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Theaflavin-3,3'-di-*O*-gallate (PubChem CID: 135536164)

ABSTRACT

The *in vivo* anti-hyperglycemic effect of non-absorbable theaflavins, polyphenolic ingredients of black tea, was evaluated in spontaneously diabetic Torii (SDT) rats. A mixture of theaflavins (25 mg/kg/day) was administered to 8-week (wk)-old SDT rats for 20 weeks, showing to improve the impaired glucose tolerance of 22-wk old pre-diabetic SDT rats in both an oral glucose tolerance test and in a plasma insulin evaluation. At 28-wk diabetic SDT rats presented with increased fasting blood glucose levels (139 ± 23 mg/dL), and the intake of the theaflavins significantly ($p < 0.05$) reduced this effect (74 ± 11 mg/dL), demonstrating an anti-diabetic effect *in vivo*. The 20 week administration of theaflavins induced increased incretin secretion compared to that of the control, while there were no changes neither in dipeptidyl peptidase-IV activity nor glucose transporter expression. Collectively, these findings demonstrate that non-absorbable theaflavins exert anti-pre-diabetic and anti-diabetic effects by improving impaired insulin secretion.

1. Introduction

Type II diabetes mellitus (T2DM), characterized by deficiencies in insulin secretion or inappropriate insulin utilization, is one of the leading causes of mortality and morbidity (Zheng, Ley, & Hu, 2018). The formal diagnostic criteria for T2DM include: (1) a glycated hemoglobin (HbA_{1c}) level of ≥6.5%, (2) a fasting blood glucose level (BGL) of ≥126 mg/dL (7.0 mmol), or (3) a BGL at 2 h after oral glucose loading of ≥200 mg/dL (11.1 mmol). Normal postprandial BGL in T2DM patients can be achieved using insulin injection or the application of insulin stimulating drugs, such as sulfonylureas and metformin (Asrafuzzaman et al., 2017). However, common side effects deriving from such drugs, such as hypoglycemia and lactic acidosis (Riddle, 2000), led us to consider alternative-medicinal interventions to treat or prevent hyperglycemia at the pre-diabetes stage via the intake of food-derived bioactive. Pre-diabetes, typically defined as impaired fasting glucose and/or impaired glucose tolerance, is a high-risk state for the development of T2DM (Grundy, 2012).

Theaflavins, one of the typical dimers formed from the catechins in fermented teas, have no ability to cross the intestinal membrane (Mulder, van Platerink, Wijnand Schuyl, & van Amelsvoort, 2001; Takeda, Park, Kunitake, Yoshiura, & Matsui, 2013) but demonstrate various anti-hyperglycemic effects including the inhibition of intestinal maltase activity (Matsui et al., 2007), downregulation of sodium-dependent glucose transporter 1 (SGLT1) expression in Caco-2 cells (Li et al., 2020), and suppression of type I diabetes-induced intestinal Na^+/

Abbreviations: AMPK, AMP-dependent kinase; AUC, area under the curve; BGL, blood glucose level; DPP-IV, dipeptidyl peptidase-IV; GLP-1, glucagon-like peptide-1; GIP, glucose-dependent insulinotropic polypeptide; GLUT2, glucose transporter 2; HOMA-IR, homeostatic model assessment for insulin resistance; HPLC, high performance liquid chromatography; OGTT, oral glucose tolerance test; SDT, spontaneous diabetic Torii; SGLT1, sodium-dependent glucose transporter 1; T2DM, Type II diabetes mellitus; TF, theaflavin; TF3G, TF-3-*O*-gallate; TF3′G, TF-3′-*O*-gallate; TFdG, TF-3,3′-di-*O*-gallate; wk, week.

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K^+-ATPase disturbances (Qu et al., 2019). Although these reports suggested that theaflavin-rich foods lead to hyperglycemic effects, no *in vivo* animal studies on the preventive effect of theaflavins in pre-diabetic T2DM models have been reported. In this study, we investigated whether theaflavins could improve impaired glucose tolerance at pre-diabetic stages by using spontaneously diabetic Torii (SDT) rats, an inbred strain of Sprague-Dawley (SD) rats, behave as a non-obese spontaneously diabetic model of T2DM (Masuyama et al., 2003), and can develop hyperglycemia as a result of defective insulin secretion after 20 weeks of age with an incident ratio reaching 100% at 40 weeks of age (displaying long-term pre-diabetic periods) (Chen, Aikawa, Yoshida, & Matsui, 2015; Ishii et al., 2010), suggesting that this is an ideal animal model for studies focused on pre-diabetes. In recent report (Li et al., 2020), we demonstrated that theaflavins possess the ability to inhibit the intestinal transport of glucose through the downregulation of glucose transporter SGLT1 via AMP-activated protein kinase (AMPK) phosphorylation (p-AMPK) across Caco-2 cell monolayers following their successive stimulation for 24 h. Thus, this study aimed to investigate whether daily intake of theaflavins with no intestinal absorption (Park, Kunitake, & Matsui, 2013) and *in vitro* inhibitory potential of glucose transport (Li et al., 2020) might improve impaired glucose tolerance in pre-diabetic and diabetic SDT rats. The underlying mechanisms of the theaflavin physiological actions in the intestinal membrane were also examined.

2. Materials and methods

2.1. Preparation of theaflavin extracts

A mixture of theaflavins used in this study was obtained by purifying a commercially available black tea product (TF40, Yaizu Suisankagaku Ind., Shizuoka, Japan). Briefly, 10 g of the black tea product was dissolved in 500 mL of 6% ethanol containing 0.1% phosphoric acid. After filtration, this solution was applied to a YMC-DispoPackAT ODS-40 (30 × 130 mm, YMC Co., Ltd., Kyoto, Japan) using a mobile phase of 5% ethanol/0.1% phosphoric acid to exclude catechins and caffeine, followed by elution in 35% ethanol. The eluent was then evaporated to dryness for animal experiments. The separation was performed on a Prominence LC-20A HPLC system (Shimadzu Co., Kyoto, Japan), with a Cadenza CL-C18 column (100 mm × 4.6 mm, 3 μm, Imtakt Co., Kyoto, Japan) at 40 °C and a UV detection wavelength at 280 nm. Elution was employed, using 0.1% formic acid in 20% acetonitrile, at a flow rate of 1.0 mL/min, with 10 μL sample injection. The composition analysis of theaflavin mixture was then quantified using individual theaflavin standards, including theaflavin (Lot: WDE1540, FUJIFILM Wako Co., Osaka, Japan); TF-3-*O*-gallate (Lot: PTR2501, FUJIFILM Wako Co., Osaka, Japan); TF-3′-*O*-gallate (Lot: CAP5181, FUJIFILM Wako Co., Osaka, Japan) and TF-3,3′-di-*O*-gallate (Lot: PTQ2878, FUJIFILM Wako Co., Osaka, Japan). Briefly, the retention time was matched with each standard (Fig. S1) and the peak area of each theaflavin in extract was compared with that of 1.0 mM standard. As shown in Fig. S1, the mixture of theaflavins was composed of 12% theaflavin, 24% TF-3-*O*-gallate, 6% TF-3′-*O*-gallate, and 33% TF-3,3′-di-*O*-gallate (others: theasinensins and thearubigins, a complex mixture of uncharacterized catechin oxidation products with relatively high molecular weights) (Tanaka & Matsuo, 2020).

