

## Go-sha-jinki-Gan (GJG), a traditional Japanese herbal medicine, protects against sarcopenia in senescence-accelerated mice



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### ABSTRACT

Sarcopenia is characterized by age-associated skeletal muscle atrophy and reduced muscle strength; currently, no pharmaceutical treatment is available. Go-sha-jinki-Gan (GJG) is a traditional Japanese herbal medicine that is used to alleviate various age-related symptoms, especially motor disorders. Here, we investigated the effect of GJG on aging-associated skeletal muscle atrophy by using senescence-accelerated mice (SAMP8). Immunohistochemical and western blotting analyses clearly showed that GJG significantly reduced the loss of skeletal muscle mass and ameliorated the increase in slow skeletal muscle fibers in SAMP8 mice compared to control mice. The expression levels of Akt and GSK-3 $\beta$ , the phosphorylation of FoxO4, and the phosphorylations of AMPK and mitochondrial-related transcription factors such as PGC-1 $\alpha$  were suppressed, while the expression of MuRF1 increased in SAMP8 mice, but approximated that in senescence-accelerated aging-resistant (SAMR1) mice after GJG treatment. We demonstrate for the first time that GJG has a therapeutic effect against sarcopenia.

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### Introduction

As mammals age, muscle mass and strength decrease progressively, a phenomenon known as sarcopenia (Wickham et al. 1989). Sarcopenia is characterized by the reduction in the size and number of muscle fibers, muscle mass, and the ratio of slow-twitch muscle fibers to fast-twitch muscle fibers (Lexell et al. 1988). Sarcopenia is a major determinant of the decline in physical function in older adults (Cruz-Jentoft et al. 2010). Although some trials have aimed at reversing the reduction in muscle mass, there is currently no effective pharmaceutical treatment for sarcopenia (Sayer et al. 2013). Multiple factors appear to be involved in the development of sarcopenia: changes in insulin-like growth factor (IGF-1), changes in the mitochondrial network, and chronic inflammation are followed by alterations in signaling pathways in the muscle (Bonaldo and Sandri 2013).

IGF-1 activates phosphatidylinositol-3-kinase (PI3K), resulting in Akt activation. Akt inhibits protein degradation by repressing the forkhead box protein (FoxO) family, leading to expression of atrogen-1/Muscle Atrophy F-box (MAFbx) and Muscle RING-Finger Protein-1 (MuRF1) (Brunet et al. 1999; Franke et al. 1997). Akt stimulates pro-

tein synthesis by regulating glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) (Moule et al. 1997). It has been shown that lower plasma concentrations of IGF-1 and higher plasma concentrations of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) are associated with lower muscle mass and strength in the elderly (Donahue et al. 1990; Visser et al. 2002).

Go-sha-jinki-Gan (GJG) is a traditional Japanese herbal medicine composed of 10 herbal drugs in fixed proportions (Usuki et al. 1991). This medicine has been used to alleviate various types of age-related conditions in the locomotor apparatus. Previous studies have not reported any severe adverse effects of GJG in humans (Launer et al. 1990). Despite the potential of GJG as an anti-aging drug, few studies have clarified its effect on senescent skeletal muscle. Therefore, we investigated whether GJG can protect against sarcopenia by using senescence-accelerated mice (SAMP8), which exhibit several accelerated aging characteristics, are widely used in aging research (Takeda et al. 1997), and have been reported to be a cost-effective model for muscular aging studies (Derave et al. 2005). Here, we show that GJG markedly protects against sarcopenia in SAMP8 mice.

### Materials and methods

#### Preparation of GJG extract and animals

Spray-dried, water-extracted GJG powder was obtained from Tsumura & Co. (Tokyo, Japan). GJG was approved in 1986 as a drug for

