EUR REV MED PHARMACOL SCI
2025; 29: 425-433

MULBERRY LEAF EXTRACTS AND QUERCETIN GLYCOSIDES PROMOTE GLYCOGEN ACCUMULATION IN ASTROCYTES

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ABSTRACT – *Objective:* The astrocyte–neuron lactate shuttle is the mechanism by which astrocytic lactate is transferred to neurons to maintain brain function and is modulated by astrocytic glycogen content. The aims of this study were to determine whether mulberry leaf extracts promote astrocytic glycogen accumulation and to explore the active compounds of mulberry leaf extracts.

Materials and Methods: Glycogen content was determined in human-induced pluripotent stem cell-derived astrocytes treated with mulberry leaf extracts or their active compounds. To determine the involvement of active compounds in glycogen synthesis and degradation, human-induced pluripotent stem cell-derived astrocytes were treated with glucose-free medium containing ^{13}C-glucose, and the amounts of ^{12}C- and ^{13}C-glucose in glycogen were quantified.

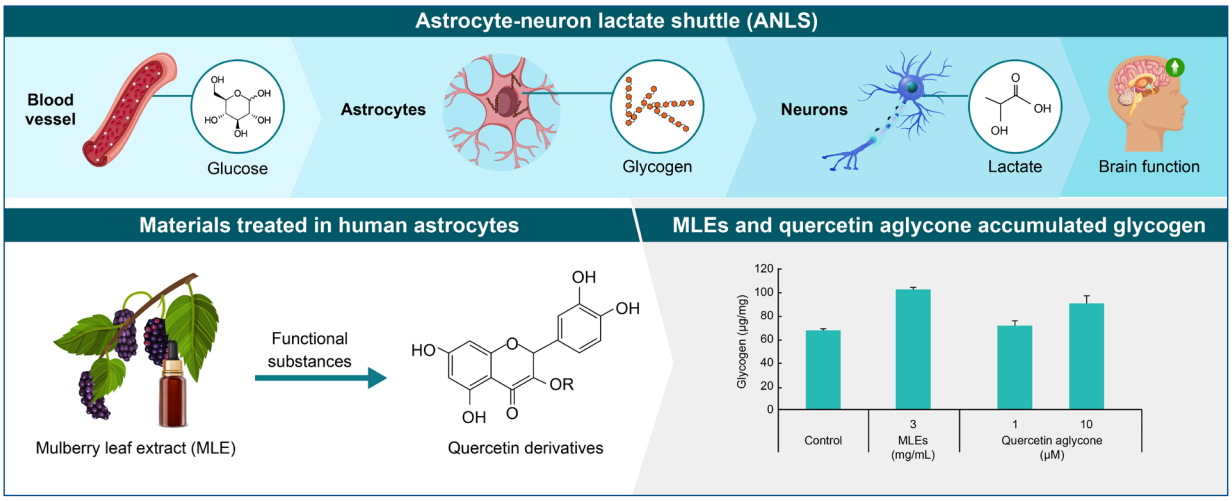
Results: Mulberry leaf extracts promoted astrocytic glycogen accumulation. Iminosugars in mulberry leaf extracts did not significantly increase the glycogen content of astrocytes. In contrast, several quercetin glycosides exhibited some of the activities of the mulberry leaf extracts. Quercetin aglycone, the basic backbone structure without glycosides, increased astrocytic glycogen content. Additionally, quercetin aglycone increased both ^{12}C- and ^{13}C-glucose contents, meaning it promoted glycogen synthesis and inhibited glycogen breakdown.

Conclusions: In this study, quercetin glycosides were identified as active compounds present in mulberry leaf extracts. The principal compound responsible for the activity was identified as quercetin aglycone. Mulberry leaf extracts and quercetin analogs may induce astrocyte–neuron lactate shuttling by promoting the accumulation of glycogen in astrocytes.

KEYWORDS: Quercetin, Mulberry leaf, Astrocyte–neuron lactate shuttle, Glucose metabolism, Brain function, Aging, Cognitive decline.

INTRODUCTION

Age-related cognitive decline and dementia, leading causes of disability and dependency among older adults, are a growing public health concern devastating the lives of those affected, their caregivers, and their families. According to the World Health Organization, the number of people living with dementia is expected to reach 82 million by 2030 and 152 million by 2050[1]. Dementia affects between 5% and 8% of the population aged 60 years and over.



Graphical Abstract. Astrocytes produce lactic acid from glycogen, which is synthesized from glucose, and supply it to neurons in accordance with their energy demands *via* the astrocyte-neuron lactate shuttle (ANLS) system. Mulberry leaf extracts (MLEs) and quercetin analogs as functional substances promote glycogen accumulation in astrocytes, thereby potentially enhancing ANLS.