2.2. Animals and diets

8-week-old (wk) male SDT rats purchased from CLEA Japan (Tokyo, Japan) were housed individually and acclimatized under laboratory conditions (temperature, 22 ± 2 °C; humidity, 60 ± 5%; 12 h lighting cycle) for 1 week before the experiments, fed on a laboratory diet (MF, Oriental Yeast, Tokyo, Japan), and given water *ad libitum*. All experimental rats were of the same age, sex, breed, and similar body mass (336 ± 12 g). The 8-wk SDT rats were randomly divided into two groups using a random number table: the control ($n = 4$, average body mass = 336.5 g) and theaflavin group ($n = 4$, average body mass = 337.0 g). A single oral administration of 2 mL of theaflavin extract in 0.5% carboxymethyl cellulose (CMC) solution (25 mg/kg/day) was performed daily from 9 to 28-wk old SDT rats (control group: CMC solution). Body weight and food intake of rats were measured weekly.

All animal experiments were performed in accordance with the guidelines set by the Guidance for Animal Experiments in the Faculty of Agriculture and in the Graduate Course of Kyushu University in accordance with the Law (No. 105, 1973) and Notification (No. 6, 1980, of the Prime Minister's Office) issued by the Japanese Government. All experimental protocols were reviewed and approved by the Animal Care and Use Committee at Kyushu University (permit number: A30-015).

2.3. Oral glucose tolerance test

An oral glucose tolerance test (OGTT) was performed every two weeks to evaluate fasting BGL in SDT rats. Glucose (2 g/kg) was injected to the rats fasted for 16 h (from 5p.m. to 9 a.m.), according to a reported protocol (Rakvaag, Lund, Wiking, Hermansen, & Gregersen, 2019). Approximately 300 μL of blood from the portal vein was collected before and after administration (0, 30, 60, 90, and 120 min), and some blood was immediately used for evaluating BGL by a disposable BGL sensor (Glutest Pro, Sanwa Chemical Research Co., Tokyo, Japan).

2.4. Plasma insulin assay

Blood samples taken at the above time intervals were placed in tubes containing EDTA-2Na (15105-22, Lot: M2P3825, Nacalai Tesque Inc., Kyoto, Japan) and protease inhibitors [0.1 mg aprotinin (016-11836, Lot: WDL7896, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and 0.1 mg chymostatin (4063, Lot: 680802, Peptide Institute Inc., Osaka, Japan)]. These tubes were centrifuged at 3500*g* for 15 min at 4 °C to obtain the plasma samples and stored at −80 °C. Plasma insulin levels (ng/mL) were measured using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (UE type, AKRIN-130, 630-05589, FUJIFILM Wako Shibayagi Co., Gunma, Japan) according to the manufacturer's instructions.

2.5. Preparation of portal blood and intestinal tissues

Blood from the portal vein was collected at 30 min after glucose loading in 28-wk SDT rats and then used in the dipeptidyl peptidase-IV (DPP-IV) assay. Intestinal tissues from the distal duodenum, upper jejunum, and mid-ileum were also taken from the SDT rats, flushed with cold PBS buffer (pH 7.4) containing phenylmethylsulfonyl fluoride (PMSF, 1 mmol) (06297-02, Lot: V4P4512, Nacalai Tesque Inc.) and EDTA-2Na (2 mmol). These tissues were then immediately frozen in liquid nitrogen and stored at –80 °C until biochemical assays. Given the fact that glucose-dependent insulinotropic polypeptide (GIP) is produced in enteroendocrine K cells located in the proximal region, while glucagon-like peptide-1 (GLP-1) is produced in the L cells in the distal region of the small intestine we were forced to use both the distal duodenum and mid-ileum tissues in the GIP and GLP-1 incretin assays, respectively (Deacon, 2004). Additionally, the upper jejunum, which acts as a critical point in glucose absorption, was used in the SGLT1 and glucose transporter 2 (GLUT2) expression assays (Barik et al., 2019).

2.6. Capillary electrophoresis-based immunoassay analysis

The mucosa samples from the upper jejunum were evaluated for SGLT1 and GLUT2 using a capillary electrophoresis-based immunoassay which were run on a Wes instrument (ProteinSimple Co., San Jose, CA, USA). The samples were homogenized using a microtube homogenizer (POLYTRON PT 1200 E Handheld Dispersing System, Bohemia, NY, USA) in cold RIPA buffer containing 0.1% (v/v) Nonidet P-40 (25223-

04, Lot: M7B7874; Nacalai Tesque Co.), 1% (v/v) protease inhibitor cocktail (25955-24, Lot: L9T1242; Nacalai Tesque Co.) and then centrifuged at 16,000*g* for 15 min and 4 °C. The supernatant was then collected and diluted to 2.0 and 0.5 μg/μL of protein for SGLT1 and GLUT2 assays, respectively, in a 0.1 × Sample Diluent buffer (ProteinSimple Co.). Four volumes of the diluted samples were mixed with one volume of 5 × Fluorescent Master Mix containing 5% SDS and 200 mmol dithiothreitol, and denatured at 37 °C for 20 min (SGLT1) or 95 °C for 5 min (GLUT2), respectively.

The Wes measurements were performed using a 12–230 kDa Separation Module (8 × 25 mm capillary cartridge, ProteinSimple Co.), according to the previously described method (Tanaka et al., 2020) with several modifications. Wes reagents (biotinylated ladder and primary antibodies) were dispensed in a microplate and added to the Wes automated capillary electrophoresis. These samples were then subjected to an automated immune detection using a horseradish peroxidase-conjugated anti-rabbit secondary antibody and a chemiluminescent substrate. Primary antibodies against SGLT1 (1:20 dilution, ab14686, Lot: GR161462-6; Abcam, Cambridge, UK) and GLUT2 (1:200 dilution, ab192599, Lot: GR3213891-2; Abcam) were used. Total protein was then detected using a pentafluorophenyl ester-biotin labeling reagent, which is capable of attachment to the applied proteins. At the end of the run, the chemiluminescent signal was displayed as a virtual blot-like image, and an electropherogram was generated based on molecular weights, using ProteinSimple Compass software. Protein expression of SGLT1 or GLUT2 was then normalized against the area of the electropherogram for the total protein in each lane, and the data were expressed as the ratio of the control.

