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Faeces tea of cherry caterpillar (larvae of *Phalera flavescens*) promotes differentiation into myotubes, activates mitochondria, and suppresses the protein expression of ubiquitin ligase in C2C12

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Abstract

Sarcopenia is a syndrome characterised by progressive and systemic loss of skeletal muscle mass and strength. In order to prevent sarcopenia and lead a healthy life, it is necessary to maintain muscle mass and prevent loss of muscle mass. Insect faeces have long been consumed as tea in China, both as a medicine and as a functional food. In the present work, we investigated the efficacy of cherry caterpillar faeces tea (PT) for treating sarcopenia, particularly concerning muscle building and atrophy suppression using C2C12 cells. PT treatment (0.2 mg/mL) increased myotube widths by approximately 40%, and increased the expression levels of *Myod*, *Myog*, and *MYHC*. Additionally, *PGC1α*, *TFAM*, *SDHA*, *BCAT*, and *BCKDH* were upregulated in a PT concentration-dependent manner. For *PGC1α*, which is the transcription coactivator, the protein expression level also increased in a concentration-dependent manner. The findings demonstrated that PT could stimulate *PGC1α* and activate mitochondria via branched-chain amino acid metabolism and the electron transport chain in C2C12 myoblasts. Furthermore, PT suppressed LPS-induce expression of IL6 and TNFα, and reduced the protein expression levels of the ubiquitin ligases *Atrogin-1* and *MuRF*, which are major cause of muscle atrophy. These results indicated that PT could be effective for muscle building and suppression of atrophy.

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Introduction

Sarcopenia is a disorder characterised by progressive and generalised reduction in skeletal muscle mass due to aging and illness, thus resulting in muscle weakness and deterioration of physical function. Since sarcopenia was proposed as a formal disease in 1989, muscle loss due to aging and disease has become a major problem in an aging society worldwide (Ali and Kunugi, 2020). Skeletal muscle grows and enlarges through the action of satellite cells in muscle fibres. Stimulated satellite cells differentiate into progenitor cells known as myoblasts, which proliferate via division and then differentiate into myocytes. Subsequently, myocytes fuse to form myotubes, leading to skeletal muscle growth by fusing with existing muscle fibres (Hartman and Spudich, 2012). Differentiation into myotubes is primarily regulated by transcription factors in the myogenic regulatory factor (MRF) family, including myoblast determination protein (*Myod*), myogenin (*Myog*), myogenic factor 6 (MRF4), and myogenic factor 5 (MRF5) (Hernández-Hernández *et al.*, 2017). Among these, *Myod* and MRF-5 determine whether satellite cells differentiate into myoblasts, and play an important role in their maintenance. *Myog* exerts an essential function in the early stages of differentiation into myotubes, whereas MRF4 is expressed in the late stages of differentiation and maintains myotubes (Hughes *et al.*, 1993). Treatment for sarcopenia must not only promote the growth of new muscle mass to replace that which has been lost, but also should prevent further muscle atrophy. A cause of sarcopenia is the degradation of skeletal muscle due to inflammation, which causes mitochondrial dysfunction and activates the ubiquitin ligases, *MuRF* and *Atrogin-1* (Bodine and Baehr, 2014). Consequently, upregulated muscle degradation by proteasome leads to decreased muscle mass.

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Sarcopenia induces the development of diabetes and pneumonia (Mesinovic *et al.*, 2019; Okazaki *et al.*, 2020). Quercetin, catechin, and resveratrol, which have anti-inflammatory effects in foods, reportedly prevent muscle atrophy by suppressing the expression of these ubiquitin ligases (Hemdan *et al.*, 2009; Mukai *et al.*, 2010). Sufficient dietary protein intake is critical to the growth of muscle mass. In recent years, foods that activate MRFs, and show a muscle-building effect have attracted attention as a preventive and countermeasure for sarcopenia. Catechin, which is present in green tea, promotes the expression of *Myog* by activating TAZ, a transcription coactivator of *Myod* (Kim *et al.*, 2017). Folic acid is abundant in spinach and chicken liver, and promotes the expression of *Myod* and *Myog* by regulating the protein kinase B (AKT) signalling pathway (Hwang *et al.*, 2015). Moreover, insulin-like growth factor 1 (*IGF-1*) stimulates myoblast proliferation and differentiation. In addition, plum-derived polyphenols increase *IGF-1* levels and promote muscle hypertrophy (Alsolmei *et al.*, 2019). C2C12 cells, which are typical myoblasts, are typically used in *in vitro* skeletal muscle differentiation experiments. A few studies have identified functional foods that promote differentiation into myotubes using C2C12 cells, which has the potential to ameliorate sarcopenia by promoting muscle growth. Although exercise is the best approach for gaining muscle mass, this is not always viable (particularly in elderly patients with reduced mobility), and the promotion of muscle growth through proper diet remains an important treatment strategy.