Glucose metabolism decreases with aging and in dementia[2]. In a cohort study[3], glucose hypometabolism was associated with an increased risk of mild cognitive impairment. Therefore, improving glucose metabolism may be important in treating and preventing dementia in older adults. The astrocyte–neuron lactate shuttle (ANLS) is crucial for cognition[4-7] and has been proposed as the mechanism by which astrocytic lactate is supplied as an energy substrate to neurons[8]. Astrocytes convert some glucose into glycogen in a steady state. As the energy demands of neurons increase, glycogen is converted to lactate and transported to neurons as a source of energy through the mitochondrial tricarboxylic acid cycle. Glycogen-derived lactate from astrocytes is crucial for memory consolidation and long-term potentiation synaptic plasticity[9,10]. Conditional glycogen synthase-1 knockout mice lacking brain glycogen exhibit impaired long-term memory formation[11]. The glycogen phosphorylation inhibitor CP-316819 induces glycogen accumulation in the brain of rats and sustains neuronal activity under hypoglycemic conditions[12]. Rats subjected to moderate exercise for 4 weeks show an increase in hippocampal glycogen levels and enhanced memory function[13]. Therefore, it is indicated that a steady-state increase in astrocytic glycogen involved in ANLS is important for improving glucose metabolism in patients with dementia.

In this study, we focused on mulberry leaf extracts (MLEs), which are rich in phenolic compounds, such as flavonoids and iminosugars[14], and have been shown to ameliorate cognitive decline in mice with cognitive deficits[15]. Neuronal protection through antioxidant activity and the enhancement of acetylcholine by polyphenols in MLEs enhances memory. Conversely, MLEs promote glycogen storage in the skeletal muscle of diabetic mice and the liver of diabetic rats[16,17]. However, whether MLEs induce glycogen accumulation in the brain and astrocytes remains unknown. Therefore, we evaluated the effect of MLEs on astrocytes and explored the active compounds in human-induced pluripotent stem cell (hiPSC)-derived astrocytes expressing characteristic proteins, such as S100 calcium-binding protein B and glial fibrillary acidic protein, and secreting trophic factors upon stimulation[18].

MATERIALS AND METHODS

Materials

The MLEs were purchased from Toyotama International Inc., Tokyo, Japan. CP-316819, 1-deoxynojirimycin (DNJ), 2-*O*-α-D-galactopyranosyl-DNJ (Gal-DNJ), and fagomine were purchased from Biodynamic Plant Institute Co. Ltd., Hokkaido, Japan. 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) (SC-220553) was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Isoquercitrin (HY-N1445), rutin (HY-N0148), and quercetin aglycone (HY-188085) were purchased from MedChemExpress, Monmouth Junction, NJ, USA. Quercetin 3-*O*-(6”-*O*-malonyl)-β-D-glucoside (16733) was purchased from Sigma-Aldrich, Darmstadt, Germany. iCell Astrocytes (01434) were purchased from FUJIFILM Cellular Dynamics Inc., Madison, WI, USA. Matrigel (354230) was purchased from Corning, New York, NY, USA. Astrocyte Growth Medium (AGM) (CC-3186) was purchased from Lonza Walkersville Inc., Walk-

ersville, MD, USA. Glucose-free Dulbecco's Modified Eagle's Medium (DMEM) (042-32255) and ^{13}C-glucose (574-69731) were purchased from FUJIFILM Wako Pure Chemical Co., Ltd., Osaka, Japan.

MLEs

The MLEs were standardized to contain at least 0.4% DNJ, an iminosugar and inhibitor of disaccharide-degrading enzymes[19], as the active ingredient.

Measurement of phenolic compounds in the MLEs

Targeted iminosugars (DNJ, Gal-DNJ, fagomine, and DAB) and flavonoids [isoquercitrin, rutin, quercetin 3-*O*-(6"-*O*-malonyl)-β-D-glucoside, and quercetin aglycone] were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). High-performance liquid chromatography (HPLC) separation was performed using a Nexera X2 system (Shimadzu Co., Kyoto, Japan) equipped with a Gelpack GLHilicAex column (5 µm particle size, 2.1 mm inner diameter × 150 mm) (Resonac Techno Service Co., Ibaraki, Japan) and an ExionLC AC system (SCIEX, Framingham, MA, USA) equipped with a Triart C18 column (1.9 µm particle size, 2.0 mm inner diameter × 150 mm) (YMC, Kyoto, Japan) for the introduction of iminosugars and flavonoids to the mass spectrometer, respectively. Mobile phases comprised 40 µM ammonium hydrogen carbonate (iminosugars) or 0.1% (v/v) formic acid (flavonoids) in H_2O as solvent A and acetonitrile (iminosugars) or methanol (flavonoids) as solvent B, with a flow rate of 0.3 and 0.2 mL/min, respectively. The gradient was as follows: 0-10 min, 90-40% B; 10-15 min, 100% A (iminosugars) or 0-3 min, 2% B; 3-10 min, 2-15% B; 10-20 min, 15-50% B; 20-25 min, 50-98% B; 25-30 min, 98% B (flavonoids). The column temperature was set to 40°C, and the injection volume was 2 µL. For LC-MS/MS analysis, the HPLC system was coupled with a QTRAP 6500 mass spectrometer (SCIEX) equipped with an electrospray ionization source operating in positive mode (iminosugars) or an X500R QTOF mass spectrometer (SCIEX) equipped with an electrospray ionization source operating in negative mode (flavonoids). Data were collected in the multiple reaction monitoring (MRM) mode. The MRM transitions were (+) 164 > 69, (+) 326 > 164, (+) 148 > 86, and (+) 134 > 68 for DNJ, Gal-DNJ, fagomine, and DAB and (–) 463.0877 > 300.0270, (–) 609.1456 > 300.0270, (–) 549.0880 > 300.0270, and (–) 301.0348 > 151.0031 for isoquercitrin, rutin, quercetin 3-*O*-(6"-*O*-malonyl)-β-D-glucoside, and quercetin aglycone, respectively.