2.7. Incretin secretion in intestinal membranes

The mucosa samples from the distal duodenum and mid-ileum were homogenized in an ethanol-acid solution (ethanol/water/12 mol/L HCl, 74:25:1; 5 mL/g of tissue) and incubated overnight at 4 °C. After centrifugation at 12000*g* for 10 min at 4 °C, the supernatant was used in ELISA assays against GIP (YK251, Lot: 190411, Yanaihara Institute Inc., Shizuoka, Japan) and GLP-1 (299-7550, Lot: SKM4770, FUJIFILM Wako Shibayagi Co., Gunma, Japan) according to the manufacturer's instructions. Measurements were performed at 450 nm using a Wallac 1420-microplate reader (PerkinElmer Life Science, Tokyo, Japan) and both the GIP and GLP-1 concentrations were expressed as pmol/g.

2.8. Measurement of plasma DPP-IV activity

The plasma DPP-IV activity in the portal blood was assayed using a colorimetric DPP-IV/CD26 assay kit (BML-P189-9091, Enzo Inc., Farmingdale, NY, USA) with 2 mmol/L H-glycyl-prolyl-7-amino-4-methylcoumarin (Gly-Pro-AMC) as the substrate, according to the manufacturer's instructions. Measurements were performed at 460 nm using a Wallac 1420-microplate reader, and the amount of the released 7-amino-4-methylcoumarin (AMC) released from the substrate was calculated from a standard curve. DPP-IV activity is expressed as mmol/min.

2.9. Statistical analysis

A sample size was determined based on previous studies (Chen, Aikawa, Yoshida, Kawaguchi, & Matsui, 2017; Chen et al., 2015). Results are expressed as the mean ± standard error of the mean (SEM). All data were tested for normality distribution before selecting the appropriate statistical method using the Kolmogorov-Smirnov test. When the experimental data conforms to the normality distribution, statistical differences among groups were evaluated by two-way analysis of variance (ANOVA), followed by Bonferroni's post-hoc test. Statistical evaluation of the differences between two groups was completed using a Student's *t*-test. In addition, for other data that does not match normality distribution, a Mann-Whitney *U* test was used to evaluate statistical differences. Statistical significance was set at $p < 0.05$. These analyses were performed using Prism, version 5 (GraphPad Software Inc., San Diego, CA, USA) and IBM SPSS Statistics 25.0 (SPSS, IBM Corp. Armonk, NY, USA).

3. Results and discussion

3.1. Effect of theaflavins on the biological parameters of SDT rats

Food intake and body weight in the control and theaflavin groups were comparable between 10 and 22 weeks of age, indicating that a daily intake of theaflavins at a dose of 25 mg/kg did not affect the growth of the SDT rats. At > 23 weeks of age, logical growth behaviors were observed in the control group, namely, the tendency of decreased food intake and decreased body weight (Fig. 1A and B). These phenomena are typical for SDT rats at progressive pre-diabetes stages (Masuyama et al., 2004). In contrast, the absence of unusual growth in theaflavin group suggested that theaflavins might prevent or at least delay the progression of pre-diabetes in these animals.

The effect of the theaflavins on fasting BGL was investigated over an 18-wk protocol using SDT rats. Fasting BGL was shown to gradually increase with age in the control group from 24 weeks of age (Fig. 1C), and at 28 weeks of age, SDT rats entered the initial diabetic phase (fasting BGL: 139 ± 23 mg/dL). In contrast, the intake of theaflavins completely inhibited this increase in fasting BGL over the entire duration of this protocol with none of the theaflavin-treated SDT rats developing clinical diabetes (fasting BGL at 28 wk: 74 ± 11 mg/dL, $p < 0.05$ vs. control). To our knowledge, there have been some reports on the anti-diabetic effect of natural compounds, in which daily intake of sea buckthorn juice at a dose of 100 mg/kg for 10 weeks resulted in reduced fasting BGL in T2DM db/db mice (Xue et al., 2015). Rose-hip extract was also reported to be effective in the prevention of diabetes in SDT rats at a dose of 100 mg/kg/day (Chen et al., 2017). However, the reduced dose of the theaflavins (25 mg/kg/day) may suggest that theaflavins have a more potent anti-diabetic effect than other previously evaluated compounds. It seems that the specific structure-activity relationship (SAR) of theaflavins may be responsible for the anti-diabetic effect (Fig. 1C), however, due to the limitations of the experiment, the theaflavin extract used is a mixture, but not a pure compound. Therefore, the in-depth mechanism of theaflavin-induced anti-diabetic effect still needs further research, including the analysis of theaflavin derivatives (Gothandam, Ganesan, Ayyasamy, & Ramalingam, 2019) and the design of a positive control group of standard theaflavins.

3.2. Effect of theaflavins on glucose tolerance and plasma insulin secretion in SDT rats

To evaluate the effect of the theaflavins on glucose tolerance and thus evaluate their anti-diabetic effect in SDT rats, we went on to investigate changes in the glucose tolerance and fasting BGL in these animals. We evaluated impaired glucose tolerance using an OGTT assay every two weeks up to 24-wk old SDT rats. Fig. 2A shows the results of OGTT experiments at 10-, 16-, 22-, and 24-wk old SDT rats. At 10 and 16 weeks of age the SDT rats in the control group did not display any impaired glucose tolerance, while after 22 weeks of age, an elevated BGL was reported in response to an impaired glucose tolerance result at 120 min after glucose loading. This indicated that, under these experimental conditions, SDT rats entered the pre-diabetic state at > 22 weeks of age. In contrast, it was clear that theaflavin-dosed SDT rats still maintained normal glucose clearance even at the pre-diabetic stage, >22 weeks of age. The significantly lower area under the curve ($AUC_{0\text{-}120\ min}$) for the BGL response in the theaflavin group (Fig. 2B) suggests that theaflavins may ameliorate the progressive impaired glucose tolerance associated with the pre-diabetic stages of SDT rat development ($AUC_{0\text{-}120\ min}$ at 22-wk: control group, 598 ± 6 mg·h/dL; theaflavin group, 455 ± 18 mg·h/

