t*-test (*p<0.05).

Text for graphical abstract

The specific effect of apples on constipation is due to reduced expression of ASBT, which increases intestinal BAs, thereby promoting motility of the gut and water content, resulting in the relief of constipation.

Figure 1

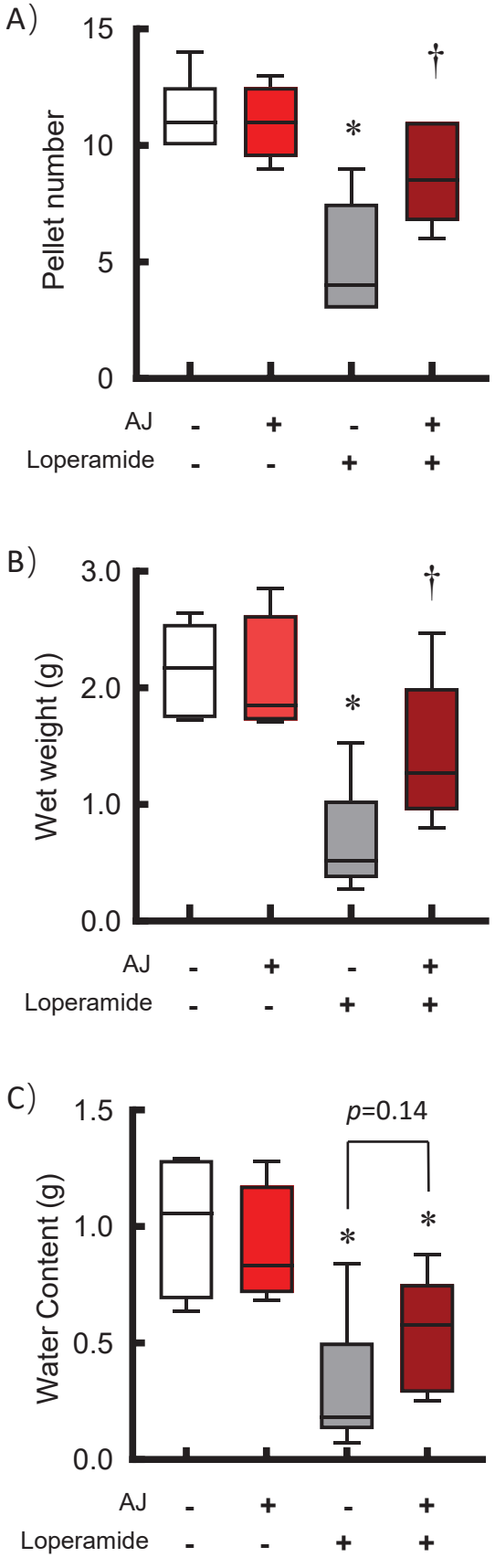

A)
Pellet number
15
10
5
0
*
†
AJ - + - +
Loperamide - - + +
B)
Wet weight (g)
3.0
2.0
1.0
0.0
*
†
AJ - + - +
Loperamide - - + +
C)
Water Content (g)
1.5
1.0
0.5
0.0
p=0.14
*
*
AJ - + - +
Loperamide - - + +