hiPSC-derived astrocytes

iCell Astrocytes, a highly pure population of hiPSC-derived astrocytes[15], were plated in 48-well plates coated with Matrigel and cultured in AGM in an incubator at 37°C with 5% CO_2. Half of the medium was replaced every 2-3 days for 2 weeks.

Determination of glycogen content

hiPSC-derived astrocytes were incubated overnight with different concentrations (0.3, 1, and 3 mg/mL) of the MLEs (Figure 1A). The glycogen phosphorylase inhibitor CP-316819 was used as a positive control. Glycogen content was determined following the manufacturer's protocol (MAK016; Sigma-Aldrich). Cells were homogenized with H_2O on ice, boiled for 10 min to inactivate enzymes, and centrifuged at 18,000 × g for 10 min to remove insoluble material. The supernatants were analyzed using a colorimetric assay to quantify glycogen levels. Protein concentrations were determined using the bicinchoninic acid method (23250; Thermo Fisher Scientific, Waltham, MA, USA).

Determination of ^{12}C- and ^{13}C-glucose contents in glycogen

hiPSC-derived astrocytes were treated with quercetin aglycone in glucose-free DMEM containing 25 mM ^{13}C-glucose overnight. The supernatants, including astrocytic glycogen, were prepared as described above, hydrolyzed with an enzyme mix (MAK016; Sigma-Aldrich), and passed through a solid-phase extraction column [Strata-X 33U Polymeric Reversed-Phase (10 mg/mL)] (8B-S100-AAK; Phenomenex, Torrance, CA, USA). The glucose fraction was eluted in 200 µL of H_2O. LC-MS/MS analysis was performed on an LC-MS-8030 mass spectrometer (Shimadzu Co., Kyoto, Japan) with a Shodex Asahipak NH2P-50 2D column (5 µm particle size, 2.0 mm inner diameter × 150 mm) (Resonac Techno Service Co.) and an isocratic elution solvent (aqueous solution of 75% acetonitrile and 0.05% NH_3) at a flow rate of 0.2 mL/min. The glucose retention time was 5.6 min. Quantification was done using the MRM method. The MRM transitions were (–) 179.05 > 89.10 and (–) 185.10 > 92.05 for ^{12}C- and ^{13}C-glucose, respectively.

Statistical analysis

Data are presented as mean ± SEM. Comparisons between two groups were performed using Student's *t*-test, whereas multiple comparisons were performed using Dunnett's test or Tukey-Kramer's test. Statistical analyses were conducted using JMP (version 16.0; SAS Institute,