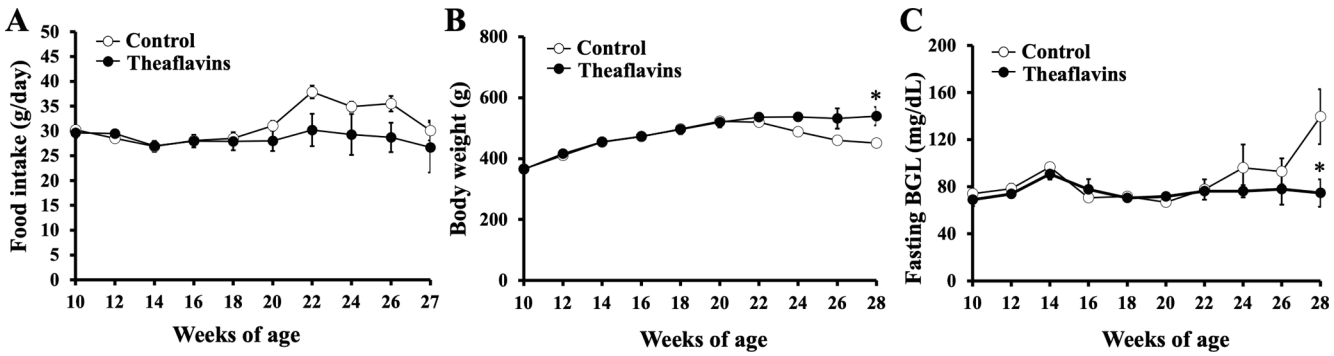

Fig. 1. Effect of theaflavin supplementation on the biological parameters of SDT rats. Changes in food intake and body weight in the SDT rats with or without theaflavin supplementation (25 mg/kg/day) were evaluated every week. Changes in fasting BGL were measured every two weeks during the 18-wk protocol. Results are expressed as the mean ± SEM ($n = 4$). Statistical differences among the groups were evaluated by two-way analysis of variance (ANOVA), followed by Bonferroni's post-hoc test, or the Mann-Whitney *U* test. *$p < 0.05$.

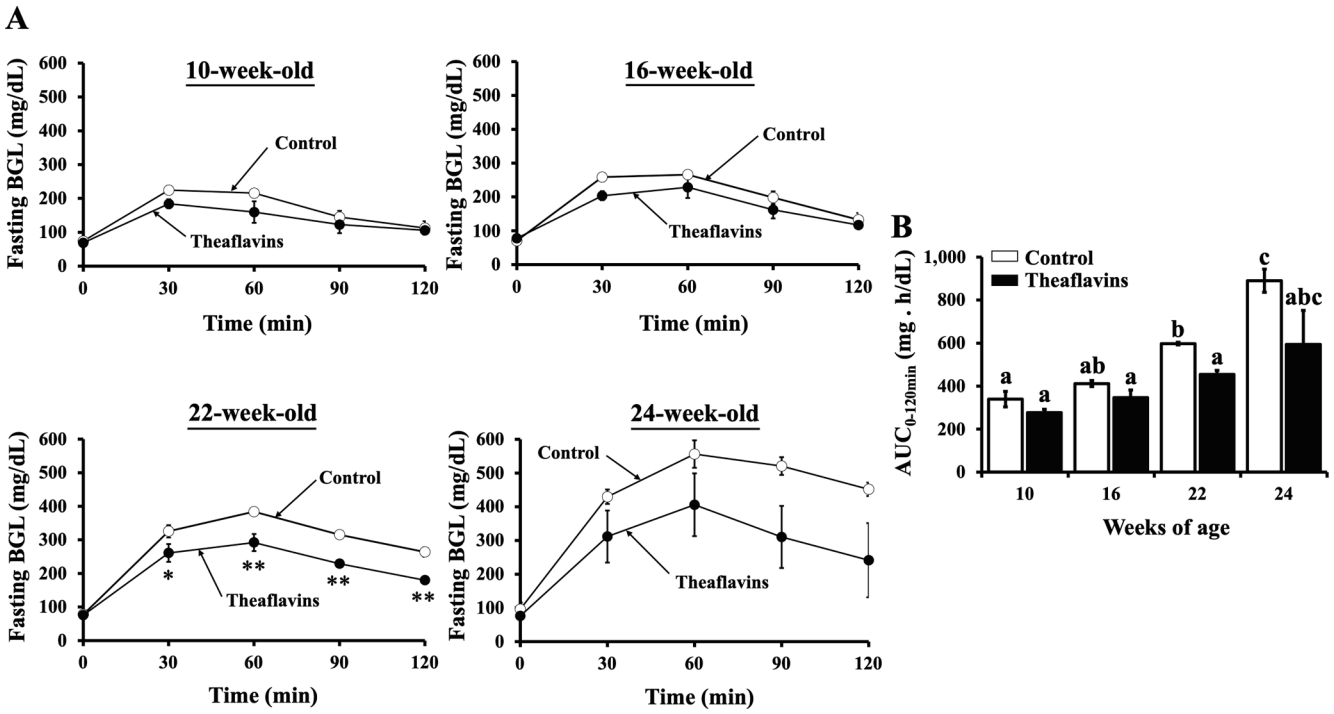

Fig. 2. Effect of theaflavin supplementation on glucose tolerance in SDT rats. OGTT experiments (2 g/kg glucose load) were performed on 10-, 16-, 22-, and 24-wk old SDT rats with or without theaflavin supplementation (25 mg/kg/day). BGL was measured before and after administration (0, 30, 60, 90, and 120 min), as described in the Section 2.3. Results are expressed as the mean ± SEM ($n = 4$). Statistical differences among the groups were evaluated by two-way analysis of variance (ANOVA), followed by Bonferroni's post-hoc test, or the Mann-Whitney *U* test. Different letters represent statistically significant differences at $p < 0.05$. *$p < 0.05$, **$p < 0.01$.

dL, $p < 0.01$).

OGTT experiments were performed in 10-, 16-, 22-, and 24-wk old SDT rats and then used to evaluate plasma insulin levels. As shown in Fig. 3A, plasma insulin levels in the theaflavin group of fasting pre-diabetic SDT rats (0 min) did not differ from those in the control group, which indicates that at pre-diabetic stages, there is no evidence of insulin resistance in these SDT rats as evaluated by a lack of change in the homeostatic model assessment for insulin resistance (HOMA-IR). However, 22-wk old SDT rats (Fig. 2A) in both groups had a demonstrated increase in BGL in response to OGTT. The theaflavin group, however, showed a significantly lower BGL response to OGTT at 30, 60, 90, and 120 min, when compared to the control group. In addition, the insulin concentrations significantly increased in the theaflavin group at the 60 min and 90 min post glucose loading when compared to the control (Fig. 3A), indicating the development of pre-diabetes in control rats, and the improved insulin production in theaflavin treated animals. At 24 weeks of age (Figs. 2A, 3A), the theaflavin group demonstrated an overall increase in insulin response and a reduction in the glucose response compared with those of the control group, but these values did not reach statistical significance. There exist several hypotheses regarding this phenomenon, including firstly, theaflavin-induced increases in insulin secretion might be masked by weakened ß-cell function, which may trigger via a complex mechanism following the onset of hyperglycemia (Kuo, Kim-Muller, McGraw, & Accili, 2016); secondly, theaflavin-treatment may increase the activity of insulin-degrading enzyme, which aims to mediate insulin catabolism. In detail, theaflavins can promote the secretion of insulin, however, the increase of insulin-degrading enzyme activity might accelerate the degradation of insulin, so that it may offset the effect of theaflavin-induced insulin secretion promotion (Leissring et al., 2010); thirdly, insulin receptor or