Figure 2

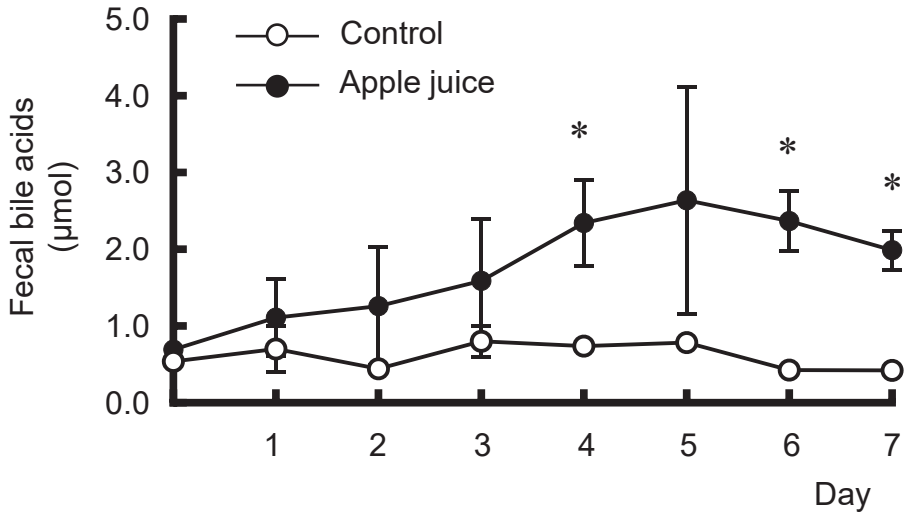

5.0
4.0
3.0
2.0
1.0
0.0
Fecal bile acids (μmol)
Control
Apple juice
*
*
*
1
2
3
4
5
6
7
Day