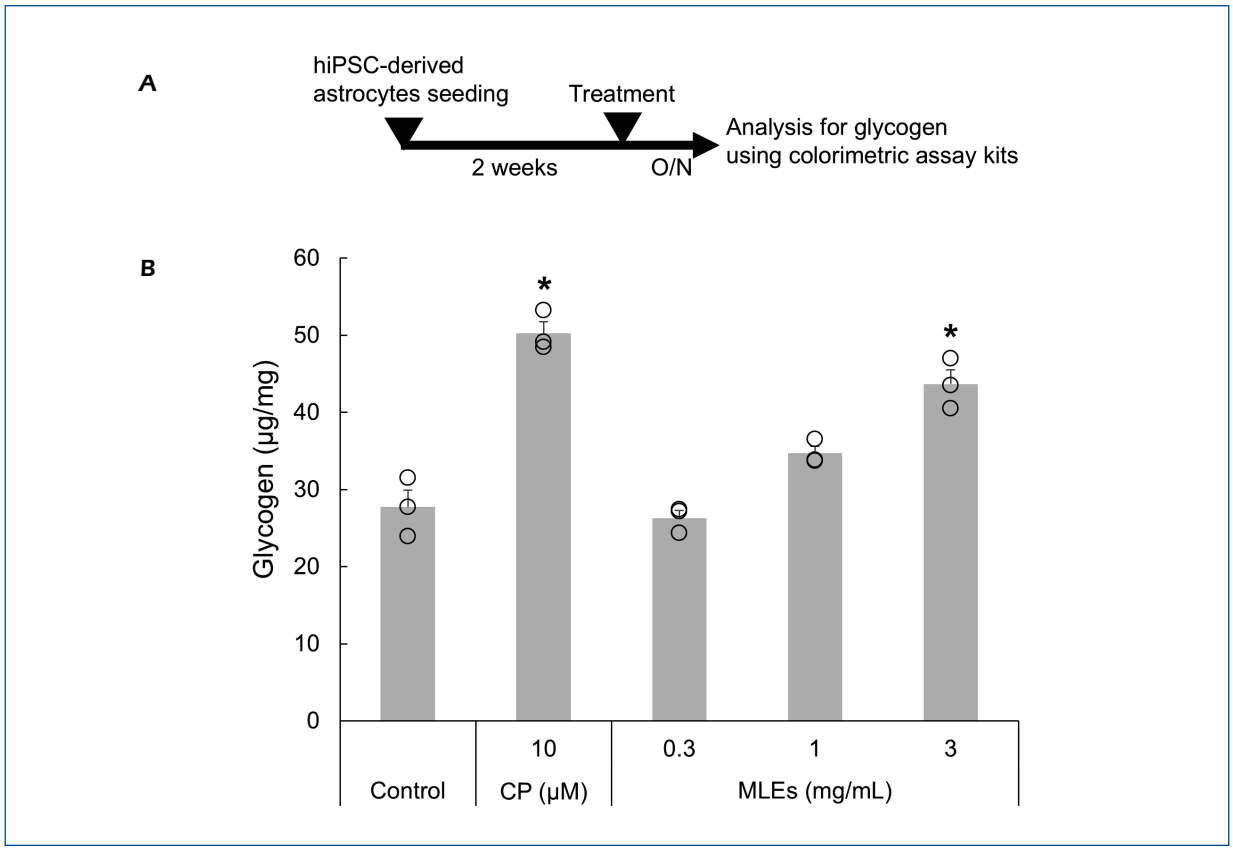


Figure 1. MLEs promoted glycogen storage in hiPSC-derived astrocytes in a dose-dependent manner. **A**, Schematic protocol for glycogen analysis. hiPSC-derived astrocytes were seeded in 48-well plates and incubated for 2 weeks. Cells were treated with CP-316819 or the MLEs overnight. **B**, Glycogen levels obtained by colorimetric assay. Data are presented as mean ± SEM (N = 3). *$p < 0.05$ *vs.* control (Dunnett's test). CP, CP-316819; hiPSC, human-induced pluripotent stem cell; MLE, mulberry leaf extract; O/N, overnight.

Cary, NC, USA). $p < 0.05$ indicated a statistically significant result.

RESULTS

MLEs promoted glycogen accumulation in hiPSC-derived astrocytes

Cells treated with CP-316819 showed an increase in glycogen content compared to control cells (Figure 1B). MLEs induced glycogen accumulation in a dose-dependent manner.

Quercetin glycosides exhibited MLE activity

Iminosugars are specific to MLEs[20,21] and have been shown to promote glycogen accumulation by inhibiting glycogen breakdown in primary rat hepatocytes[22]. The concentrations of targeted iminosugars in the MLEs were determined (DNJ, 4.62 mg/g; Gal-DNJ, 8.22 mg/g; fagomine, 0.65 mg/g; DAB, 2.87 mg/g) (Figure 2A). The effect of iminosugars was tested; however, treatment with iminosugars at a concentration equivalent to 3 mg/mL of the MLEs did not significantly increase the glycogen content of astrocytes (Figure 2B).

Flavonoids are the dominant class of phenolic compounds in the MLEs[14]. Therefore, we measured the contents of specific quercetin glycosides, which are major flavonoids. The MLEs contained isoquercitrin, rutin, and quercetin 3-*O*-(6"-*O*-malonyl)-β-D-glucoside at concentrations of 4.23, 4.22, and 1.29 mg/g, respectively (Figure 3A). Next, we evaluated the effects of quercetin glycosides on glycogen accumulation. Each compound exhibited less than 30% of the activity of the MLEs, with a mixture of these compounds exhibiting 38.2% of the activity of the MLEs (Figure 3B). Quercetin glycosides were identified as active compounds.

Quercetin aglycone, the basic backbone structure without glycosides, increased astrocytic glycogen content

Quercetin glycosides share the same basic backbone structure. Therefore, we hypothesized that the principal compound responsible

