Sarcopenia is a disease whose symptoms include decreased muscle mass and weakened muscle strength with age. In sarcopenia, decreased production of insulin-like growth factor-1 (IGF-1) increases ubiquitin ligases, such as Atrogin1 and Muscle RING-Finger Protein-1 (MuRF1), by activating forkhead box O (FOXO), and inflammatory cytokines and oxidative stress increase the expression of ubiquitin ligases by activating the transcription factor nuclear factor-kappa B (NF-κB). In addition, increased levels of ubiquitin ligases cause skeletal muscle atrophy. Conversely, sirtuin 1 (Sirt1) is known to regulate the expression of ubiquitin ligases by suppressing the activities of NF-κB and FOXO. In this study, we evaluated the effect that juzentaihoto hot water extract (JTT) has on skeletal muscle atrophy and motor function by administering it to senescence-accelerated mouse prone-8 (SAMP8). The group treated with JTT displayed larger gastrocnemius muscle (GA) and extensor digitorum longus (EDL) weights, larger GA muscle fiber cross-sectional areas, and motor function decline during rota-rod tests. JTT also increased IGF-1 serum levels, as well as mRNA Sirt1 levels in GA. Serum levels of tumor necrosis factor-α, interleukin-6, and mRNA levels of Atrogin1 and MuRF1 in GA were reduced by JTT. The muscle fiber cross-sectional area of GA was correlated with the mRNA levels of Sirt1 in GA. The results of this study suggested that JTT administration suppresses skeletal muscle atrophy and motor function decline in SAMP8 mice. This effect may be associated with the increased expression levels of Sirt1 and IGF-1 by JTT.

Key words senescence-accelerated mouse prone-8 (SAMP8); sarcopenia; sirtuin 1 (Sirt1); juzentaihoto

INTRODUCTION

Sarcopenia is a disease that impairs motor function in patients, and significantly reduces their QOL. Symptoms include decreased muscle mass and weakened muscle strength with age. The number of patients is increasing with the aging of the population, which is a serious social problem, particularly in developed countries. One of the several factors associated with sarcopenia is the increase in inflammatory cytokines and oxidative stress due to aging. Increased inflammatory cytokines and oxidative stress induce ubiquitin ligases such as Atrogin1 and Muscle RING-Finger Protein-1 (MuRF1) in skeletal muscle, which causes muscle fibers atrophy. In addition, aging-related decreases in anabolic hormone secretion also affects the onset of sarcopenia. In particular, insulin-like growth factor-1 (IGF-1) is known to act on muscle cells to create an anabolic action. In addition, IGF-1 decreases ubiquitin ligases by suppressing the activities of transcription factor Forkhead box O (FOXO). Thus, decreased IGF-1 secretion promotes muscle fiber atrophy.

Juzentaihoto is a herbal medicine clinically used to help treat symptoms of weakness and fatigue, and has been reported to have anti-inflammatory, anti-cancer, anti-obesity, and immunostimulatory effects. In previous studies, the administration of juzentaihoto hot water extract (JTT) to streptozotocin-induced type 1 diabetes mouse alleviates skeletal muscle atrophy by increasing sirtuin 1 (Sirt1) expression. In addition, it was reported that JTT and its constituent medicines, namely Glycyrrhiza Radix, Astragalus Radix, and Cinnamomi Cortex, promoted Sirt1 transcriptional activity in C2C12 cells derived from mouse skeletal muscle. Sirt1 is located upstream of the transcription factor nuclear factor-kappa B (NF-κB), which regulates the expression of inflammatory cytokines, such as tumor necrosis factor alpha (TNFα) and interleukin (IL)-6. In addition, Sirt1 is known to regulate ubiquitin ligase expression by suppressing the activities of NF-κB and FOXO, and thus, it is believed to alleviate muscle atrophy. Therefore, JTT is expected to improve muscle atrophy by increasing Sirt1 expression.

Senescence-accelerated mouse prone-8 (SAMP8) and senescence-resistant inbred strain 1 (SAMR1) mice are frequently used as models of muscular atrophy associated with aging. It has been reported that SAMP8 undergoes early aging and skeletal muscle atrophy. SAMR1 was used as its normal control. In this study, JTT was administered to SAMP8 and SAMR1 to investigate its effect on skeletal muscle mass and motor function.