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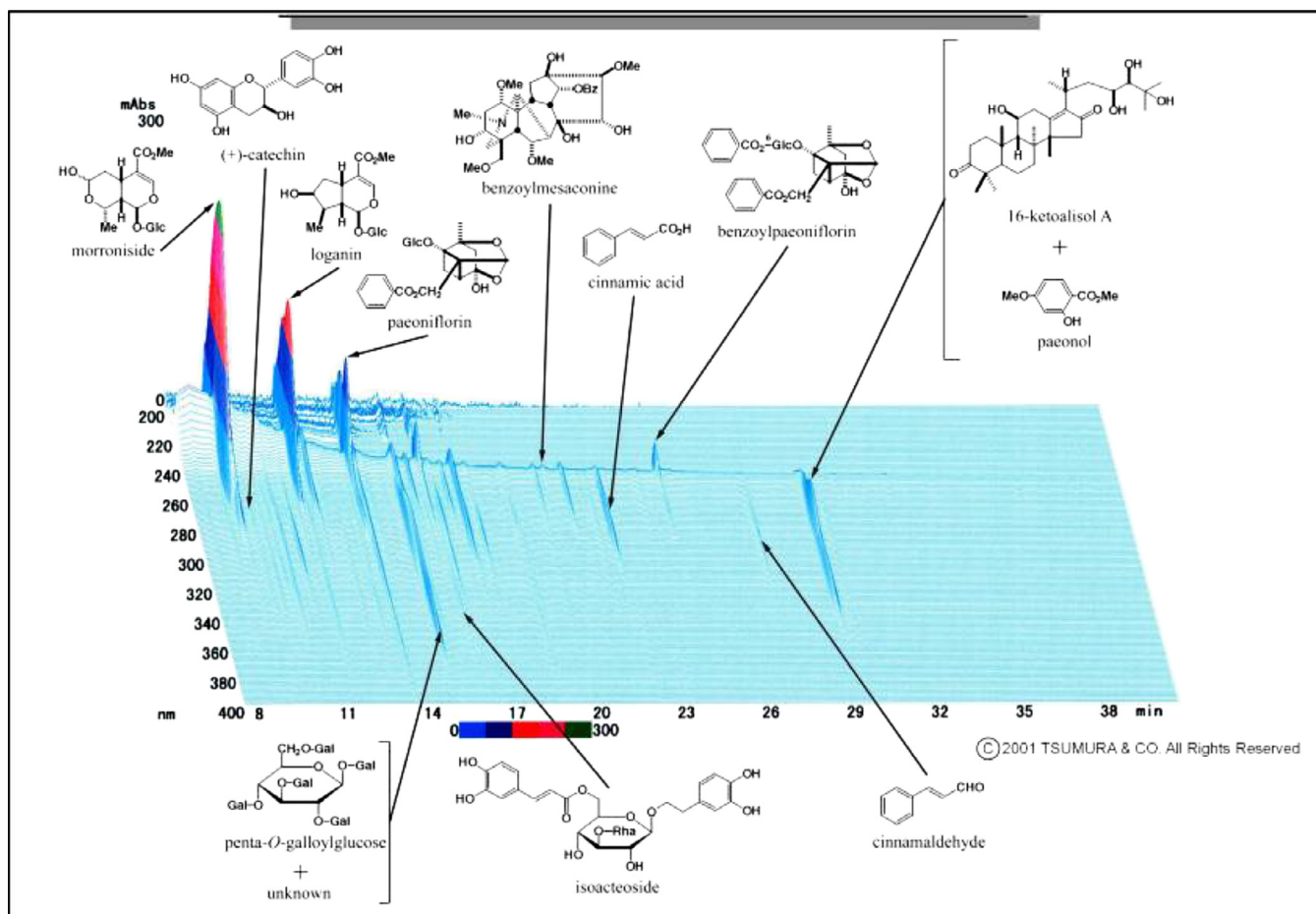


Fig. 1. HPLC analysis of GJG.