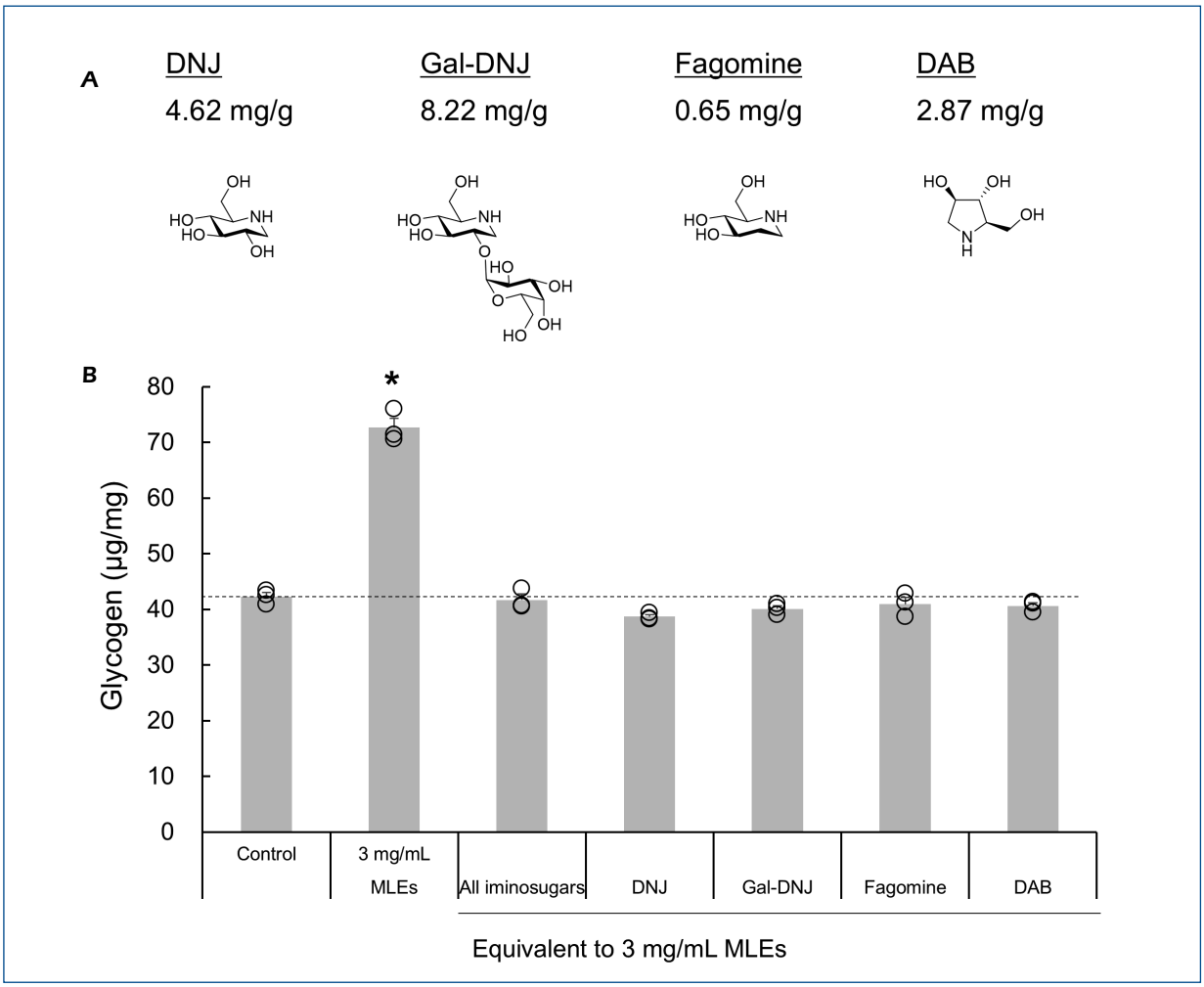


Figure 2. Iminosugars in the MLEs did not significantly increase the glycogen content of astrocytes. **A**, LC-MS/MS analysis of the iminosugars and their content in 1 g of the MLEs. **B**, hiPSC-derived astrocytes treated with the MLEs or iminosugars at a concentration equivalent to 3 mg/mL of the MLEs overnight. Glycogen content was determined using a colorimetric assay. "All iminosugars" indicates a mixture of iminosugars. Data are presented as mean ± SEM (N = 3). *$p < 0.05$ *vs.* control (Dunnett's test). DAB, 1,4-dideoxy-1,4-imino-D-arabinitol; DNJ, 1-deoxynojirimycin; Gal-DNJ, 2-*O*-α-D-galactopyranosyl-DNJ; hiPSC, human-induced pluripotent stem cell; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MLE, mulberry leaf extract.

for the activity of quercetin glycosides is quercetin aglycone. The tested concentrations of quercetin glycosides ranged from approximately 1-10 µM; therefore, quercetin aglycone was tested at concentrations of 1 and 10 µM. Figure 4A shows that treatment with 10 µM of quercetin aglycone significantly increased astrocytic glycogen content.

Then, we investigated whether quercetin aglycone affected glycogen synthesis or degradation using glucose-free DMEM containing ^{13}C-glucose (Figure 4B). ^{13}C-glucose is incorporated into glycogen in astrocytes *via* the glycogen synthesis pathway. Therefore, the involvement of quercetin aglycone in glycogen synthesis can be evaluated by measuring ^{13}C-glucose content in glycogen, with an increase in ^{13}C-glucose indicating glycogen synthesis pathway activation. The amount of ^{12}C-glucose in glycogen represents the accumulated glucose before the addition of ^{13}C-glucose and can be used to determine the involvement of quercetin aglycone in glycogen degradation. A higher ^{12}C-glucose content than that in the control group indicated inhibition of the glycogen degradation pathway. hiPSC-derived astrocytes were incubated with quercetin aglycone in a medium supplemented with ^{13}C-glucose, and the ^{12}C- and ^{13}C-glucose contents of glycogen were measured (Figure 4C). Quercetin aglycone increased the ^{12}C- and ^{13}C-glucose contents in glycogen compared with the control (Figure 4D). Quercetin aglycone treatment enhanced glycogen storage, as calculated from the sum of the ^{12}C- and ^{13}C-glucose contents (Figure 4D).