MATERIALS AND METHODS

Preparation of JTT Extracts-Containing Chow

Juzentaihoto was provided by Tsumura Co., Ltd. (Tokyo, Japan), with 5.0 g of extract obtained from 28.5 g of crude juzentaihoto. JTT consists of the constituent crude drug shown in Table 1. In a previous study, JTT alleviated skeletal muscle atrophy in mice with streptozotocin-induced type 1 diabetes.
diabetes. In that study, the beneficial effect was observed in mice that received 4% (w/w) JTT mixed feed.\textsuperscript{19,20} Therefore, in this study, JTT was mixed with normal feed (CLEA, Shizuoka, Japan) at 4% (w/w).

**Animals** Animal experiments were conducted with permission from the Kochi University animal experiment ethics committee (Approval No. M-00031). SAMP8 and SAMR1 mice (male) were purchased from Japan CLEA. SAMP8 mice were randomly divided into 2 groups at 18 weeks of age. Each group was fed either a normal (CE-2) or a JTT mixed diet until they were 36 weeks of age. SAMR1 mice received a normal diet (CE-2) (SAMP8, n = 6; SAMP8 + JTT, n = 7; SAMR1, n = 6). Body weight was measured every 14 d during the breeding period.

**Rota-Rod Performance Test** Motor function was measured using rota-rod (MK-670, Muromachikai, Tokyo, Japan). The rotor speed was equally accelerated at a rate of 0.3 rotations/s until it reached 80 rotations per minute, at which point the rotation speed was kept constant until the mouse dropped, and the time was recorded. Each mouse was tested three times, at intervals of 10 min, and the average time of the three measurements was taken as the result. The rota-rod test was performed every 14 d.

**Extraction of Skeletal Muscle and Blood Sample** Mice underwent laparotomy under anesthesia at 36 weeks of age. Whole blood was collected from the descending vena cava as blood samples for enzyme-linked immunosorbent assay (ELISA). Thereafter, the gastrocnemius muscle (GA), extensor digitorum longus (EDL), and soleus (SOL) were removed which point the rotation speed was kept constant until the muscle fiber area and used this in our analysis.

**Quantitative RT-PCR of mRNA in Gastrocnemius** GA was cut to a width of 0.1 cm, and then homogenized with POLYTRON RT-MR 3100 (Central Science Trade, Tokyo, Japan). Total RNA was collected from the homogenized gastrocnemius using an RNAasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany). The collected RNA was reverse transcribed using a PrimeScript RT reagent Kit (TaKaRa Bio, Otsu, Japan) to obtain cDNA. TaqMan quantitative PCR was performed using StepOnePlus Real-time PCR Systems (Applied Biosystems, CA, U.S.A.). MuRF1, Atrogin1 and Sirt1 mRNA expression levels were normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression levels. PCR primers with TaqMan probes for GAPDH (Mm99999915_g1), MuRF1 (Mm01185221_m1), Atrogin1 (Mm00499523_m1) and Sirt1 (Mm01168521_m1), and Taq Man Universal PCR Master Mix were purchased from Applied Biosystems (Foster City, CA, U.S.A.).

**ELISA** The blood sample was centrifuged (8000 rpm, 4 °C) and the resulting supernatants were used as serum samples. The serum level of IGF-1 was measured by IGF-1 ELISA kit (Proteintech Group, Rosemont, IL, U.S.A.). The serum level of TNFα was measured by a TNFα ELISA kit (R & D Systems, Minneapolis, MN, U.S.A.). The serum level of IL-6 was measured by an IL-6 ELISA kit (R & D systems).

**Statistical Analysis** All statistical analyses were performed using Stat Flex program (View Flex, Tokyo, Japan). Data are expressed as mean ± standard deviation (S.D.). Body weight and rota-rod test results were analyzed using two way repeated measured ANOVA, while the other experimental results were analyzed using one-way ANOVA. After variance analysis, multiple comparison tests were performed using Bonferroni post hoc test. The correlation between the mRNA expression level of Sirt1 and the cross-sectional area of the GA muscle fiber was evaluated using the Pearson’s test. A p-value of <0.05 was considered statistically significant.

## RESULTS

**Body Weight** Body weights were measured every 2 weeks, in order to investigate the effect of JTT on body weight. In SAMR1 mice, body weight was significantly higher than SAMP8 mice at 18–36 weeks (p < 0.01). In the SAMP8 + JTT group, body weight was not altered by the administration of JTT at 18–36 weeks (Fig. 1). Two way repeated measured ANOVA results indicate that these effects were significant with regard to time (F\textsubscript{9,189} = 47.8, p < 0.001), group (F\textsubscript{2,189} = 17.5, p < 0.001), and interactions between the time and the group (F\textsubscript{18,189} = 5.36, p < 0.001).