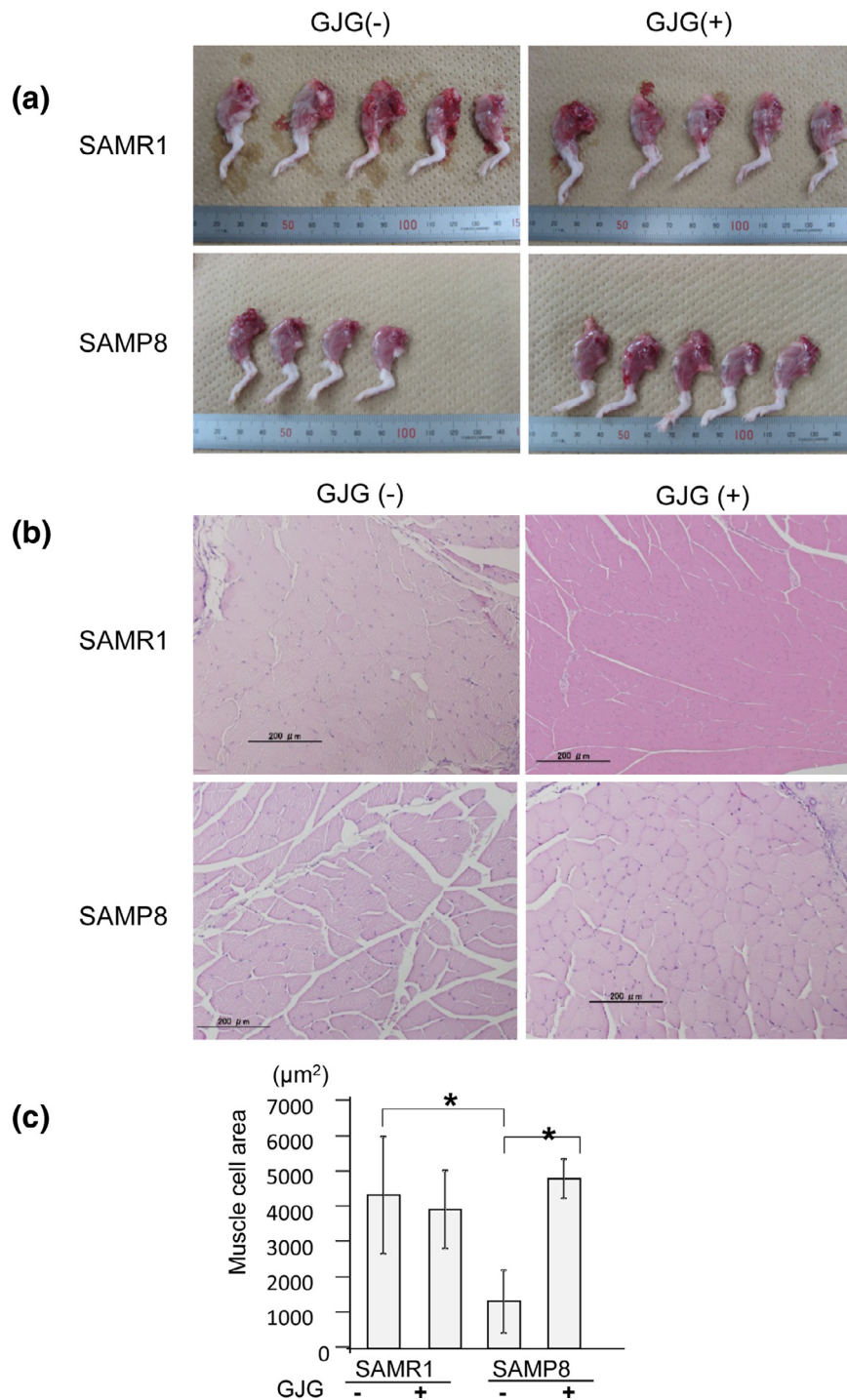
clinical use by the Japanese Ministry of Health, Labour and Welfare. It is produced at the Shizuoka plant which meets Japanese pharmaceutical GMP (good manufacturing practice). The local pharmaceutical administration of Shizuoka Prefecture assesses the GMP status of the plant every 5 years. The plant has had permission for pharmaceutical production for more than 30 years, and the production process has been well validated. Since active substances are still ambiguous, quality control is conducted by quantitation of major components. In the case of GJG, paeoniflorin (moutan bark), loganin (*Rehmannia* root), and total alkaloids (processed aconite root) are chosen as marker compounds for quality control. Paeoniflorin, loganin, and total alkaloids in 1 g of GJG extract powder used in our experiments were 2.11, 1.58, and 0.11 mg, respectively. In 10 lots (a total of 20 lots) produced before and behind this lot, paeoniflorin, loganin, and total alkaloids were within  $\pm 10\%$  of the range of this content, and quality was managed satisfactorily. Other physicochemical properties, e.g. loss on drying, water content, ash, heavy metals, etc., were also examined in all lots. GJG extract is listed in the Japanese Pharmacopoeia, and the material used in this study met that description. The general manufacturing procedure of GJG extract powder is as follows. Ten kinds of botanical raw materials are crushed and then weighed in accordance with the mixing ratio as shown in Table S1. The mixture of botanical raw materials is extracted 12 times with ion-exchanged water for 60 min at 100 °C. The extract is centrifuged to obtain a supernatant, which is then concentrated *in vacuo*. The concentrated extract solution is dried by a spray dryer. The standard yield of extract powder is around 16% of the total weight of botanical raw materials. A three-dimensional high-performance liquid chromatography (HPLC) profile of a methanol solution of GJG was performed according to our previous procedure (Hattori et al. 2010) and is shown in Fig. 1.

3D-HPLC analysis and LC/MS analysis of the crude drugs involved in GJG are shown in Figs. S1–S3.

Seven-week-old male SAMP8 mice were purchased from SLC, Inc. (Shizuoka, Japan) and divided into 2 groups: those fed a normal diet (powdered mouse food; Oriental Yeast Co. Ltd. (Tokyo, Japan; P8 + N group;  $n = 10$ )); and those fed a normal diet supplemented with 4% (w/w) GJG (P8 + GJG group;  $n = 10$ ). As controls, 7-week-old male SAMR1 mice were purchased from SLC and also divided into 2 groups: those fed a normal diet (R + N group;  $n = 10$ ) and those fed a normal diet supplemented with 4% (w/w) GJG (R + GJG group;  $n = 11$ ). General conditions and body weight were recorded for all mice. The housing care rules and experimental protocol were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the Animal Care and Use Committee of Osaka University.