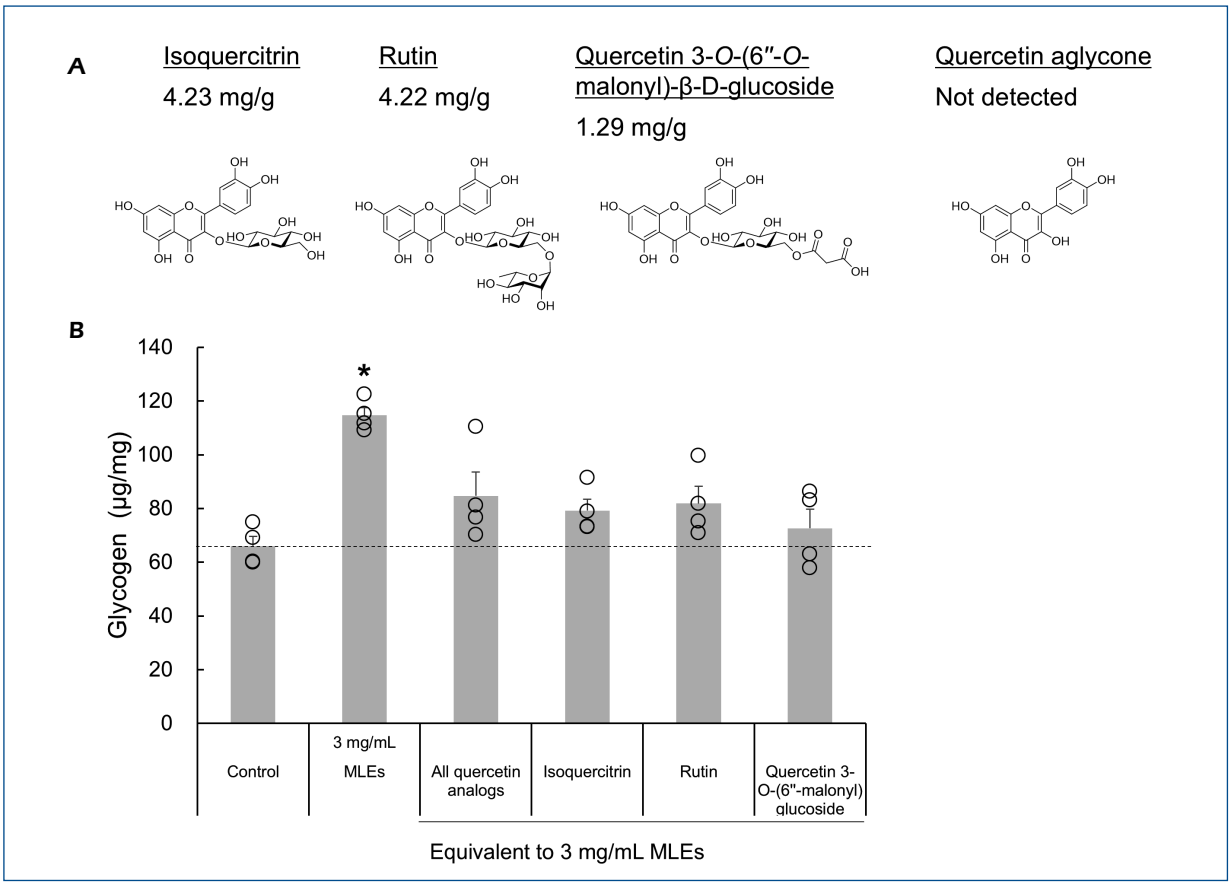


Figure 3. Quercetin glycosides exhibited partial activity of the MLEs. **A**, LC-MS/MS analysis of the quercetin analogs and their content in 1 g of the MLEs. **B**, hiPSC-derived astrocytes were treated with the MLEs or quercetin analogs at a concentration equivalent to 3 mg/mL of the MLEs overnight. Glycogen content was determined using a colorimetric assay. "All quercetin analogs" indicates a mixture of quercetin analogs. Data are presented as mean ± SEM (N = 4). *$p < 0.05$ *vs.* control (Dunnett's test). hiPSC, human-induced pluripotent stem cell; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MLE, mulberry leaf extract.

DISCUSSION

We found that MLEs could promote glycogen accumulation in hiPSC-derived astrocytes. Quercetin glycosides were identified as active compounds, exhibiting over 30% of the activity of the MLEs. Quercetin aglycone, the basic backbone of quercetin glycosides, promoted astrocytic glycogen accumulation, suggesting that quercetin aglycone, a shared structure, is responsible for the activity of quercetin glycosides. To our knowledge, this is the first study to show that MLEs and quercetin analogs promote glycogen accumulation in astrocytes.