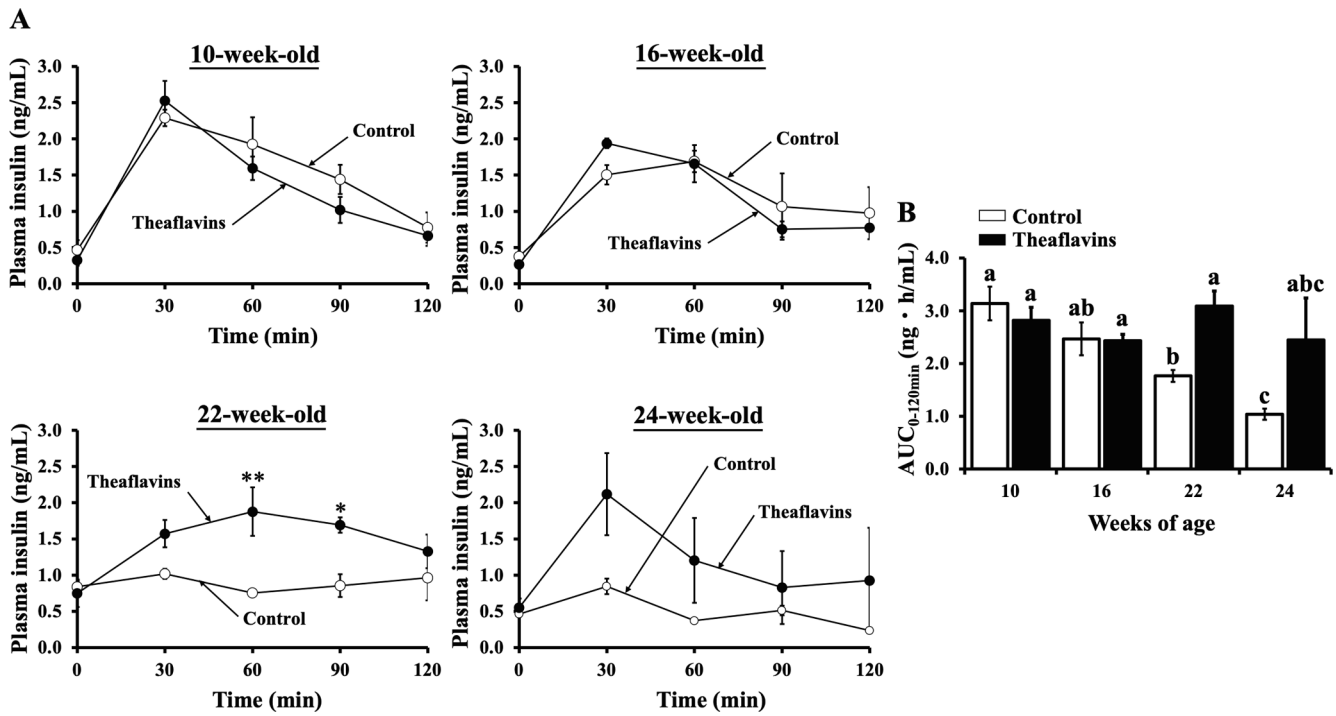

Fig. 3. Effect of theaflavin supplementation on plasma insulin level by OGTT in SDT rats. OGTT experiments (2 g/kg glucose load) were performed on 10-, 16-, 22-, and 24-wk old SDT rats with or without theaflavin supplementation (25 mg/kg/day). Plasma insulin levels were measured using an ELISA insulin assay kit and the results are expressed as the mean ± SEM ($n = 4$). Statistical differences among the groups were evaluated by two-way analysis of variance (ANOVA), followed by Bonferroni's post-hoc test, or the Mann-Whitney *U* test. Different letters represent statistically significant differences at $p < 0.05$. *$p < 0.05$, **$p < 0.01$. *N.S.*: not significant at $p < 0.05$.

related signaling pathway might be stimulated by theaflavin-treatment, thereby mediating further cellular actions of insulin (Wilcox, 2005). Taking together, further studies need to be conducted for clarifying the underlying mechanisms.

Alternatively, the improvements in the impaired glucose tolerance in the theaflavin group (Fig. 2B) may be the result of maintained insulin secretion, but not altered insulin resistance (insulin at 60 min of 22-wk: control group, 0.75 ± 0.02 ng/mL; theaflavin group, 1.88 ± 0.34 ng/mL, $p < 0.01$) (Fig. 3A), which is in accordance with the data for the 24-wk old SDT rats.

3.3. *Effect of theaflavins on the expression of glucose transporters in SDT rats*

To clarify the anti-pre-diabetic (or improved glucose tolerance) mechanism underlying the effects of the theaflavin supplementation in SDT rats, the expression of SGLT1 and GLUT2 in the upper jejunum were assayed by a Wes measurement. According to our previous study in Caco-2 cells, non-absorbable TF3′G reduced SGLT1 expression via AMPK activation (Li et al., 2020). Therefore, we speculated that daily intake of theaflavins might affect glucose transporter expression in the intestine following its long-term administration in SDT rats. However, no significant reduction in SGLT1 or GLUT2 expression was observed in the theaflavin group (25 mg/kg/day) when compared to the control (Fig. 4). The inconsistencies between the *in vitro* and *in vivo* results describing glucose transporter expression might be due to differences in the stimulation times (successive 24-h stimulation in Caco-2 cells or a daily single oral administration in SDT rats) and intrinsic differences in the model.