In 2013, the Food and Agriculture Organization of the United Nations suggested the proactive use of insects as food for humans, and as feed for animals, and in 2015, the European Union also regulated insects as a novel food source. Despite official recognition, the consumption of insects remains rare in Europe. However, insects as food contain large amounts of important nutrients such as lipids, fatty acids, and vitamins, in addition to high levels of proteins (de Castro *et al.*, 2018; Kim *et al.*, 2019). The medicinal properties of edible insects have been studied, demonstrating that they have antioxidant effects, and can reduce blood pressure (Vercruysse *et al*., 2005; Dutta *et al*., 2016).

"Faeces tea" is prepared using insect faeces in China (Xu *et al.*, 2013). After feeding, the moth larvae crushed naturally fermented leaves that are further fermented by enzymes in the insect digestive tract (Xu *et al.*, 2013). Faeces tea contains not only several nutrients such as amino acids, minerals, and fatty acids, but also antioxidants, including polyphenols (Xu *et al.*, 2013; Zhao *et al.*, 2018). Various studies have explored the medicinal properties of faeces tea such as anti-inflammatory and anti-obesity effects (Zhao *et al.*, 2017; 2018). In Japan, it is customary to eat salted cherry leaves because of their pleasant scent. Cherry leaves are a primary food source for the larvae of *Phalera flavescens*, also known as the cherry caterpillar. These larvae, and the faeces they deposit on the leaves, carry this scent and are not considered to detract from the food. However, the properties of cherry caterpillar faeces tea have not been examined. In the present work, we investigated the efficacy of cherry caterpillar faeces tea for treating sarcopenia, particularly concerning muscle building and atrophy suppression. We used C2C12 cells, which are mouse striated muscle cells that are frequently used in skeletal muscle differentiation experiments *in vitro*.

Materials and methods

Preparation of faeces tea of P. flavescens

The faeces used in the present work were those of cherry caterpillars feeding on cherry (*Prunus yedoensis* Matsumura) leaves on the Yamaguchi University campus (Yamaguchi, Japan). The *P. flavescens* larvae were bred at room temperature. Faeces were collected during breeding. Faeces tea of *P. flavescens* (1 g) was extracted using boiled water (20 mL) for 10 min. The faeces solution was centrifuged at 10,000 *g* for 10 min and filtered through a 0.2-µm pore Steradisc (Kurabo, Osaka, Japan). The filtrate obtained (~20 mg faeces/mL) was used as the *P. flavescens* tea (PT). The test solutions with 0.05, 0.1, and 0.2 mg faeces/mL were prepared by diluting PT with assay medium, and used for *in vitro* assays involving C2C12 cells.

Cell culture and differentiation

C2C12 cells were obtained from the European Collection of Authenticated Cell Cultures, and cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/mL penicillin, and 100 U/mL streptomycin (Sigma-Aldrich) at 37°C under 5% CO_2. Penicillin and streptomycin were added to all

culture media used thereafter. Cells were seeded at 1.5×10^4 cells per well in 24-well plates, and cultured in growth medium for 3 d until reaching 100% confluency. The medium was changed to differentiation medium (Dulbecco's Modified Eagle's Medium supplemented with 2% foetal horse serum (Gibco)) to induce differentiation. The cytotoxicity of PT on C2C12 cells was examined using a Cell Counting Kit-8 following the manufacturer's instructions (Dojindo Laboratories, Kumamoto, Japan).

Immunocytochemistry

The primary antibody used for immunostaining was monoclonal anti-slow skeletal myosin heavy chain (ab11083; Abcam, Cambridge, UK; 1:300), and the secondary antibody was anti-IgG + IgM (H + M), mouse, goat-poly, fluorescein isothiocyanate (Funakoshi, Tokyo, Japan). The cells were fixed in 4% formaldehyde for 15 min after 3 d of culture in differentiation medium containing PT (0.05, 0.1, or 0.2 mg/mL), washed with phosphate-buffered saline (PBS), and blocked in 2% foetal bovine serum at room temperature (20 - 30°C) for 1 h. The primary antibody was incubated overnight at 4°C. After several washes with PBS, the secondary antibody was incubated for 1 h at room temperature (20 - 30°C). The cells were then washed with PBS, and counterstained with DAPI Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Fluorescence images were captured using a microscope equipped with CellSens Pro (Olympus, Tokyo, Japan). The width of the immunostained myotubes was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The average width was obtained from five randomly selected fields for each treatment, and the experiments were performed independently in triplicate.