In this study, MLEs and quercetin analogs promoted glycogen accumulation, potentially activating ANLS. As indicated in the introduction, MLEs improve cognitive function[15]. Quercetin administration has been shown to ameliorate cognitive dysfunction in rodents[23]. Additionally, quercetin glycoside intake from beverages[24] or quercetin-rich onions[25] significantly improves the cognitive function of healthy older adults. Several neuroprotective mechanisms have been proposed, including antioxidant and anti-inflammatory effects, a reduction in phosphorylated tau and amyloid plaques, and an improvement in mitochondrial damage[23]. Whether ALNS is a crucial factor in the amelioration of cognitive decline following MLE or quercetin treatment is an emerging research topic.

Using ^{13}C-glucose, we showed that quercetin aglycone promoted glycogen synthesis and inhibited glycogen degradation. The exact mechanism by which quercetin analogs promote glycogen accumulation is unknown, but they may activate Akt signaling in astrocytes. Akt promotes glycogen synthesis and inhibits glycogen degradation[26-28]. Akt activation inactivates glycogen synthase kinase-3β, further activating glycogen synthase. Akt inactivates glycogen phosphorylase, thereby preventing glycogen degradation. Both signaling pathways promote glycogen accumulation. In support of this hypothesis, quercetin

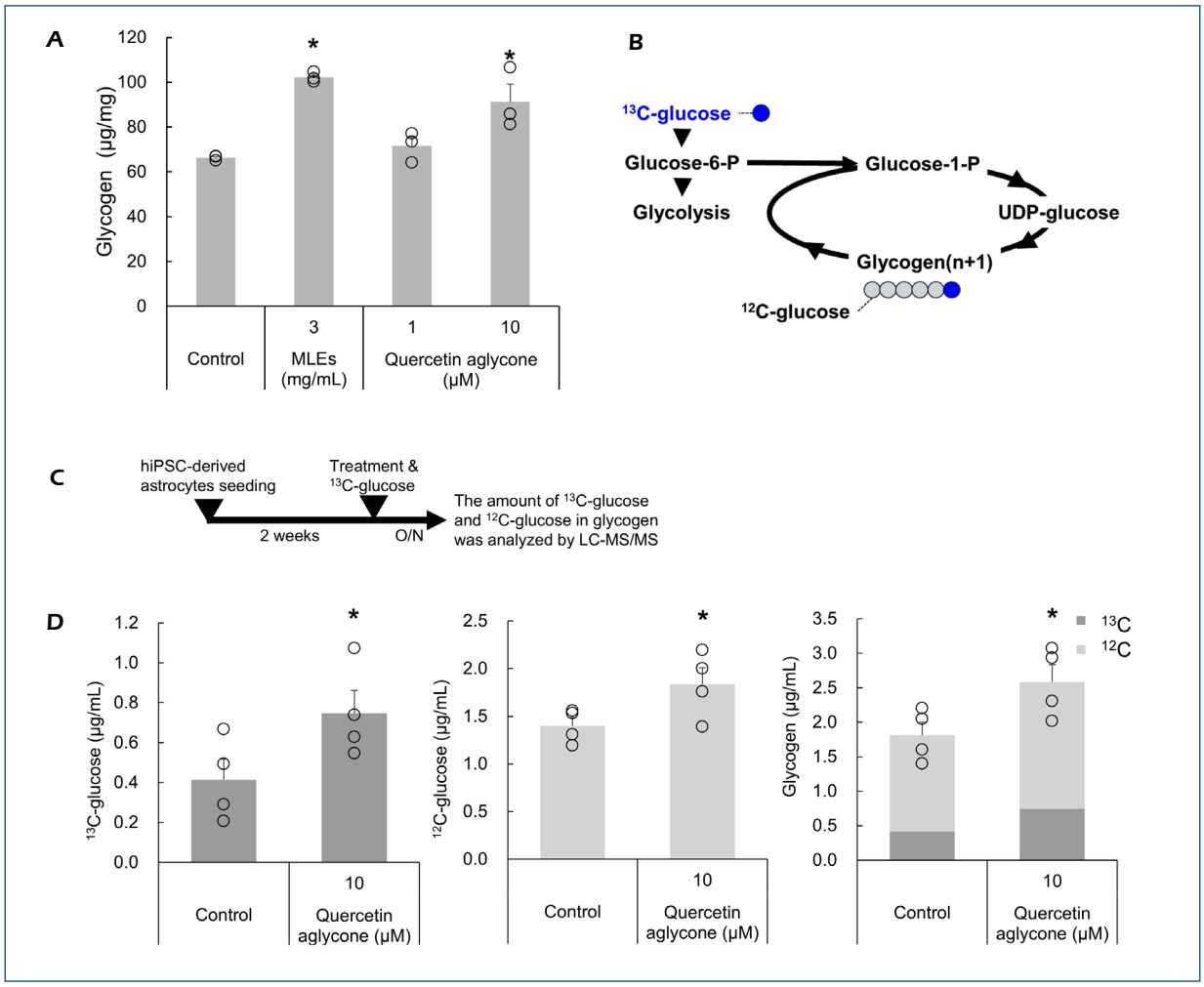


Figure 4. Quercetin aglycone significantly increased astrocytic glycogen content. **A**, hiPSC-derived astrocytes were treated with the MLEs or quercetin aglycone overnight. Glycogen content was determined using a colorimetric assay. Data are presented as mean ± SEM (N = 3). *$p < 0.05$ *vs.* control (Dunnett's test). **B**, Glycogen synthesis pathway. The growth medium contained ^{13}C-glucose, which was incorporated into glycogen in astrocytes. **C**, Scheme of the experimental protocol. hiPSC-derived astrocytes were treated with quercetin aglycone and cultured in a medium containing ^{13}C-glucose. **D**, After overnight incubation, glycogen collected from cells was hydrolyzed, and ^{12}C- and ^{13}C-glucose contents were analyzed using LC-MS/MS. Glycogen content was estimated as the sum of ^{12}C- and ^{13}C-glucose contents. Data are presented as mean ± SEM (N = 4). *$p < 0.05$ *vs.* control (Student's *t*-test).

analogs affect glycogen metabolism through Akt signaling. Studies[29,30] of streptozotocin-induced diabetic rats suggest that quercetin aglycone or quercetin glycoside administration increases hepatic glycogen levels *via* Akt signaling.

Glycogen accumulation induced by MLEs or quercetin analogs may be broken down to meet the energy demands of neurons. CP-316819 inhibits glycogen phosphorylase. This inhibitory effect is lost at low glucose concentrations, allowing for glycogen utilization at low glucose concentrations in astrocytes and hepatocytes[12,31]. Elevated brain glycogen levels support neuronal function under hypoglycemic conditions in rats administered CP-316819[12]. These studies suggest that a similar mode of action may be at play when astrocytes are treated with MLEs or quercetin analogs.

Limitations