3.4. *Effect of theaflavins on incretin secretion and DPP-IV activity in SDT rats*

Our data show that SDT rats fed with theaflavins displayed an overall increase in plasma insulin levels in response to change in the BGL concentration (Figs. 2B and 3B), which may contribute to incretin stimulation (Jens & Jesper, 2004). In addition, it has been reported that blackcurrant polyphenol extract ameliorated glucose tolerance by increasing insulin secretion and regulating glucose control (Iizuka, Ozeki, Tani, & Tsuda, 2018) in T2DM mice. Therefore, in order to clarify the underlying anti-pre-diabetic (or improved glucose tolerance) mechanism of theaflavins during the pre-diabetic stages of SDT rats, the effect of theaflavin intake on incretin secretion in the intestines of these animals was investigated. SDT rats fed with theaflavins experienced a significant increase in both GIP and GLP-1 (Fig. 5) in the intestinal membrane, compared to the control group (GIP: control group, 55.1 ± 6.3 pmol/g-tissue; theaflavin group, 124.9 ± 28.0 pmol/g-tissue, $p < 0.05$, GLP-1: control group, 161 ± 41.8 pmol/g-tissue; theaflavin group, 271.4 ± 15.5 pmol/g-tissue, $p < 0.05$). This clearly indicates that daily intake of theaflavins promotes intestinal production of incretin which acts as a stimulator of insulin secretion from the islets of Langerhans (Nauck, Vardarli, Deacon, Holst, & Meier, 2011). It has been reported that in male Wistar rats treated with an AMPK activator (AICAR, 250 mg/kg) experienced an increase in plasma GLP-1 expression over a 2 h period (Andreozzi et al., 2016). Yu et al. also reported that the addition of an AMPK inhibitor (compound C, 2 μmol) inhibited berberine (100 μmol)-mediated GLP-1 secretion in NCl-H716 cells (Yu et al., 2010). These reports suggest that there is a close relationship between AMPK activation and incretin production in the intestinal membrane. Taking these reports and the previous results describing TF3′G-induced AMPK activation in Caco-2 cells (Li et al., 2020), into consideration we can presume that the daily intake of theaflavins in SDT rats provides significant health benefits preventing pre-diabetes via the maintenance of insulin secretion by AMPK-promoted incretin production in the intestine. Furthermore, it has been reported that theaflavins are supposed to activate bitter taste receptors, which are known to participate in the secretion of incretins such as GLP-1 and cholecystokinin (CCK), in HEK293T cells (Yamazaki et al., 2014). Thus, further studies on

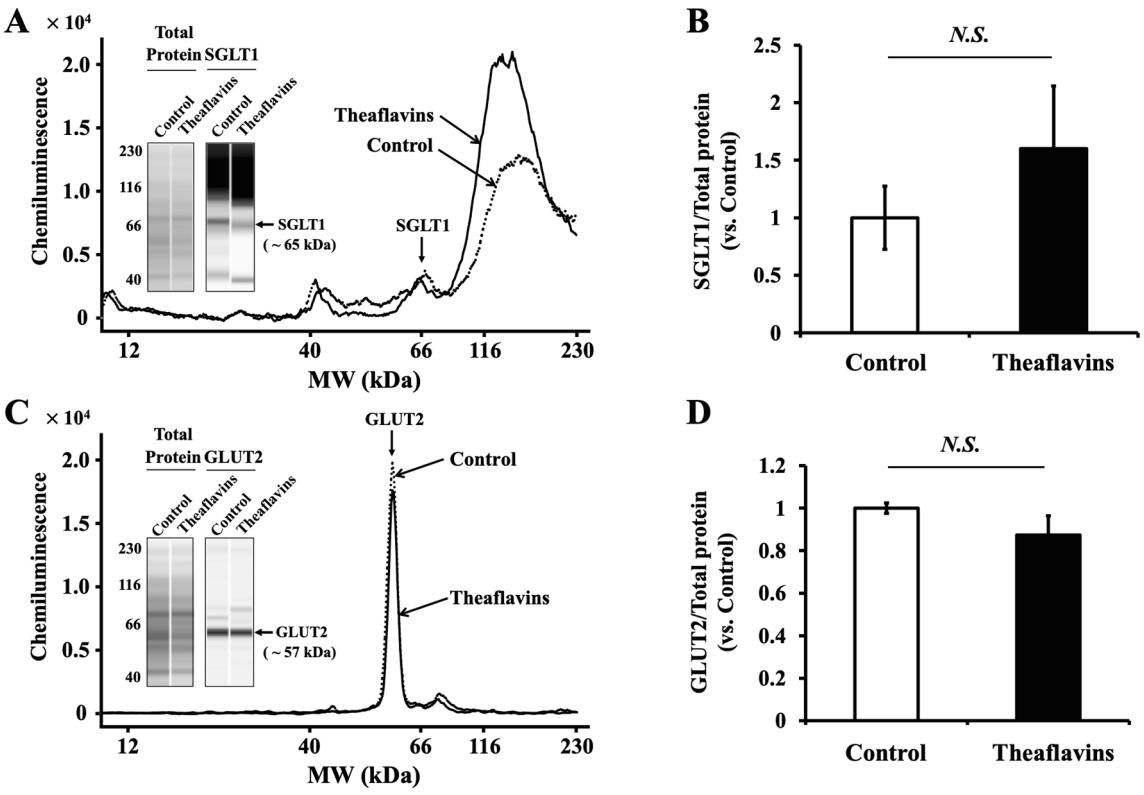

Fig. 4. Analysis of the effect of theaflavin supplementation on the expression of SGLT1 and GLUT2 in the upper jejunum region of 28-wk old SDT rats. We collected mucosa samples from the upper jejunum of 28-wk old SDT rats with or without theaflavin supplementation (25 mg/kg/day). These samples were then subjected to a capillary electrophoretic-based immunoassay on a Wes instrument, as described in the Section 2.7. The chemiluminescent signal was displayed as a virtual blot-like image, and an electropherogram was generated based on the molecular weights. The expression of SGLT1 (B) and GLUT2 (D) was normalized against the total area in the electropherogram peak area representing the total protein applied in each lane. Results are expressed as the mean ± SEM ($n = 4$) and statistical differences between the two groups were evaluated using the Mann-Whitney *U* test. *N.S.*: not significant at $p < 0.05$.