mRNA preparation and real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

C2C12 cells in a 24-well plate were incubated in differentiation medium containing PT (0.05, 0.1, or 0.2 mg/mL). The cells were harvested 2 d after inducing differentiation, and total RNA was extracted from the cells using an RNeasy Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. cDNA was synthesised using random primers and the PrimeScript Reverse Transcriptase kit (Takara Bio, Shiga, Japan). An aliquot of cDNA was used as a template for qRT-PCR in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The target cDNAs were amplified using Fast SYBR Green Master Mix (Applied Biosystems) with the following gene-specific primers: *Myod* (MA128901; Takara Bio), *Myog* (MA127738; Takara Bio), *MYHC* (MA117697; Takara Bio), *IGF-1* (MA109541; Takara Bio), *TFAM* (MA027412; Takara Bio), *ESRα* (MA128422; Takara Bio), *ACAD* (MA078709; Takara Bio), *ACOX* (MA061629; Takara Bio), *CPT1* (MA031170; Takara Bio), *BCAT* (MA102865; Takara Bio), *BCKDH* (MA111227; Takara Bio), *SDHA* (MA115726; Takara Bio), and *PGC1α* (sense, 5′-CACCAAACCCACAGAAAACAG-3′; antisense, 5′-GGGTCAGAGGAAGAGATAAAGTTG-3′). The relative expression level of each mRNA was normalised to that of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; MA050371, Takara Bio).

Protein quantification by the Jess Simple Western System

Protein quantification was performed with the Jess Simple Western System (ProteinSimple, San Jose, CA, USA). Proteins extracted from C2C12 cells with cOmplete™ Lysis-M (Sigma-Aldrich), and protein lysates (0.5 mg/mL) were mixed with fluorescent master mix, and heated at 95°C for 5 min. Then, mixed protein lysates, wash buffer, target primary antibody and secondary-HRP (Anti-Mouse Secondary Antibody or Anti-Rabbit Secondary Antibody; ProteinSimple), and chemiluminescent substrate were dispensed into designated wells in a 12 - 230 kDa Pre-filled Plates (ProteinSimple). Primary antibody against *GAPDH* was purchased from Cell Signalling Technology (MA, Danvers, USA), and diluted 1:25; primary antibody against *PGC1α* was purchased from Proteintech (IL, Rosemont, USA) and diluted 1:100. A Muscle Atrophy Ubiquitin Ligase Antibody Sampler Kit (ECM biosciences, KY, Versailles, USA) was used as the primary antibody. Primary antibody against *Atrogin-1* and *MuRF* were diluted 1:50. The plate and the attached 25 capillary cartridges was loaded into the instrument. Data were obtained using Compass for Simple Western software (ProteinSimple). The relative expression level of each protein was normalised to that of the housekeeping protein *GAPDH*.

Mitochondrial staining

Active staining of mitochondria was performed using the JC-1 MitoMP Detection Kit (Dojindo Laboratories). After 2 d of incubation in differentiation medium containing PT (0.05, 0.1, or 0.2 mg/mL), the medium was changed to 20 µmol/L JC-1, and the cells were incubated at 37°C under 5% CO_2 for 1 h. After washing the cells with PBS, the imaging buffer provided with the kit was added to the cells. Fluorescence images were captured using a microscope equipped with CellSens Pro.

Lipopolysaccharide (LPS)-induced inflammation in C2C12 cells

C2C12 cells in a 24-well plate were incubated in differentiation medium at 37°C under 5% CO_2. Five days after inducing differentiation, the medium was changed to 500 µL differentiation medium containing PT (0.1 or 0.2 mg/mL), and pre-incubated for 2 h. The cells were incubated with the addition of 2 µg of LPS, for 2 h. The cells were then harvested, and the total RNA was extracted using an RNeasy Kit. The expression levels of *Atrogin-1*, *MuRF*, TNFα, and IL-6 were examined by qRT-PCR. *Atrogin-1* (MA117269, Takara), *MuRF* (MA061093, Takara), TNFα (MA031450, Takara), and IL6 (MA152279, Takara) were used as primers. When examining the protein expression levels of Atrogin1 and *MuRF*, the same procedure was performed, cells were collected 24 h after LPS addition, and their expression levels were measured by the Jess Simple Western System.

Statistical analysis

Data were analysed by one-way analysis of variance followed by Dunnett's test using GraphPad Prism (GraphPad, Inc., San Diego, CA, USA). Statistical significance was denoted by values of $p < 0.01$ or $p < 0.05$.

Results

Effect of P. flavescens larval faeces tea (PT) treatment on C2C12 cell differentiation into myotubes

The effect of PT on morphological changes in C2C12 cells related to the differentiation process was investigated by immunostaining of the myosin heavy chain (Figure 1A). The width and number of immunostained myotubes were measured to determine the effects of PT treatment on myogenic differentiation. Results showed that the width of myotubes increased in a concentration-dependent manner. Particularly, at a PT concentration of 0.2 mg/mL, the width increased by approximately 40% (Figure 1B). There was no significant difference in the number of cells; although, a concentration-dependent increase was observed (Figure 1C). These suggested that PT treatment could promote the myogenic differentiation of C2C12 cells. There were no significant differences in cell viability between different concentrations of PT.