#### Immunohistochemistry, histological analysis, and PAS staining

At 38 weeks, the mice were euthanized and tibiae were removed. Samples from 4 or 5 mice were photographed. Samples from 3 mice were fixed in 10% formalin, and the other samples were frozen at  $-80$  °C until required for use. Samples were embedded in paraffin. They were stained with hematoxylin and eosin (H&E) and the soleus muscles were evaluated microscopically to confirm the state of the muscles. The area of a muscle fiber was measured by evaluating 300 fibers that were randomly selected using WinROOF software (Mitani-Corp, Fukui, Japan). For the sarco/endoplasmic  $\text{Ca}^{2+}$ -dependent ATPase-driven pump 1 (SERCA1) gene expressed in fast-twitch muscle (type II) fibers (Periasamy and Kalyanasundaram 2007),



**Fig. 2.** GJG improved muscle fiber size. (a) Photographs of lower extremities. (b) H&E staining of the soleus muscles of the mice. The bar indicates 200 μm. Magnification: 100×. (c) The area of muscle fibers was measured. These data are expressed as means ± SD. \* $p < 0.0001$  vs. P8 + N group (one-way ANOVA).

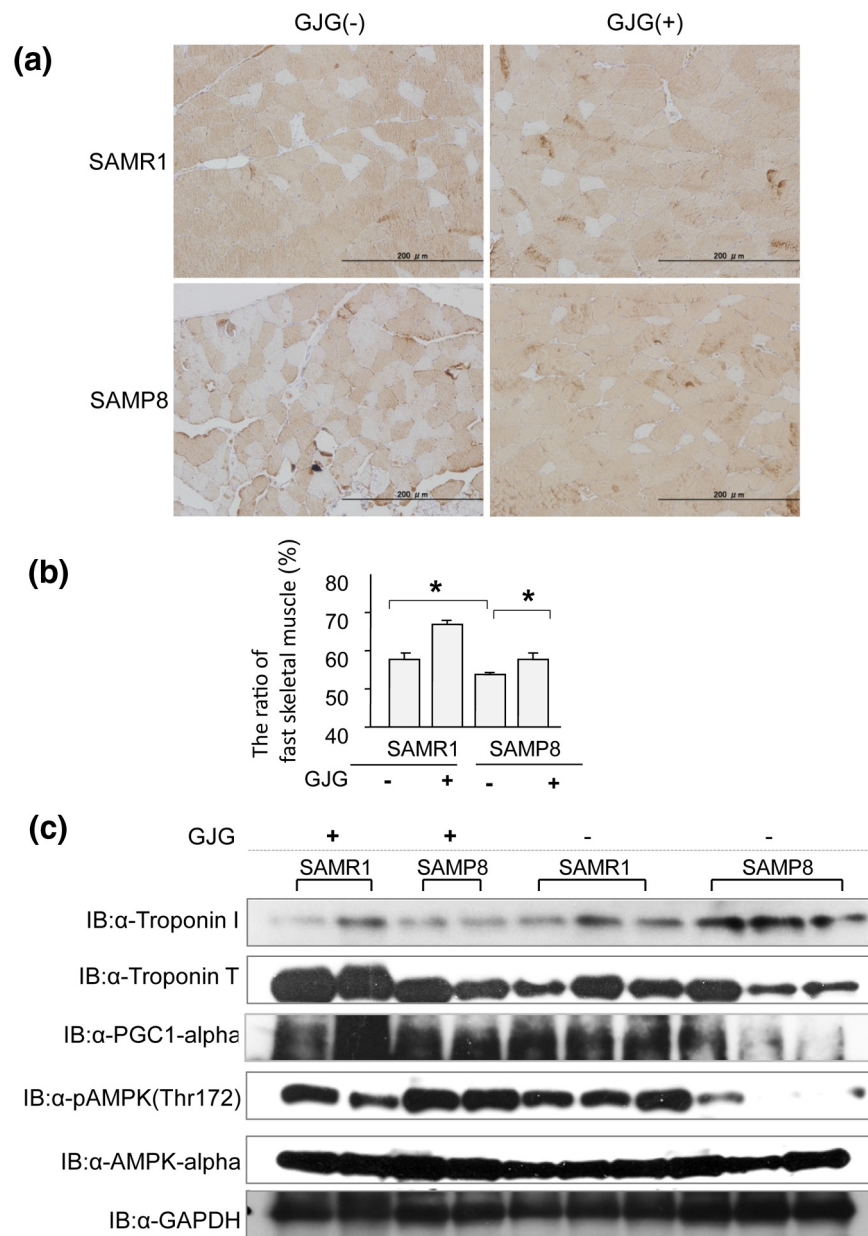
anti-SERCA1 ATPase (Abcam Cambridge, MA, USA) was visualized using 3,3-diaminobenzidine (DAB) with counterstaining by eosin. Fiber type distribution as a percentage was calculated. Periodic acid-Schiff (PAS) staining was performed using a PAS kit (Muto, Tokyo, Japan) according to the manufacturer's protocol.