Figure 3

A) Liver

B) Ileum

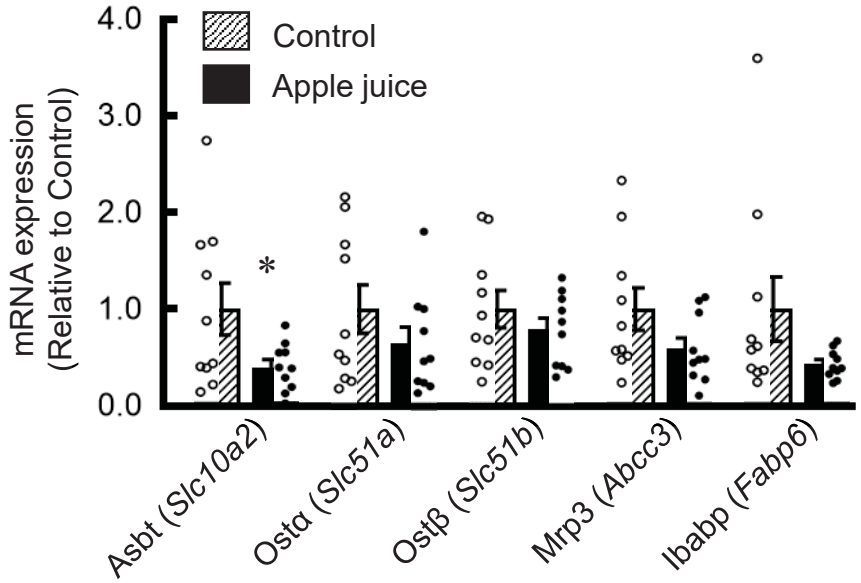


Figure 4

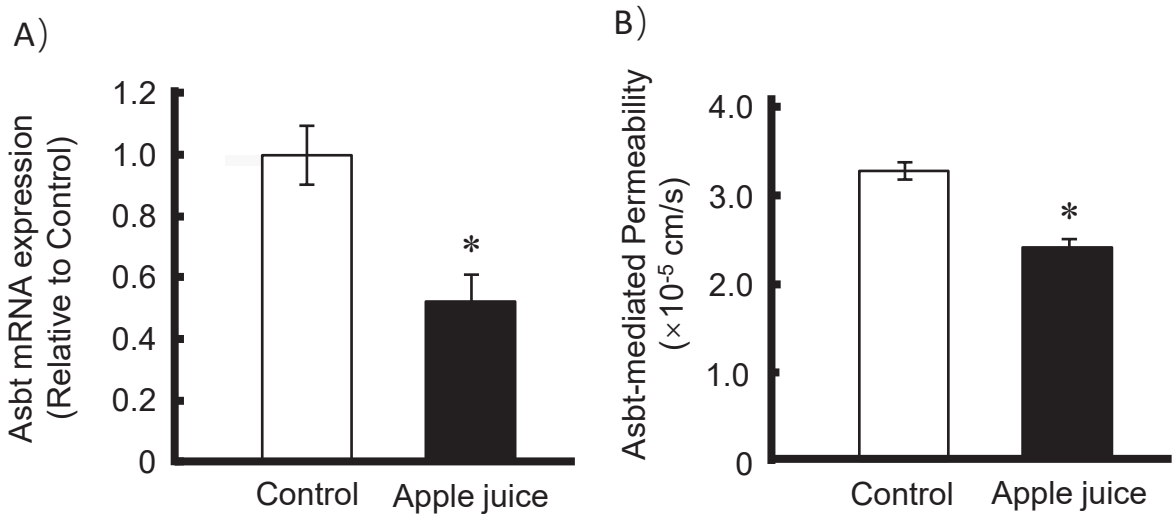

A)
Asbt mRNA expression
(Relative to Control)
1.2
1.0
0.8
0.6
0.4
0.2
0
*
Control
Apple juice
B)
Asbt-mediated Permeability
(×10-5 cm/s)
4.0
3.0
2.0
1.0
0
*
Control
Apple juice

Figure 5

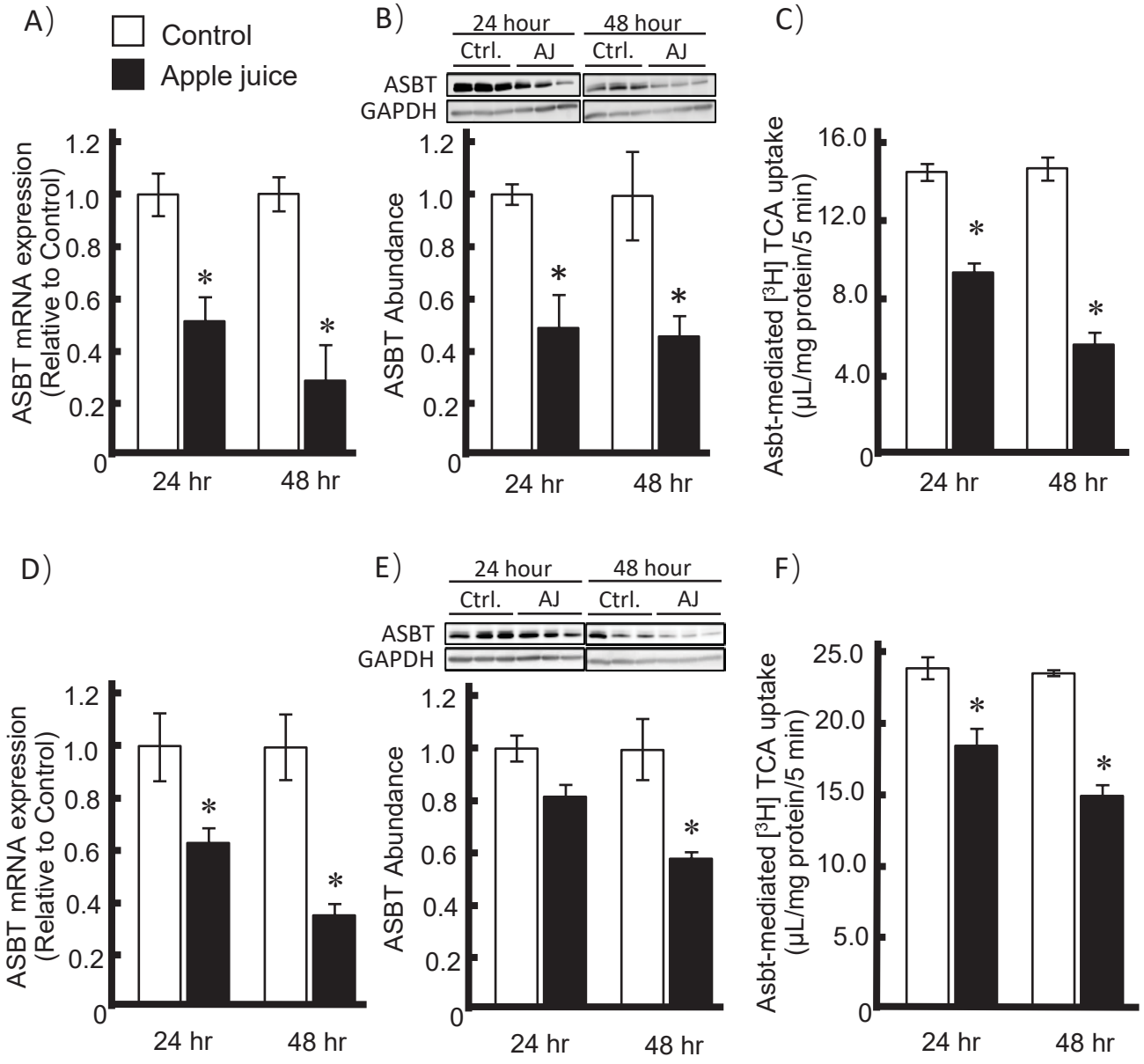
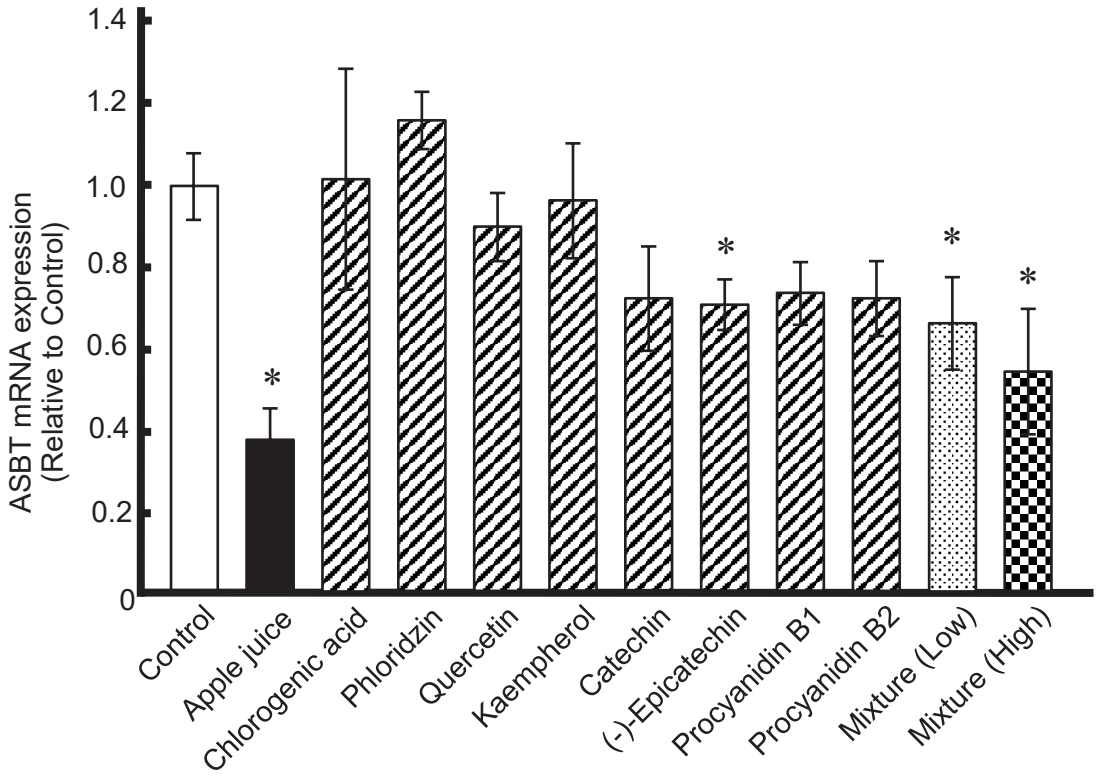

A)
Control
Apple juice
ASBT mRNA expression (Relative to Control)
1.2
1.0
0.8
0.6
0.4
0.2
0
24 hr
48 hr
B)
24 hour
48 hour
Ctrl.
AJ
Ctrl.
AJ
ASBT
GAPDH
ASBT Abundance
C)
Asbt-mediated [3H] TCA uptake (µL/mg protein/5 min)
16.0
12.0
8.0
4.0
D)
E)
F)
25.0
20.0
15.0
10.0
5.0

Figure 6


ASBT mRNA expression
(Relative to Control)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*
*
*
Control
Apple juice
Chlorogenic acid
Phloridzin
Quercetin
Kaempherol
Catechin
(-)-Epicatechin
Procyanidin B1
Procyanidin B2
Mixture (Low)
Mixture (High)