Effect of PT treatment on gene expression of MRFs and energy metabolism in C2C12 cells

The expression of *Myod* and *Myog* coding for MRFs and *MYHC* was examined by qRT-PCR 2 d after the initiation of differentiation of cells treated with PT (Figure 2A). The expression levels of all three genes increased in a concentration-dependent manner following PT addition. In addition to MRFs, energy metabolism is also involved in the gain of muscle mass (Gill *et al.*, 2018); therefore, we investigated the expression levels of genes related to energy metabolism via qRT-PCR 2 d after the initiation of differentiation of cells treated with PT (Figure 2B). We found that the addition of PT increased the expression levels of *PGC1α* and *TFAM*, as well as the β-oxidation-related genes *ESRα* and *CPT1*. There was no change in the expression of *ACOX* and *ACAD*. Additionally, PT induced a concentration-dependent increase in the expression of *SDHA*, which is involved in the electron transport chain. Furthermore, there were significantly increased expression levels of *BCAT* and *BCKDH*, factors in branched-chain amino acid (BCAA) metabolism. Finally, PT increased the protein expression level of *PGC1α*, a transcription coactivator, using the Jess Simple Western System in a concentration-dependent manner (Figure 2C).

Effect of PT treatment on mitochondria in C2C12 cells

To confirm whether PT activated mitochondria, active staining of mitochondria in C2C12 cells was performed 2 d after the initiation of differentiation in cells treated with PT (Figure 3). The intensity of the red colour clearly increased in cells treated with PT as compared to control cells, thus indicating that PT activated mitochondrial biosynthesis and metabolism.

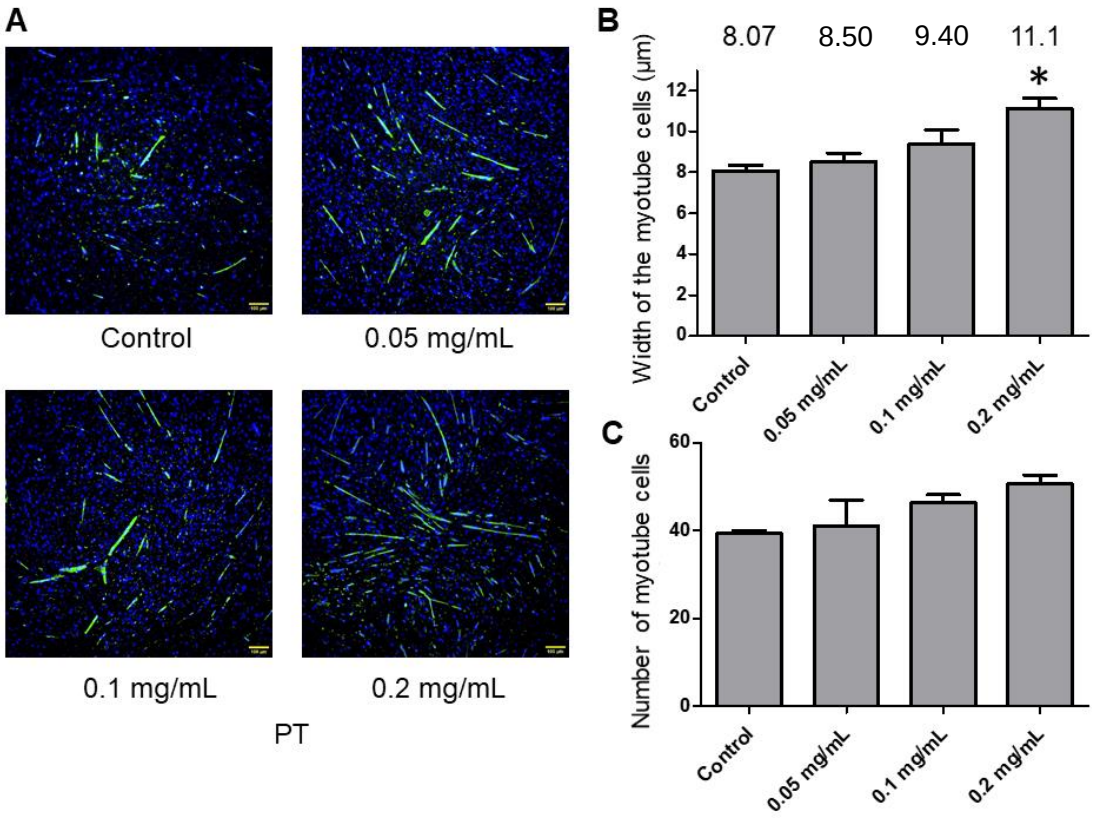

Figure 1. Effects of PT treatment on C2C12 myoblast differentiation. **(A)** Representation images of differentiated cells after treatment with several concentrations of PT showing nuclei stained in blue and myotubes stained in green. Pictures were taken using an Olympus fluorescence microscope. The bar represents a length of 100 µm. **(B)** The width of the immunostained myotubes from five random fields measured using the ImageJ software. The numbers above the bars indicate the average width (µm). **(C)** The effect of PT treatment on the number of myotubes. Values are expressed as the mean ± standard error of the mean (n = 3). *p < 0.05 as compared to the control. PT, *Phalera flavescens* larval faeces tea.