**Each Skeletal Muscle Weights** To investigate the effect on skeletal muscle atrophy by administration of JTT, the wet weight of GA, EDL, and SOL muscles excised from the lower limbs were measured. In the SAMR1 group, weights for all three muscles were significantly higher than for the SAMP8 group (GA, EDL, SOL: p < 0.01). In the SAMP8 + JTT group,
GA and EDL weights were significantly larger than in the SAMP8 group (GA, EDL: \( p < 0.05 \)) (Table 2). SOL muscle weight did not change under JTT administration (Table 2). One-way ANOVA results indicate that the effect was significant (GA: \( F_{2,16} = 72.2, \ p < 0.001 \); EDL: \( F_{2,16} = 12.6, \ p < 0.001 \); SOL: \( F_{2,16} = 4.73, \ p < 0.05 \)).

**Table 2. Effect of JTT for Skeletal Muscle Weight**

|               | SAMR1          | SAMP8          | SAMP8 + JTT     |
|---------------|----------------|----------------|-----------------|
| Gastrocnemius (mg) | 209.6 ± 14.1** | 134.8 ± 13.3   | 150.5 ± 5.9*    |
| Extensor digitorum longus (mg) | 20.0 ± 1.8**   | 14.2 ± 1.8     | 16.9 ± 1.4*     |
| Soleus (mg)    | 6.1 ± 0.7**    | 5.4 ± 0.5      | 5.5 ± 0.5       |

JTT administration for mice was started at the age of 18 weeks. Gastrocnemius, extensor digitorum longus and soleus were removed from mice, and these wet weights were measured at the age of 36 weeks. These data were expressed as mean ± S.D. (\( n = 6–7 \)). *\( p < 0.05 \), **\( p < 0.01 \) vs. SAMP8 JTT no treated group evaluated by one way ANOVA and followed by Bonferroni post hoc test.

**Fig. 1. Effect of JTT on Body Weight**

JTT administration was started at 18 weeks. Skeletal muscle weights were measured at 18–36 weeks. These data were expressed as mean ± S.D. (\( n = 6–7 \)) and were evaluated using two way repeated measured ANOVA, which was followed by the Bonferroni post hoc test. **\( p < 0.01 \) vs. SAMP8 JTT no treated group evaluated by two way repeated measured ANOVA and followed by Bonferroni post hoc test.

**Fig. 2. JTT Improved Motor Function**

JTT administration for mice started at the age of 18 weeks. And, rota-rod tolerance test was performed at the age of 18–36 weeks. These data were expressed as mean ± S.D. (\( n = 6–7 \)). **\( p < 0.01 \) vs. SAMP8 JTT no treated group evaluated by two way repeated measured ANOVA and followed by Bonferroni post hoc test.

GA and EDL weights were significantly larger than in the SAMP8 group (GA, EDL: \( p < 0.05 \)) (Table 2). SOL muscle weight did not change under JTT administration (Table 2). One-way ANOVA results indicate that the effect was significant (GA: \( F_{2,16} = 72.2, \ p < 0.001 \); EDL: \( F_{2,16} = 12.6, \ p < 0.001 \); SOL: \( F_{2,16} = 4.73, \ p < 0.05 \)).

**Rota-Rod Test** The rotor-rod test was conducted every 2 weeks from the start of JTT administration, in order to evaluate motor function. The time it took for the mouse to drop from the rotor was measured. Endurance was significantly longer in SAMR1 mice, than in SAMP8 or the SAMP8 + JTT group at 18–36 weeks of age (\( p < 0.01 \)). In the SAMP8 + JTT group, endurance was significantly longer than in the SAMP8 group at 34–36 weeks of age (\( p < 0.01 \)) (Fig. 2). Two way repeated measured ANOVA results indicate that these effects were significant regarding time (\( F_{9,189} = 2.87, \ p < 0.005 \),
group ($F_{2,189} = 25.3, \ p < 0.001$), and interactions between the
time and the group ($F_{18,189} = 1.92, \ p < 0.05$).

**Cross-Sectional Area of GA** In order to measure skeletal
muscle atrophy, cross-sectional areas of GA were excised from
the lower limb and measured. In SAMR1 mice, these cross-
sectional areas of GA were significantly bigger than in the
SAMP8 group ($p < 0.01$). In the SAMP8 + JTT group, cross-
sectional areas of GA were significantly larger than in the
SAMP8 group ($p < 0.05$) (Fig. 3). One-way ANOVA results
indicate that the effect was significant ($F_{2,16} = 26.9, \ p < 0.001$).