This study has some limitations that warrant consideration. The extent to which MLEs or quercetin analogs contribute to cognitive function by activating ANLS remains to be elucidated. This can be clarified by examining whether learning and memory are affected when glycogenolysis is simultaneously inhibited with MLEs or quercetin analog treatment *in vivo*. Alternatively, ANLS involvement can be evaluated by assessing neural activity in co-cultures of neurons and astrocytes. As glucose metabolism in the brain declines with age[2,3], a corresponding decline in ANLS is plausible. Clinical trials focusing on cognitive function in older adults, with MLEs or quercetin glycosides as the intervention, would be a significant contribution to the field. Incidentally, we did not

show that quercetin aglycone increases glycogen levels in astrocytes *via* Akt activation, which further promotes glycogen synthesis and inhibits glycogen degradation. A comprehensive analysis of the activation of p-Akt, in conjunction with the evaluation of Akt signaling using Akt inhibitors or knockdown, is imperative for elucidating the underlying mechanisms of quercetin-induced glycogen accumulation in astrocytes. In this study, quercetin glycosides exhibited more than 30% of the activity of the MLEs and were identified as active compounds. In addition to quercetin glycosides, MLEs contain other phenolic compounds, which may also promote glycogen accumulation, including benzofurans, phenolic acids, coumarins, chalcones, and stilbenes[14]. Therefore, further research regarding the identification of other active compounds is warranted.

CONCLUSIONS

MLEs and their active compounds promote glycogen accumulation in hiPSC-derived astrocytes. Quercetin aglycone, the basic backbone of glycosides, promotes astrocytic glycogen accumulation, suggesting that quercetin aglycone, a shared structure, is responsible for the activity of quercetin glycosides. MLEs and quercetin analogs may induce ANLS by promoting the accumulation of glycogen in astrocytes.

Conflict of Interest
Hideyuki Arie, Kentaro Nishioka, Toshiaki Azuma, Yumi Nakamura, Toshihide Suzuki, and Norihito Murayama are employees of the Suntory Global Innovation Center Limited. The remaining authors declare that they have no conflict of interest to disclose.

Ethics Approval
The study was approved by the Ethics Committee at Suntory, with protocol number (20194), dated March 3, 2022.

Informed Consent
Not applicable.

Funding
This research received no external funding.

Authors' Contributions
Hideyuki Arie: conceptualization, data curation, investigation, writing – original draft preparation. Kentaro Nishioka: conceptualization, data curation, investigation, writing – review and editing. Toshiaki Azuma: investigation, writing – original draft preparation. Tohru Yamagaki: investigation, writing – original draft preparation. Toshihide Suzuki: conceptualization, writing – review and editing. Yumi Nakamura: writing – review and editing. Norihito Murayama: conceptualization, writing – review and editing.

Data Availability
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgements
We are grateful to Kazuyoshi Kirimura and Yoshiko Shimizu for their assistance with the experiments. We would like to thank Editage (www.editage.jp) for English language editing and their assistance in creating the graphical abstract for this paper.

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