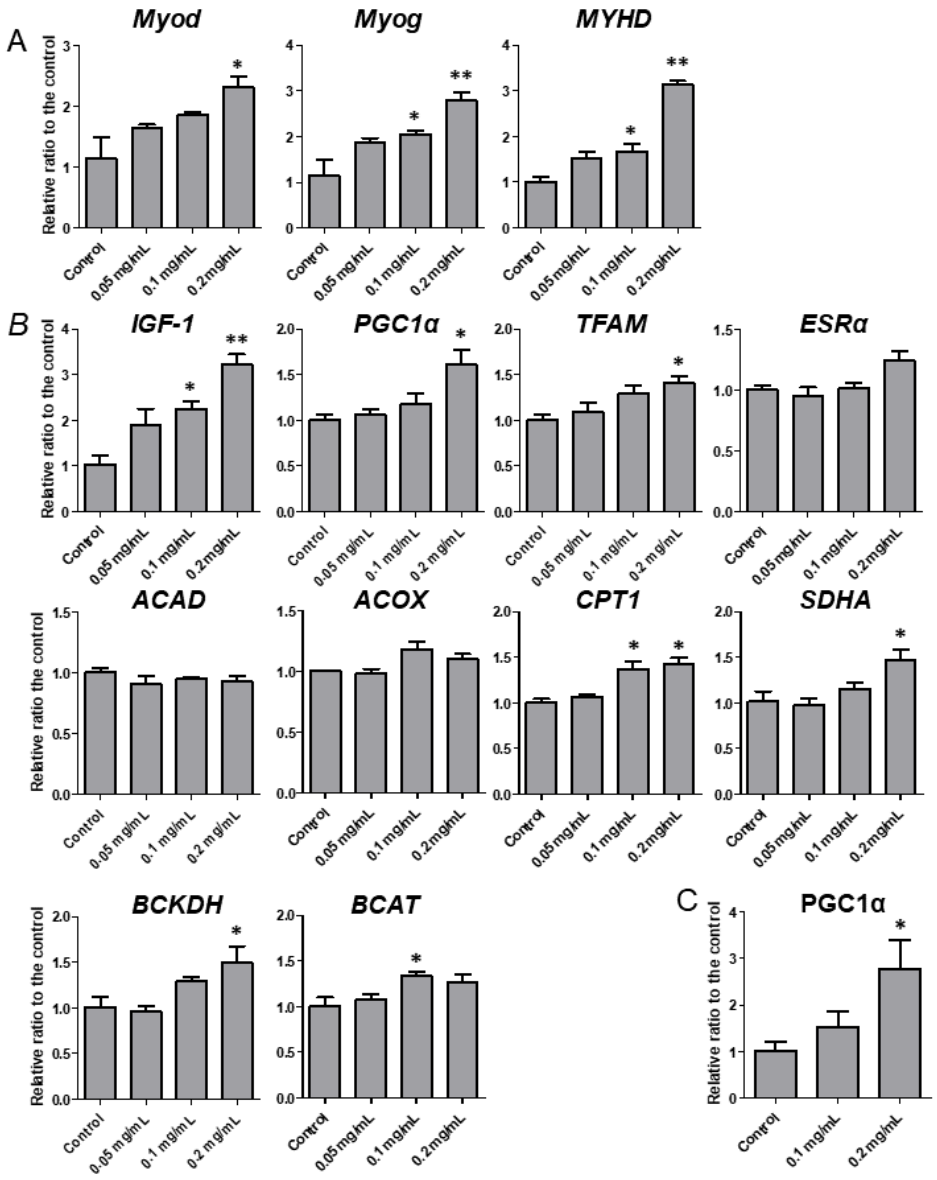

Figure 2. Effect of PT treatment on the expression of myogenic regulatory factor family genes and energy metabolism genes, and the protein expression of *PGC1α* in C2C12 cells. **(A)** and **(B)**: The expression of each gene was examined via real-time PCR two days after the initiation of differentiation of cells treated with PT. **(A)** The effect of PT treatment on the expression of myogenic regulatory factor family genes; **(B)** The effect of PT treatment on the expression of energy metabolism genes. Each mRNA level was normalised to the *GAPDH* level. **(C)** The protein on of *PGC1α* was examined via the Jess Simple Western System two days after the initiation of differentiation of cells treated with PT. Protein level was normalised to the *GAPDH* level. Values are expressed as the mean ± standard error of the mean (n = 3). *$p < 0.05$ and **$p < 0.01$ as compared to the control group as the mean ± standard error of the mean (n = 3). PT, *Phalera flavescens* larval faeces tea; PCR, polymerase chain reaction; *Myod*, myoblast determination protein; *Myog*, myogenin; *MYHC*, Myosin heavy chain; *IGF-1*, insulin growth factor 1; *PGC1α*, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; *TFAM*, mitochondrial transcription factor A; *ESRα*, oestrogen-related receptor alpha; *ACAD*, acyl-CoA dehydrogenase; *ACOX*, acyl-CoA oxidase; *CPT1*, carnitine/choline acetyltransferase 1; *SDHA*, succinate dehydrogenase complex subunit A flavoprotein variant; *BCAT*, branched-chain aminotransferase; and *BCKDH*, α-ketoacid dehydrogenase.