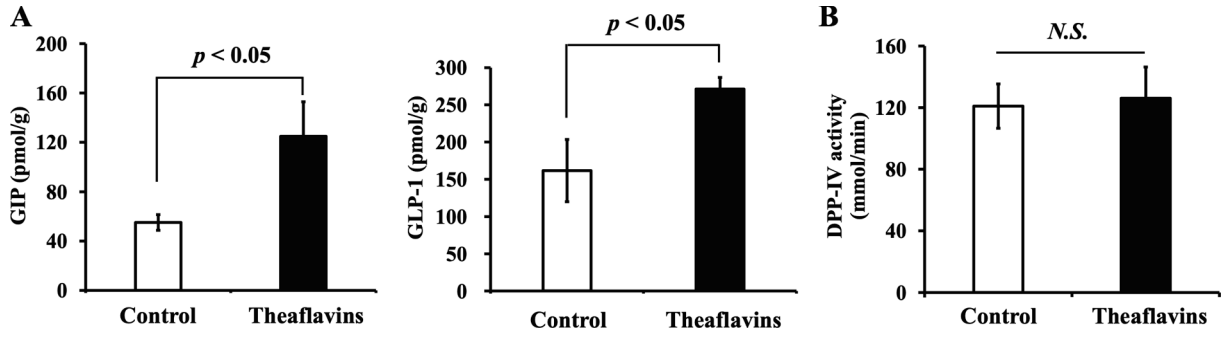

Fig. 5. Effect of theaflavin supplementation on intestinal incretin secretion in the duodenum and ileum regions and DPP-IV activity in the portal blood of 28-wk old SDT rats. We collected mucosa samples from the intestinal duodenum (for GIP, pmol/g-tissue) and ileum (for GLP-1, pmol/g-tissue) of 28-wk old SDT rats treated with or without theaflavin supplementation (25 mg/kg/day) and then used these samples to complete GIP or GLP-1 ELISA assays. We also collected blood samples from the portal vein of these animals at 30 min after glucose loading and used these samples to complete a colorimetric DPP-IV/CD26 assay. Results are expressed as the mean ± SEM ($n = 4$). Statistical differences between the two groups were evaluated using the Mann-Whitney *U* test. *N.S.*: not significant at $p < 0.05$.

clarifying underlying mechanisms of theaflavin-induced incretin secretion related to AMPK phosphorylation or bitter taste receptors activation need to be conducted *in vitro* experiments using e.g., NCI-H716 cells.

Prior to the conclusion that theaflavin-induced anti-pre-diabetic effect was due to increased incretin production in the intestine of SDT rats, the effect of theaflavins on plasma DPP-IV activity was examined. It is well known that DPP-IV, a serine aminopeptidase, plays a major role in not only glucose metabolism as it facilitates *N*-terminal truncation and incretin inactivation (Deacon, 2004). As the latter action of DPP-IV is associated with degraded insulin secretion, sitagliptin, a DPP-IV inhibitor drug, has been successfully developed as an efficient supplemental therapy for increasing insulin expression via the inhibition of GIP and GLP-1 degradation (Karasik, Aschner, Katzeff, Davies, & Stein, 2008; Shimizu, Hara, & Hira, 2021). In this study, DPP-IV activity in the portal blood of SDT rats fed with theaflavins was comparable to that of the control animals (Fig. 5B) and our data firmly show that theaflavins could indirectly ameliorate glucose tolerance by increasing incretin production .

4. Conclusion

This study evaluated the *in vivo* anti-hyperglycemic effects of the

long-term administration of theaflavin extracts in SDT rats. These results revealed that the daily intake of theaflavins significantly improved the impaired glucose tolerance response to OGTT at the pre-diabetic stage. At 28 weeks of age, the fasting BGL of SDT rats in the control group was $>$ 126 mg/dL, while the animals in the theaflavin group did not reach the BGL threshold for diabetic fasting, suggesting that this intervention delays or prevents the onset of diabetes in these animals. In addition, our evaluations revealed that this effect was likely the result of increased incretin secretion (Fig. 5A). Taken together these results suggest that non-absorbable theaflavins, mainly derived from black tea beverage, could provide a new source of anti-hyperglycemic food compounds for application in the prevention and management of diabetes.

5. Ethics statement

All animal experiments were performed in accordance with the guidelines set by the Guidance for Animal Experiments in the Faculty of Agriculture and in the Graduate Course of Kyushu University in accordance with the Law (No. 105, 1973) and Notification (No. 6, 1980, of the Prime Minister's Office) issued by the Japanese Government. All experimental protocols were reviewed and approved by the Animal Care and Use Committee at Kyushu University (permit number: A30-015).

Author statement

Baorui Li and Lei Fu equally contribute to this study. Baorui Li, Lei Fu, and Ruchia Kojima performed experiments and analyzed data; Ayaka Yamamoto, and Tomoya Ueno prepared the extract sample; Baorui Li, Lei Fu, and Toshiro Matsui wrote and revised the manuscript. Toshiro Matsui designed the study, interpreted, analyzed data, and reviewed and edited the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2021.104702.

References

Andreozzi, F., Raciti, G. A., Nigro, C., Mannino, G. C., Procopio, T., Davalli, A. M., … Folli, F. (2016). The GLP-1 receptor agonists exenatide and liraglutide activate glucose transport by an AMPK-dependent mechanism. *Journal of Translational Medicine, 14*(1). https://doi.org/10.1186/s12967-016-0985-7.

Asrafuzzaman, M.d., Cao, Y., Afroz, R., Kamato, D., Gray, S., & Little, P. J. (2017). Animal models for assessing the impact of natural products on the aetiology and metabolic pathophysiology of type 2 diabetes. *Biomedicine & Pharmacotherapy, 89*, 1242–1251.

Barik, S. K., Russell, W. R., Moar, K. M., Cruickshank, M., Scobbie, L., Duncan, G., & Hoggard, N. (2019). The anthocyanins in black currants regulate postprandial hyperglycaemia primarily by inhibiting α-glucosidase while other phenolics modulate salivary α-amylase, glucose uptake and sugar transporters. *The Journal of Nutritional Biochemistry, 78*, Article 108325.

Chen, S. J., Aikawa, C., Yoshida, R., Kawaguchi, T., & Matsui, T. (2017). Anti-prediabetic effect of rose hip (*Rosa canina*) extract in spontaneously diabetic Torii rats. *Journal of the Science and Food Agriculture, 97*(12), 3923–3928.

Chen, S. J., Aikawa, C., Yoshida, R., & Matsui, T. (2015). Methylglyoxal-derived hydroimidazolone residue of plasma protein can behave as a predictor of pre-diabetes in spontaneously diabetic Torii rats. *Physiological Reports, 3*(8), e12477. https://doi.org/10.14814/phy2.12477.

Deacon, C. F. (2004). Circulation and degradation of GIP and GLP-1. *Hormone and Metabolic Research, 36*(11/12), 761–765.

Gothandam, K., Ganesan, V. S., Ayyasamy, T., & Ramalingam, S. (2019). Antioxidant potential of theaflavin ameliorates the activities of key enzymes of glucose metabolism in high fat diet and streptozotocin-induced diabetic rats. *Redox Report, 24*(1), 41–50.

Grundy, S. M. (2012). Pre-diabetes, metabolic syndrome, and cardiovascular risk. *Journal of the American College of Cardiology, 59*(7), 635–643.

Iizuka, Y., Ozeki, A., Tani, T., & Tsuda, T. (2018). Blackcurrant extract ameliorates hyperglycemia in type 2 diabetic mice in association with increased basal secretion of glucagon-like peptide-1 and activation of AMP-activated protein kinase. *Journal of Nutritional Science and Vitaminology, 64*(4), 258–264.