#### ELISA and western blotting

We measured the sera of mice in duplicate using a mouse IGF-1 ELISA system (Abcam). The limit of sensitivity for IGF-1 was 2.74 pg/ml. The soleus muscles were homogenized and analyzed by

immunoblot analysis. We used the following antibodies: anti-troponin T (fast skeletal muscle) was purchased from Abbtotec, LLC; anti-troponin I (slow skeletal muscle) was purchased from Novus Biologicals, LLC; anti-PGC-1α was purchased from Calbiochem (Darmstadt, Germany); anti-GAPDH, anti-phospho-Akt (Thr308), anti-phospho-Akt (Ser473), anti-phospho-GSK3-β, anti-GSK3-β, anti-phospho-FoxO4 (Ser193), anti-phospho-5'-AMP-activated protein kinase (AMPK) (Thr172), anti-AMPK-alpha, anti-TNF-α, and anti-Akt were purchased from Cell Signaling Technology (Danvers, MA, USA); and anti-FoxO4, anti-MAFbx, and anti-MuRF1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).





**Fig. 3.** GJG normalized the imbalance of muscle fiber in SAMP8 mice. (a) Immunohistochemical analysis of SERCA1 (fast skeletal muscle marker), visualized with DAB (brown) and counterstained with hematoxylin. The bar indicates 200 μm. Magnification: 200×. (b) The ratio of fast skeletal muscle to the soleus muscle. These data are expressed as means ± SD. \* $p < 0.0001$  vs. P8 + N group (one-way ANOVA). (c) Expression of troponin I (slow skeletal muscle marker), troponin T (fast skeletal muscle marker), PGC-1α, p-AMPK, and AMPK as determined by western blotting analysis of the soleus muscles. GAPDH is shown as a loading control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

### Statistical analysis

Data are presented as means ± standard deviation values. Groups were compared by one-way analysis of variance (ANOVA). Differences between treatment groups were considered significant at  $p < 0.05$ .