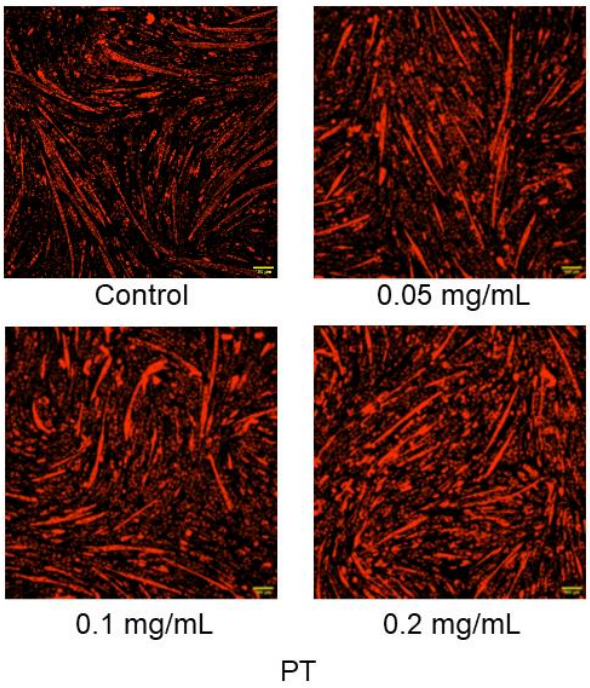

Figure 3. Mitochondria active staining via the JC-1 MitoMP Detection Kit. After two days of incubation in a differentiation medium containing PT (0.05, 0.1, or 0.2 mg/mL) and control, the medium was changed to 200 nmol/L MitoRed (Dojindo Laboratories), and the cells were incubated at 37°C under 5% CO_2 for one hour. Fluorescence images were captured using a microscope equipped with CellSens Pro. Mitochondria were stained red. PT, *Phalera flavescens* larval faeces tea.

Effect of PT on LPS-induced inflammation in C2C12 cells

LPS was added to C2C12 cells to induce inflammation to determine the effect of PT on inflammation. The gene expression levels of the inflammatory cytokines *IL-6* and *TNFα* were significantly suppressed in C2C12 cells 2 h after LPS addition, thus suggesting that PT had an anti-inflammatory effect (Figure 4A). *Atrogin-1* and *MuRF* decreased slightly but not significantly (Figure 4A). However, the expression level of these *Atrogin-1* and *MuRF* was significantly suppressed 24 h after the addition of LPS (Figure 4B).

Discussion

It has been shown that *IGF-1* promoted skeletal muscle cell differentiation by upregulating *Myog* via the phosphatidylinositol 3-kinase/AKT signalling pathway (Delling *et al.*, 2000; Machida and Booth, 2004). Additionally, daidzein, a soybean-derived isoflavone, increased muscle mass by stimulating *IGF-1* (Zheng *et al.*, 2018). Betaine present in various foods stimulated *IGF-1* to promote the differentiation of C2C12 cells (Senesi *et al.*, 2013). These studies indicated that PT induced the expression of *IGF-1* to promote differentiation. Our results also showed that PT treatment increased the expression levels of *Myod*, *Myog*, and *IGF-1* in C2C12 cells, and that PT promoted the differentiation of myoblasts. In addition to the upregulation of MRFs, we confirmed the upregulation of the gene and protein expression levels of *PGC1α* in the process of C2C12 cell differentiation via PT treatment. *PGC1α* is a major regulator of mitochondrial biosynthesis (Luo *et al.*, 2016). In addition to its role as a transcription factor, *TFAM* has a role as a structural protein that non-specifically binds to mtDNA and maintains its structure and stability, and is activated by *PGC1α* via nuclear respiratory factors, thus resulting in the activation of mitochondrial biosynthesis (Matsukawa *et al.*, 2017; Gureev *et al.*, 2019). In fact, the gene expression level of *TFAM* increased in this experiment. Despite the upregulation of *ESRα* and *CPT1*, there was no change in the expression of *ACOX* and *ACAD*. Because *ACOX* is localised in the peroxisomes, it was predicted that the addition of PT, which is thought to activate mitochondria, would not affect the expression level of *ACOX*. *ESRα* is known to activate the expression of genes involved in energy metabolism, such as those associated with β-oxidation and the electron transport chain of mitochondria in skeletal muscles (Ventura-Clapier *et*