**Sirt1 Gene Expression in Skeletal Muscle** Sirt1 is an
important factor for reducing oxidative stress. To inves-
tigate the expression level of Sirt1 in skeletal muscles of
JTT-exposed mice, mRNA expression levels were compared.

In SAMR1 mice, the mRNA expression of Sirt1 was signifi-
cantly higher than in the SAMP8 group ($p < 0.01$). In the
SAMP8 + JTT group, mRNA expression of Sirt1 was signifi-
cantly higher than in the SAMP8 group ($p < 0.05$) (Fig. 4A).
Furthermore, as a result of examining the correlation between
the cross-sectional area of GA and the mRNA expression
level of Sirt1, it was found that the cross-sectional area of GA
increased in proportion to the mRNA expression level of Sirt1
($p < 0.001$) (Fig. 4B). One-way ANOVA results indicate that
this effect was significant ($F_{2,16} = 9.50, \ p < 0.005$).

**IGF-1 Serum Levels** IGF-1 serum levels were measured.
In SAMR1 mice, IGF-1 serum levels were significantly higher
than in the SAMP8 group ($p < 0.01$). In the SAMP8 + JTT
group, IGF-1 serum levels were significantly higher than
Atrogin1 were significantly lower than in the SAMP8 group. In SAMR1 mice, mRNA expression of MuRF1 and Atrogin1 expression levels were compared using the real-time PCR method. To evaluate the expression level of ubiquitin ligase gene in skeletal muscle, MuRF1 and Atrogin1 mRNA expressions in skeletal muscle, MuRF1 and Atrogin1 mRNA were measured. These data were expressed as mean ± S.D. (n = 6–7), and evaluated using one-way ANOVA, which was followed by the Bonferroni post hoc test. *p < 0.05, **p < 0.01 between the groups.

Fig. 5. JTT Decreased IGF-1 Serum Levels in Mice
Blood was collected from mice at 36 weeks, at which point IGF-1 serum levels were measured. These data were expressed as mean ± S.D. (n = 6–7), and evaluated using one-way ANOVA, which was followed by the Bonferroni post hoc test. *p < 0.05, **p < 0.01 between the groups.

Fig. 6. JTT Decreased Serum Levels of Cytokines in Mice
Blood was collected at 36 weeks. (A) the serum levels of TNFα and (B) the serum levels of IL-6 were measured. These data were expressed as mean ± S.D. (n = 6–7), and evaluated using one-way ANOVA, which was followed by the Bonferroni post hoc test. *p < 0.05, **p < 0.01 between the groups.

Inflammatory Cytokine Serum Levels
To evaluate the effect of JTT on inflammatory cytokines, TNFα and IL-6 serum levels were measured. In the SAMR1 group, TNFα and IL-6 serum levels were significantly lower than in the SAMR1 group (TNFα: p < 0.01, IL-6: p < 0.01). In the SAMP8 + JTT group, TNFα serum levels were significantly lower than in the SAMP8 group (TNFα: p < 0.05), and the IL-6 serum levels tended to be lower than in the SAMP8 group (IL-6: p = 0.074) (Figs. 6A, B). One-way ANOVA results indicate that this effect was significant (TNFα: F_{2,16} = 6.95, p < 0.01, IL-6: F_{2,16} = 6.26, p < 0.01).

Fig. 7. JTT Increased mRNA Expression of Ubiquitin Ligase mRNA Expression
JTT administration was started at 18 weeks. Gastrocnemius muscle was removed at 36 weeks. After that, mRNA in the gastrocnemius was extracted, and mRNA expressions of (A) MuRF1 and (B) Atrogin1 were measured. These data were expressed as mean ± S.D. (n = 6–7), and evaluated by one-way ANOVA, which was followed by the Bonferroni post hoc test. *p < 0.05, **p < 0.01 between the groups.

DISCUSSION
The most important findings in this study were that the administration of JTT to SAMP8 mice alleviated skeletal muscle atrophy (Fig. 3, Table 2) and motor function declines during the rota-rod test (Fig. 2).

Muscle atrophy in SAMP8 has been reported to be dominated by fast type muscles, which is similar to sarcopenia symptoms. SAMP8 mice have been frequently used as an animal model that expresses muscle atrophy due to aging. 24–26 In this study, SAMP8 mice at 36 weeks of age had significantly smaller GA wet weights and the cross-sectional areas, and EDL wet weights (Fig. 3, Table 2). The atrophy of these muscles was significantly suppressed in the JTT-administered mice, however. SAMP8 mice had a significantly smaller SOL wet weights and the cross-sectional areas, - relative to the findings in SAMR1 mice, but the extent of atrophy was less severe than that in GA and EDL. This might explain why no change in the wet weight of SOL was observed after JTT administration.