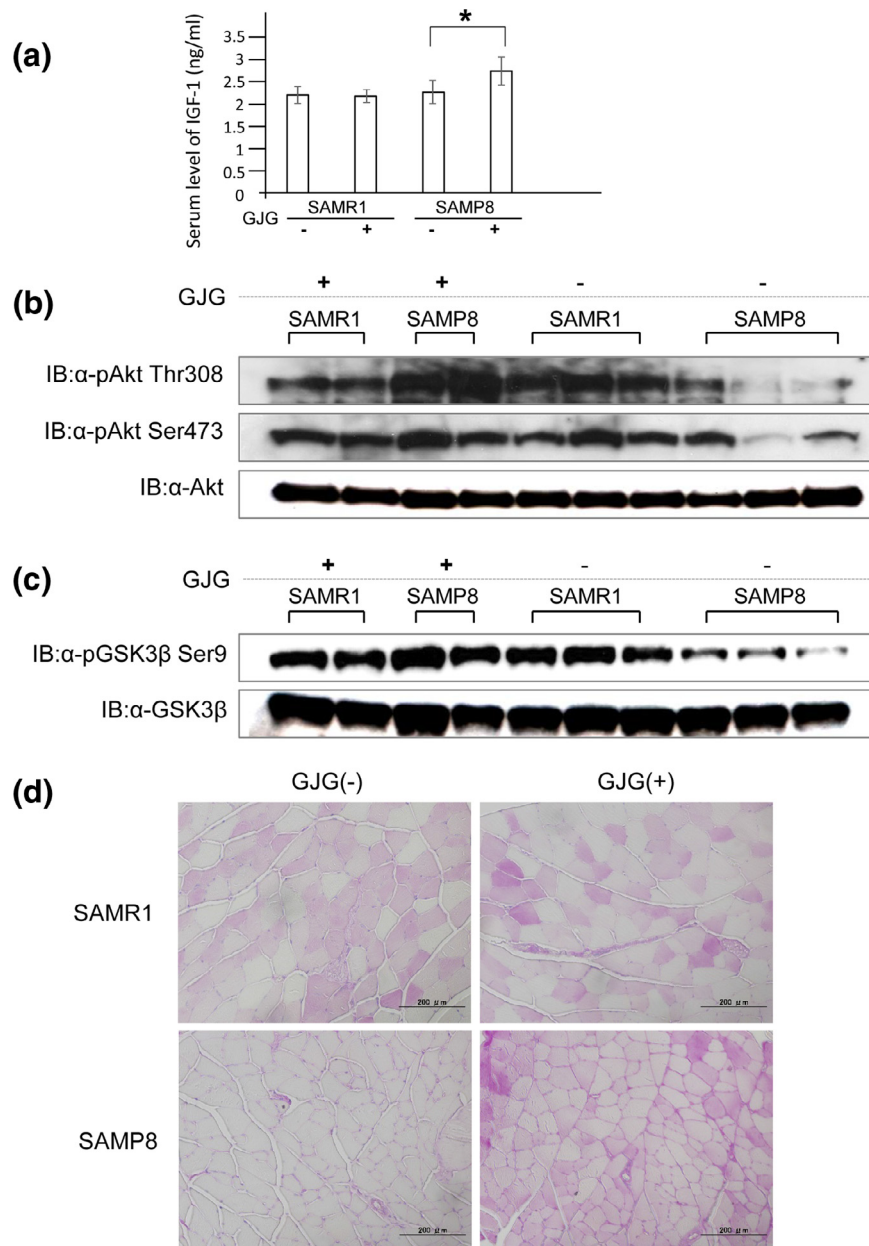
### Results

#### GJG suppressed the reduction in muscle fiber size in the soleus of SAMP8 mice

No mice in the GJG group experienced unusual activity. Within the same strain, there was no significant difference in weight regardless of whether the mice were fed GJG. No significant differences in food intake per day were found among these groups (P8 + N:  $3.9 \pm 0.7$  g,

P8 + GJG:  $3.8 \pm 0.4$  g, R + N:  $3.8 \pm 0.3$  g; R + GJG:  $3.7 \pm 0.5$  g). No significant differences in GJG intake per day were found between GJG groups (P8 + GJG:  $0.15 \pm 0.02$  g, R + GJG:  $0.15 \pm 0.02$  g).

The SAMP8 mice fed normal chow (P8 + N) group had hair loss at the time of assessment, whereas the SAMP8 mice administered GJG (P8 + GJG) group had reduced hair loss (data not shown). Photographs of lower extremities are shown in Fig. 2a. Histological analysis of soleus muscles using H&E staining revealed a marked reduction in muscle fiber size in the P8 + N group; however, we observed that muscle atrophy was strikingly alleviated in the P8 + GJG group (Fig. 2b). The area of muscle fibers in the P8 + N group was significantly less than that in the other groups; however, the area of muscle fibers in P8 + GJG was almost the same as that of the SAMR1 mice fed normal chow (R + N) and SAMR1 mice fed GJG (R + GJG) ( $p < 0.0001$ , one-way ANOVA) (Fig. 2c).



**Fig. 4.** GJG sustained glycogen storage in SAMP8 mice. (a) Serum IGF-1 as determined by ELISA. These data are expressed as means  $\pm$  SD. \* $p < 0.05$  vs. the P8 + N group (one-way ANOVA). (b through c) Western blotting analysis of soleus muscles. (b) Upper panel shows pAkt (Thr308), middle panel shows pAkt (Ser473), and bottom panel shows Akt. (c) Upper panel shows pGSK-3 $\beta$  (Ser9) and bottom panel shows GSK-3 $\beta$ . (d) PAS staining of the soleus muscles. The bar indicates 200  $\mu$ m. Magnification: 200 $\times$ . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

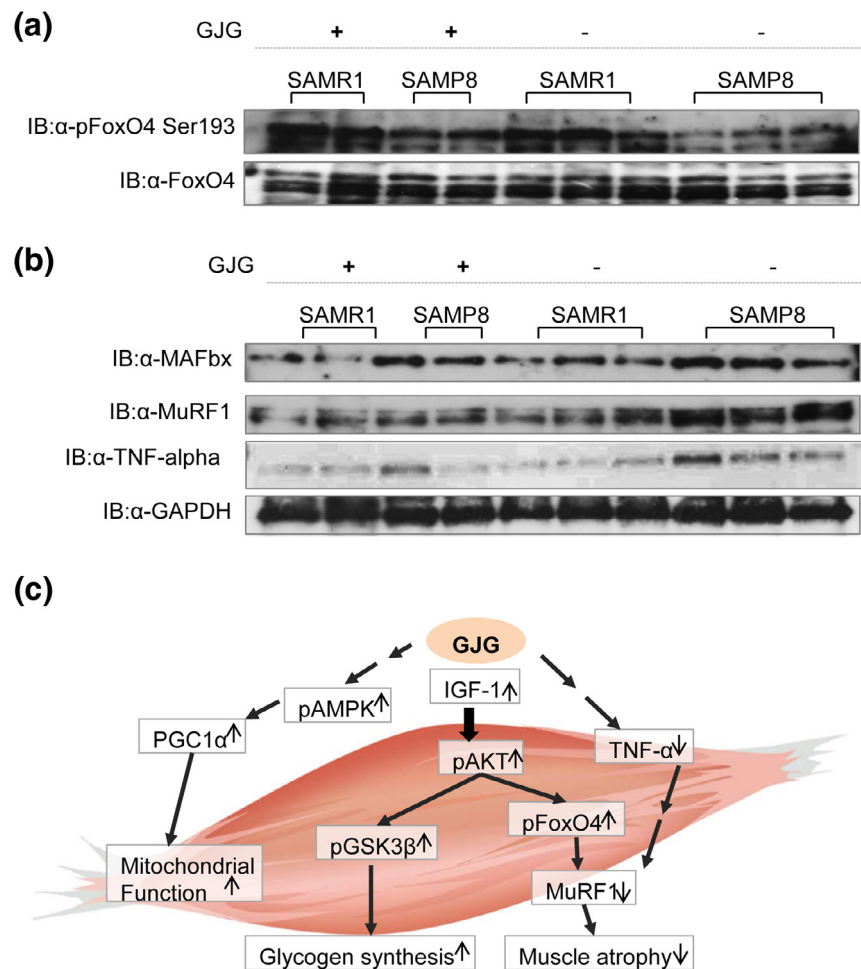
*GJG normalized the imbalance of slow and fast skeletal muscle in the soleus of SAMP8 mice via expression of peroxisome proliferator-activated receptor-co-activator 1 $\alpha$  (PGC-1 $\alpha$ )*