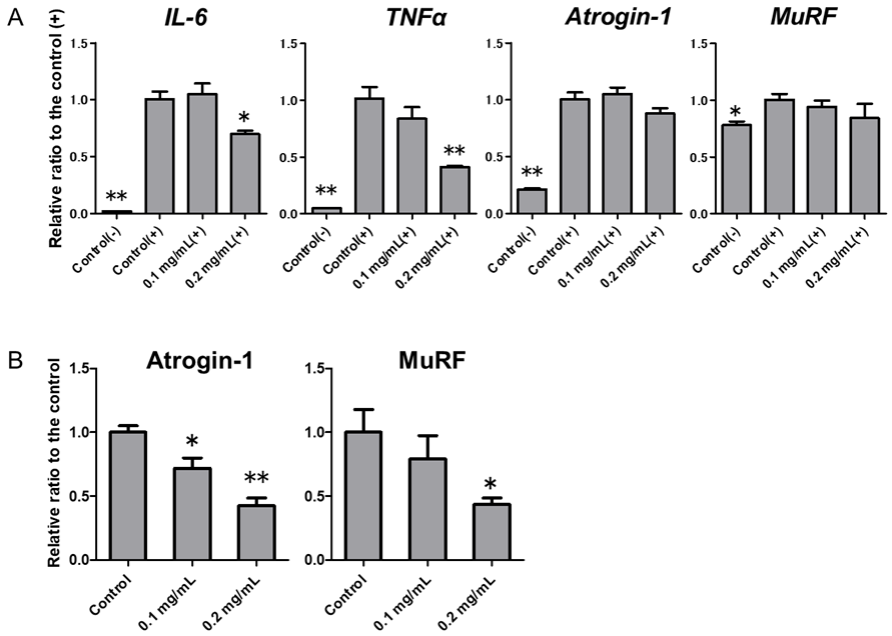

Figure 4. PT-mediated effects on LPS-treated C2C12 myotubes. The C2C12 cells were cultured in differentiation medium for five days. After two hours of pre-treatment with PT (0.1 and 0.2 mg/mL), the cells were stimulated with LPS. **(A)** Two hours after stimulated with LPS, the total RNA was extracted from the C2C12 cells, and RT-PCR for IL-6, TNFα, *Atrogin-1*, and *MuRF* were performed. Each mRNA level was normalised to the *GAPDH* level. **(B)** A day after stimulated with LPS, the protein was extracted from the C2C12 cells, and the Jess Simple Western System for *Atrogin-1* and *MuRF* were performed. Each protein level was normalised to the *GAPDH* level. Values are expressed as the mean ± standard error of the mean (**(A)**; $n = 3$, **(B)**; $n = 4$). *$p < 0.05$ and **$p < 0.01$ as compared to the control or control (+) group as the mean ± standard error of the mean. PT, *Phalera flavescens* larval faeces tea; PCR, polymerase chain reaction; IL-6, interleukin-6; TNFα, tumour necrosis factor alpha; *MuRF*, muscle specific ring finger protein; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; and LPS, lipopolysaccharide.

al., 2019). In addition, as *ESRα* targets *CPT1* (Ventura-Clapier *et al.*, 2019), *CPT1* was predicted to be upregulated via the activation of *ESRα*. However, although *CPT1* is an early initiation gene for β-oxidation localised in the outer mitochondrial membrane, we found that the expression level of *ACAD* did not change. Alternatively, PT treatment activated only the uptake of long-chain fatty acids, and did not affect β-oxidation. Furthermore, in experiments using transgenic mice overexpressing *PGC1α* in skeletal muscles, the expression levels of *BCAT* and *BCKDH* significantly increased as compared to those of wild-type mice (Hatazawa *et al.*, 2014). *BCAT* is an enzyme that transfers BCAA to branched-chain keto acids in the first step of the BCAA cycle. In the second step, the enzyme encoded as *BCKDH* decarboxylates branched-chain keto acid, and converts it to a coenzyme A compound (Karlsson *et al.*, 2006). In the present work, *BCAT* and *BCKDH* were upregulated by the activation of *PGC1α* in C2C12 cells, thus indicating that PT could affect BCAA metabolism. BCAA metabolism leads to the activation of the electron transport chain via the TCA cycle (Li *et al.*, 2017). Moreover, we found that PT activated *SDHA*, thereby affecting the electron transport chain by activating upstream BCAA metabolism. Mitochondrial activity and muscle mass are closely related, as supported by the fact that mitochondrial activation restores the differentiation ability of C2C12 cells (Watanabe *et al.*, 2020), which may lead to an increase in muscle mass. Collectively, these results suggested that PT treatment could promote the differentiation of C2C12 cells into myotubes via signal transduction, as shown in Figure 5. Plant polyphenols activate *PGC1α* in skeletal muscle (Ray Hamidie *et al.*, 2015; Wang *et al.*, 2016). There is possibility that polyphenols in PT could activate *PGC1α* and promote the differentiation of C2C12 cells.