Atrophy of skeletal muscle fibers is caused by an increase in the expression level of ubiquitin ligases such as MuRF1 and Atrogin1, which activates the ubiquitin-proteasome system in skeletal muscle. 9,27 In SAMP8 mice, the expression levels of MuRF1 and Atrogin1 were increased. 28 In addition, it has been reported that reducing the expression levels of MuRF1 and Atrogin1 suppresses skeletal muscle atrophy. 29 In this study,
the expression levels of MuRF1 and Atrogin1 in SAMP8 mice were increased compared to SAMR1 mice, and the levels were decreased by JTT administration (Fig. 7). In sarcopenia, the expression levels of MuRF1 and Atrogin1 are increased by various factors such as anabolic hormones, inflammatory cytokines, and oxidative stress.\(^6\) It has been reported that cytokines, such as TNF-\(\alpha\) and IL-6, induce the expression of MuRF1 by regulating NF-\(\kappa\)B activity, and thus induce muscle atrophy.\(^7\) On the contrary, Sirt1 reduces ROS levels by increasing the expression of antioxidant enzymes such as Mn-superoxide dismutase (SOD)\(^10\) and suppresses the production of inflammatory cytokines.\(^32\) Furthermore, the protein also reduces the expression of MuRF1 by inhibiting the activity of NF-\(\kappa\)B in skeletal muscle cells.\(^23\) Thus, Sirt1 is believed to alleviate muscle atrophy.\(^23\) It has also been reported that skeletal muscle atrophy in SAMP8 mice is suppressed by increasing Sirt1 expression.\(^33\) Furthermore, previous studies also suggested that JTT administration suppresses skeletal muscle atrophy by reducing the production of inflammatory cytokines and increasing the activity of antioxidant enzymes by elevating Sirt1 levels in streptozotocin-induced diabetic mice.\(^19,20\) In this study, JTT increased Sirt1 mRNA expression in the GA of SAMP8 mice. There was a significant correlation between Sirt1 mRNA expression, and muscle fiber cross-sectional areas of GA (\(r = 0.719, p < 0.001\)) (Fig. 4). Furthermore, the serum levels of TNF-\(\alpha\) and IL-6 were decreased significantly (Figs. 6A, B). From these results, it can be suggested that JTT’s use in SAMP8 mice reduced skeletal muscle atrophy by decreasing the expression level of inflammatory cytokines through increased Sirt1 expression. Previous studies have reported that a methanol extract of Cinamomum Cortex, Astragalli Radix, and Glycyrrhizae Radix, which is one of the crude drugs in JTT, increases the transcriptional activity of Sirt1 in C2C12 cells.\(^19\) It is expected that the components contained in these crude drugs act on the suppression of muscle atrophy in SAMP8 mice.

One of the causes of skeletal muscle atrophy in sarcopenia is a decrease in anabolic hormones. IGF-1, an anabolic hormone, is located upstream of FOXY, and it suppresses FOXY activity.\(^10\) FOXY has been reported to increase the expression of ubiquitin ligase and induce skeletal muscle atrophy.\(^24\) It has been previously reported that IGF-1 expression levels in SAMP8 mice with less anabolic hormones is lower than in SAMR1 mice, and that increasing IGF-1 suppresses skeletal muscle atrophy in SAMP8.\(^26\) In this study, JTT increased the serum IGF-1 expression (Fig. 5) in SAMP8 mice. This result indicates that JTT suppresses skeletal muscle atrophy by increasing IGF-1 expression. Regarding IGF-1/FOXY/ubiquitin ligase signal, Sirt1 is known to regulate the expression of ubiquitin ligases by suppressing the activities of FOXY.\(^22\) Therefore, JTT might also alleviate IGF-1 deficiency-induced muscle atrophy by increasing Sirt1 expression.

Thus, our results suggest that JTT alleviates muscle atrophy in SAMP8 mice by increasing Sirt1 and IGF-1 expression. Skeletal muscle atrophy in SAMP8 mice closely resembles the symptoms of sarcopenia, and JTT administration is expected to suppress the progression of sarcopenia symptoms in clinical practice. Since this study uses model mice, it is necessary to perform further research for future clinical trials.

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**Conflict of Interest** This research was conducted as a contract research by Tsumura Corporation.

**Supplementary Materials** The online version of this article contains supplementary materials.

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