Immunohistochemical analysis showed that the level of SERCA1 (fast skeletal muscle) was lower in the P8 + N group than in the other groups. However, the P8 + GJG group ameliorated the increase in slow skeletal muscle fibers (Fig. 3a and B). Western blotting analysis revealed that the expression of troponin I (slow skeletal muscle) increased in the P8 + N group, whereas it was suppressed in the P8 + GJG group. As compared with mice of the P8 + N group, mice of the P8 + GJG group demonstrated increased expression of troponin T (fast skeletal muscle; Fig. 3c). Muscle fiber type determination is regulated by PGC-1 $\alpha$  (Lin et al. 2002) which is phosphorylated by AMPK (Jager et al. 2007). Fig. 3c also shows that the expression of them

in the P8 + N group was lower than in the control groups (R + N, R + GJG). However, in the P8 + GJG group, the expression of p-AMPK and PGC-1 $\alpha$  was normal.

*GJG elevated serum IGF-1 and maintained glycogen storage via phosphorylation of Akt and GSK-3 $\beta$  in the soleus of SAMP8 mice*

Fig. 4a shows that the administration of GJG elevated the levels of serum IGF-1 in SAMP8 mice. Next, we examined signaling in skeletal muscles via western blotting. Akt is activated by phosphorylation of threonine 308 (Thr308) and of serine 473 (Ser473) (Sarbasov et al. 2005). Fig. 4b shows that phosphorylation of Akt, especially at Thr308, was significantly decreased in the muscles of P8 + N mice. This trend was corrected by the administration of GJG. Fig. 4c shows the phosphorylation levels of GSK-3 $\beta$  in the skeletal muscles of mice.



**Fig. 5.** GJG decreased the expression of MuRF1 via normalization of FoxO4 phosphorylation levels and TNF- $\alpha$  in SAMP8 mice, and schema of the signaling pathways involved in skeletal muscles of mice administered GJG. (a through b) Western blotting analysis of soleus muscles. (a) Upper panel shows pFoxO4 and bottom panel shows FoxO4. (b) Expression of MAFbx, MuRF1, and TNF- $\alpha$ . GAPDH is shown as a loading control. (c) Schema for the effect of GJG on skeletal muscle.

The levels of p-GSK-3 $\beta$  were lower in P8 + N than in the other groups, whereas treatment with GJG improved the levels of p-GSK-3 $\beta$  (Fig. 4c). Next, we evaluated the glycogen content in skeletal muscle by using PAS staining. Fig. 4d shows that the deep red regions (indicating a high glycogen content) of the soleus in the P8 + N group were much smaller than those of the other groups; however, the deep red regions of the soleus in the P8 + GJG group were markedly larger.

#### *GJG reduced the expression of MuRF1 via normalization of FoxO4 phosphorylation levels in the soleus of SAMP8 mice*

The Akt-axis stimulates phosphorylation of the FoxO family, which regulates the expression levels of atrogen-1/MAFbx and MuRF1, thereby suppressing the degradation of protein in skeletal muscle (Brunet et al. 1999; Franke et al. 1997). In our study, phosphorylation of FoxO4 markedly decreased in the P8 + N group, and administration of GJG to mice did not reduce these phosphorylation levels (Fig. 5a). We evaluated the expression of phosphorylation of FoxO1 and FoxO3, and they were slightly suppressed in SAMP8 mice (data not shown). Fig. 5b shows that the expression levels of atrogen-1/MAFbx were unaffected regardless of whether GJG was administered. However, MuRF1 expression levels were suppressed by GJG in SAMP8 mice. As TNF- $\alpha$  reportedly reduces PGC-1 $\alpha$  expression and induces MuRF1 expression (Cai et al. 2004; Remels et al. 2010), we evaluated the expression of TNF- $\alpha$  in soleus muscles. Fig. 5b also shows that the expression levels of TNF- $\alpha$  were elevated in the P8 + N group, whereas administration of GJG to mice suppressed its level.