Ishii, Y., Ohta, T., Sasase, T., Morinaga, H., Hata, T., Miyajima, K., … Matsushita, M. (2010). A high-fat diet inhibits the progression of diabetes mellitus in type 2 diabetic rats. *Nutrition Research, 30*(7), 483–491.

Jens, J. H., & Jesper, G. (2004). Role of incretin hormones in the regulation of insulin secretion in diabetic and nondiabetic humans. *American Journal of Physiology-Endocrinology and Metabolism, 287*, E199–E206.

Karasik, A., Aschner, P., Katzeff, H., Davies, M. J., & Stein, P. P. (2008). Sitagliptin, a DPP-4 inhibitor for the treatment of patients with type 2 diabetes: A review of recent clinical trials. *Current Medical Research and Opinion, 24*(2), 489–496.

Kuo, T., Kim-Muller, J. Y., McGraw, T. E., & Accili, D. (2016). Altered plasma profile of antioxidant proteins as an early correlate of pancreatic β cell dysfunction. *Journal of Biological Chemistry, 291*(18), 9648–9656.

Leissring, M. A., Malito, E., Hedouin, S., Reinstatler, L., Sahara, T., Abdul-Hay, S. O., … Liu, J. (2010). Designed inhibitors of insulin-degrading enzyme regulate the catabolism and activity of insulin. *PLoS ONE, 5*(5), e10504.

Li, B., Fu, L., Abe, C., Nectoux, A. M., Yamamoto, A., & Matsui, T. (2020). Theaflavins inhibit glucose transport across Caco-2 cells through the downregulation of the Ca^{2+}/AMP-activated protein kinase-mediated glucose transporter SGLT1. *Journal of Functional Foods, 75*, 104273. https://doi.org/10.1016/j.jff.2020.104273.

Masuyama, T., Fuse, M., Yokoi, N., Shinohara, M., Tsujii, H., Kanazawa, M., … Taniguchi, K. (2003). Genetic analysis for diabetes in a new rat model of nonobese type 2 diabetes, Spontaneously Diabetic Torii rat. *Biochemical and Biophysical Research Communications, 304*(1), 196–206.

Masuyama, T., Komeda, K., Hara, A., Noda, M., Shinohara, M., Oikawa, T., … Taniguchi, K. (2004). Chronological characterization of diabetes development in male Spontaneously Diabetic Torii rats. *Biochemical and Biophysical Research Communications, 314*(3), 870–877.

Matsui, T., Tanaka, T., Tamura, S., Toshima, A., Tamaya, K., Miyata, Y., … Matsumoto, K. (2007). α-Glucosidase inhibitory profile of catechins and theaflavins. *Journal of Agricultural and Food Chemistry, 55*, 99–105.

Mulder, T. P. J., van Platerink, C. J., Wijnand Schuyl, P. J., & van Amelsvoort, J. M. M. (2001). Analysis of theaflavins in biological fluids using liquid chromatography-electrospray mass spectrometry. *Journal of Chromatography B: Biomedical Sciences and Applications, 760*(2), 271–279.

Nauck, M. A., Vardarli, I., Deacon, C. F., Holst, J. J., & Meier, J. J. (2011). Secretion of glucagon-like peptide-1 (GLP-1) in type 2 diabetes: What is up, what is down? *Diabetologia, 54*, 10–18.

Park, H. Y., Kunitake, Y., & Matsui, T. (2013). Benzotropolone moiety in theaflavins is responsible for inhibiting peptide-transport and activating AMP-activated protein kinase in Caco-2 cells. *Functional Foods in Health and Disease, 3*(5), 111–121.

Qu, F., Liu, S., He, C., Zhou, J., Zhang, S., Ai, Z., … Ni, D. (2019). Comparison of the effects of green and black tea extracts on Na^+/K^+-ATPase activity in intestine of type 1 and type 2 diabetic mice. *Molecular Nutrition & Food Research, 63*(17), 1801039. https://doi.org/10.1002/mnfr.v63.1710.1002/mnfr.201801039.

Rakvaag, E., Lund, M. D., Wiking, L., Hermansen, K., & Gregersen, S. (2019). Effects of different fasting durations on glucose and lipid metabolism in Sprague Dawley rats. *Hormone and Metabolic Research, 51*(08), 546–553.

Riddle, M. (2000). Combining sulfonylureas and other oral agents. *The American Journal of Medicine, 108*(6), 15–22.

Shimizu, Y., Hara, H., & Hira, T. (2021). Glucagon-like peptide-1 response to whey protein is less diminished by dipeptidyl peptidase-4 in comparison with responses to dextrin, a lipid and casein in rats. *British Journal of Nutrition, 125*(4), 398–407.

Takeda, J., Park, H.-Y., Kunitake, Y., Yoshiura, K., & Matsui, T. (2013). Theaflavins, dimeric catechins, inhibit peptide transport across Caco-2 cell monolayers via down-regulation of AMP-activated protein kinase-mediated peptide transporter PEPT1. *Food Chemistry, 138*(4), 2140–2145.

Tanaka, M., Kiyohara, H., Yoshino, A., Nakano, A., Takata, F., Dohgu, S., … Matsui, T. (2020). Brain-transportable soy dipeptide, Tyr-Pro, attenuates amyloid β peptide$_{25\text{-}35}$-induced memory impairment in mice. *npj Science of Food, 4*(1), 1–4.

Tanaka, T., & Matsuo, Y. (2020). Production mechanisms of black tea polyphenols. *Chemical and Pharmaceutical Bulletin, 68*, 1131–1142.

Wilcox, G. (2005). Insulin and insulin resistance. *The Clinical Biochemist Reviews, 26*, 19–39.

Xue, Y., Miao, Q., Zhao, A.i., Zheng, Y., Zhang, Y., Wang, P., … Yang, B. (2015). Effects of sea buckthorn (*Hippophaë rhamnoides*) juice and L-quebrachitol on type 2 diabetes mellitus in db/db mice. *Journal of Functional Foods, 16*, 223–233.

Yamazaki, T., Sagisaka, M., Ikeda, R., Nakamura, T., Matsuda, N., Ishii, T., & Watanabe, T. (2014). The human bitter taste receptor hTAS2R39 is the primary

receptor for the bitterness of theaflavins. *Bioscience, Biotechnology, and Biochemistry, 78*(10), 1753–1756.

Yu, Y., Liu, L.i., Wang, X., Liu, X., Liu, X., Xie, L., & Wang, G. (2010). Modulation of glucagon-like peptide-1 release by berberine: *In vivo* and *in vitro* studies. *Biochemical Pharmacology, 79*(7), 1000–1006.

Zheng, Y., Ley, S. H., & Hu, F. B. (2018). Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. *Nature Reviews Endocrinology, 14*(2), 88–98.