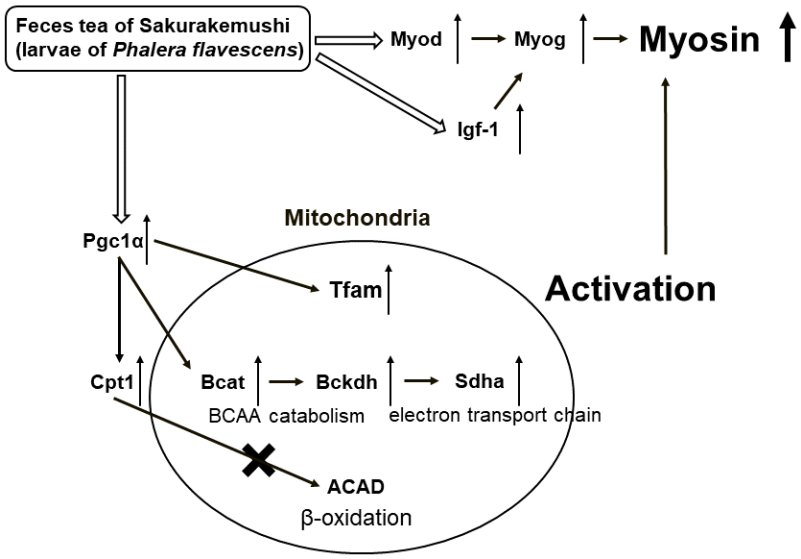

Figure 5. Schematic representation of the signal transduction activated via PT treatment of C2C12 cells. PT induced *Myod* and *IGF-1* to promote differentiation. PT stimulated *PGC1α* and activated *TFAM*. It was found that BCAA metabolism was activated by PT based on the upregulation of *BCAT* and *BCKDH*. The electron transport chain downstream of BCAA metabolism was activated based on upregulation of *SDHA*. The upward arrows indicate upregulation. PT, *Phalera flavescens* larval faeces tea; *Myod*, myoblast determination protein; *Myog*, myogenin; *MYHC*, Myosin heavy chain; *IGF-1*, insulin growth factor 1; *PGC1α*, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; *TFAM*, mitochondrial transcription factor A; *ACAD*, acyl-CoA dehydrogenase; *CPT1*, carnitine/choline acetyltransferase 1; *SDHA*, succinate dehydrogenase complex subunit A flavoprotein variant; *BCAT*, branched-chain aminotransferase; and *BCKDH*, α-ketoacid dehydrogenase.

There are many reports that plant polyphenols have anti-inflammatory and antioxidant effects, and suppress muscle atrophy (Hemdan *et al.*, 2009; Mukai *et al.*, 2010). The cherry blossoms used as feed in the present work reportedly have antioxidant and anti-inflammatory effects (Takahashi *et al.*, 2006; Lee *et al.*, 2013). In the present work, in addition to promoting the differentiation of C2C12, PT suppressed protein expression of the ubiquitin ligases *Atrogin-1* and *MuRF*. In skeletal muscle, muscle degradation is promoted by *Atrogin-1* and *MuRF* induced by signals from inflammatory cytokines such as TNF-α and IL-6 (Grounds, 2002).

A study comparing the polyphenols of Kuding tea, which used Kuding tea leaves as feed and insect faeces tea, showed that insect faeces tea had a greater antioxidant effect (Zhao *et al.*, 2018). Although these polyphenols have not been identified, it is predicted that the polyphenols of Kuding tea could have increased antioxidant activity as a result of transformation in insects. The flavonoids epigallocatechin and gallocatechin are converted by gut bacteria (*Adlercreutzia equolifaciens* JCM 14793, *Asaccharobacter celatus* JCM 14811, *Slackia equolifaciens* JCM 16059, and *S. isoflavoniconvertens* JCM 1613712) that metabolise isoflavones (Takagaki and Nanjo, 2015). For example, the soy isoflavone daidzein is metabolised in gut bacteria such as the lactic acid bacterium, *Lactococcus garvieae*, to equol, which has stronger estrogenic activity (Shimada *et al.*, 2012; Mayo *et al.*, 2019). These facts indicate that polyphenols in plants that are consumed by the insects may be converted by the insects into other active ingredients, and deposited in their faeces by gut bacteria or during the digestive process of feeding (Dillon and Dillon, 2004). Therefore, the fermented ingredients in faeces of cherry caterpillars feeding on the leaves may have contributed to the beneficial effects of PT. In fact, the leaves of cherry blossoms and the body of cherry caterpillar do not have the effect of promoting the differentiation of C2C12. In addition, as PT has

antioxidant activity, it is highly possible that it contains more effective polyphenols derived from cherry leaves. Further studies are needed to isolate and identify the active ingredients in PT to exploit them for their medicinal value.

Conclusion

Insect-based food consumption has gained attention due to its nutritional value and other benefits such as its medicinal value as a source of new bioactive molecules. The present work investigated the efficacy of PT for treating sarcopenia using C2C12 mouse myoblasts. Sarcopenia is a particular target for novel drug treatment as it frequently afflicts the elderly who are unable to counteract it with physical exercise, and instead reliant on drug treatment. The present work suggested that PT could induce the differentiation of myoblasts into myotubes, and activate C2C12 mitochondrial metabolism. Furthermore, PT could have an anti-inflammatory effect, and suppress muscle atrophy. This indicated that PT could be an effective beverage for the treatment of sarcopenia, and further study to identify its active ingredients would be fruitful.

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