## Discussion

In this study, we demonstrated that GJG prevented the progression of sarcopenia in SAMP8 mice. In addition, we showed that administration of GJG to SAMP8 mice maintained the area of muscle fibers in the soleus via normalizing signal transduction through the IGF-1-Akt axis, the suppression of inflammation, and the maintenance of mitochondrial-related transcription factors. We found that skeletal muscles in SAMP8 mice treated with GJG were comprised of more fast skeletal muscle fibers as compared to the P8 + N group. The muscle fiber type has been reported to be regulated by PGC-1 $\alpha$  (Lin et al. 2002); this protein is known to play an important role in activating mitochondrial biogenesis and oxidative metabolism (Wu et al. 1999). In the present study, the expression level of PGC-1 $\alpha$  decreased in SAMP8 mice; however, administration of GJG changed this trend.

Mitochondrial turnover changes with aging, and autophagy is sequentially decreased in atrophying muscles (Romanello et al. 2010). PGC-1 $\alpha$  is phosphorylated by AMPK (Jager et al. 2007). Koltai et al. reported that phosphorylated AMPK content decreases with aging (Koltai et al. 2012). Our study did not contradict their results. It is well known that IGF-1 is essential for growth and the promotion of skeletal muscle development (Brunet et al. 1999; Franke et al. 1997). Moreover, an age-related reduction in plasma IGF-1 concentrations is well known (Donahue et al. 1990). We showed that administration of GJG elevated serum levels of IGF-1 in SAMP8 mice. Our study also demonstrated that phosphorylation of Akt at Thr308 and Ser473 significantly declined in the muscles of SAMP8 mice,



particularly that of Thr308. GJG treatment normalized the level of phosphorylation of Akt in SAMP8 mice to that seen in SAMR1 mice. It is possible that the Akt in the skeletal muscles of P8 + GJG mice was activated mainly by phosphorylation at Thr308.

In skeletal muscles, Akt stimulates glycogen synthesis via phosphorylation of GSK-3 $\beta$  and inhibits protein degradation via phosphorylation of FoxOs (Brunet et al. 1999; Franke et al. 1997). GJG treatment restored the phosphorylation of GSK-3 $\beta$  levels in SAMP8 mice to those seen in SAMR1 mice. The present study showed that the PAS staining density of GJG-treated SAMP8 mice was significantly higher than that of SAMP8 mice fed with normal chow. Our findings suggested that GJG sustained glycogen storage in the skeletal muscles of SAMP8 mice by changing the phosphorylation levels of Akt and GSK-3 $\beta$ .

Next, we examined the phosphorylation levels of FoxOs, which are associated with skeletal muscle atrophy and is inactivated by Akt (Brunet et al. 1999; Franke et al. 1997). It has been reported that the regulation of FoxO1 and FoxO3 is different from that of FoxO4 (Senf et al. 2011). In the present study, phosphorylations of FoxO1 and FoxO3 were slightly suppressed in SAMP8 mice; however, a marked reduction in phosphorylation of FoxO4 was observed, and these levels recovered with GJG treatment. FoxOs regulate the expression levels of atrogen-1/MAFbx and MuRF1, which are up-regulated in atrophic and aged skeletal muscles (Brunet et al. 1999; Franke et al. 1997). The present study showed that the expression level of MuRF1 in the P8 + N group was higher than that in P8 + GJG, but no similar trend was observed for atrogen-1/MAFbx. On the other hand, Yoshida et al. suggested that FoxO1 does not activate transcription of MuRF1, but does activate that of atrogen-1/MAFbx (Yoshida et al. 2010). Cai et al. reported that TNF- $\alpha$  upregulates the expression of MuRF1 but not of MAFbx (Cai et al. 2004). In our study, although the expression of TNF- $\alpha$  was high in SAMP8 mice, it was suppressed by GJG. Our data thus do not contradict these previous studies.

In conclusion, we showed that GJG suppressed sarcopenia via the IGF-1/insulin pathway, maintained the expression of mitochondrial-related transcription factors, and suppressed TNF- $\alpha$  in SAMP8 mice (see Fig. 5c for a summary). Our results indicate that GJG is a promising candidate for relief from sarcopenia.

### Conflict of interest

The authors declare no conflict of interests.

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### Supplementary Materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2014.11.005.

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