Juzentaihoto Suppresses Muscle Atrophy in Streptozotocin-Induced Diabetic Mice

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In diabetic patients, skeletal muscle atrophy occurs due to increased oxidative stress and inflammation. Skeletal muscle atrophy reduces the QOL of patients and worsens life prognosis. Therefore, development of preventive therapy for muscle atrophy in hyperglycemic state is eagerly awaited. Juzentaihoto is a medicinal herb that has a function to supplement physical strength, and it is expected to prevent muscle atrophy. To determine the preventive effect of juzentaihoto on muscle atrophy in hyperglycemic state, streptozotocin (STZ) was administered to induce diabetes in mice and the preventive effect of juzentaihoto was evaluated. Mice that received juzentaihoto extract (JTT) showed that the decrease in muscle fiber cross-sectional area in the gastrocnemius muscle was reversed. Additionally, the expression level of tumor necrosis factor α (TNF-α), an inflammatory cytokine, in serum decreased, and that of ubiquitin ligase (atrogin-1, muscle RING-finger protein-1) mRNA in skeletal muscle decreased. An anti-inflammatory cytokine interleukin-10 showed increased levels in the serum and increased levels in spleen cell culture supernatant collected from mice that received JTT. JTT had no effect on the blood glucose level. These results suggest that prophylactic administration of JTT to STZ-induced diabetic mice affects immune cells such as in spleen, causing an anti-inflammatory effect and inhibiting excessive activation of the ubiquitin-proteasome system, to reverse muscle atrophy.

Key words muscle atrophy; juzentaihoto; diabetes; inflammatory

INTRODUCTION

In diabetic patients, it is reported that skeletal muscle atrophy occurs. In particular, muscle atrophy occurs markedly in type 1 diabetes. Skeletal muscle atrophy reduces the QOL of patients and worsens life prognosis.1,2) In diabetic patients, the expression level of inflammatory cytokines increases due to persistence of hyperglycemia state.3,4) Inflammatory cytokines increase the expression of ubiquitin ligase (muscle RING-finger-1 (MuRF1), atrogin-1) and induce atrophy of skeletal muscle.1,5–9)

Juzentaihoto10) is a traditional herbal medicine that originated in Ho-chi-chü-fang’s writings at the time of the Song Dynasty in China. Juzentaihoto was reported to have antioxidant, anticancer, and immunostimulatory effects,11–18) and it is also used against weakness, fatigue, and malaise. Juzentaihoto is expected to prevent muscle atrophy.

In mice intraperitoneally injected with streptozotocin (STZ), pancreatic islets of Langerhans are destroyed and insulin secretion failure occurs, resulting in symptoms similar to type 1 diabetes.15,16) In STZ-induced diabetic mice, inflammation and oxidative stress are elevated, and ubiquitin ligase expression is increased. This causes skeletal muscle atrophy.17,18) In this study, diabetic model mice were prepared by STZ injection and juzentaihoto extract (JTT) was administered prophylactically. Skeletal muscle mass and anti-inflammatory effects were then evaluated.

MATERIALS AND METHODS

Preparation of Food Containing JTT Juzentaihoto extract10) was provided as the spray-dried product by Tsumura (Tokyo, Japan). Five grams of the extract was obtained from 28.5 g of crude drug mixture shown in Table 1. JTT was mixed with normal feed (CLEA, Shizuoka, Japan) at a rate of 4% (w/w).19,20)

Animals Animal experiments were conducted with permission of Kochi University Animal Experiment Ethics Committee. ICR mice (8 weeks old, male) were purchased from Japan SLC (Shizuoka, Japan).

STZ was intraperitoneally injected at a dose of 150 mg/kg 4d before the start of JTT administration (day −4). Blood glucose and body weight were measured on the JTT administration start date (day 0), and mice were divided into two groups (STZ or STZ + JTT) to ensure that the average body weight and blood glucose levels were the same, and each group received normal feed or JTT-mixed feed. Additionally, the control group was fed normal feed.

Sample Preparation Mice underwent laparotomy under anesthesia on day 35 after starting JTT administration (day 0), and whole blood was collected from the descending vena cava. The collected blood was centrifuged (8000 rpm, 4°C) and the supernatant was used as a serum sample. After collecting the whole blood, the spleen and gastrocnemius muscle were excised and these tissue samples were analyzed.

Blood Glucose When blood glucose was measured, mice were fasted for 6 h before blood collection. Blood was collected from the tail vein and measured using Glu-TEST-STRIPS.
serum were measured using a TNF-
(Mm01185221_m1) and Atrogin1 (Mm00499523_m1) and Taq
with TaqMan probe for GAPDH (Mm99999915_g1), MuRF1
were normalized using glyceraldehyde 3-phosphate dehy-
CA, U.S.A.). MuRF1 and atrogin-1 mRNA expression levels
StepOnePlus Real-Time PCR Systems (Applied Biosystems,
obtain cDNA. TaqMan quantitative PCR was performed using
PrimeScript RT reagent Kit (TaKaRa Bio, Ohsu, Japan) to
many). The collected RNA was reverse transcribed using a
an RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Ger-
tems, Minneapolis, U.S.A.). Interleukin (IL)-10 serum and
collected, and interleukin (IL)-10 levels were measured using
ELISA kit (Invitrogen, Carlsbad, CA, U.S.A.).

Spleen Cells Culture The excised gastroc-
nemius muscle was fixed with 10% formalin, embedded in
paraffin, sectioned at 5 µm, and stained with hematoxylin and
eosin. A cross-section of the gastrocnemius muscle was traced
using computer analysis software WinROOF (Mitani Corpora-
tion, Ohtsu, Japan), and the cross-sectional area of each fiber
was measured. Cross-sectional areas of 100 muscle fibers
from each mouse were randomly measured, and the average
value was used in the analysis.

Spleen Cells Culture The excised spleen was pressed
through a 70 µm EASY strainer (Falcon, New York, U.S.A.)
to obtain a cell suspension. The obtained cell suspension was
stained with a 0.4% (w/v) trypan blue solution (FUJIFILM
Wako, Osaka, Japan) and the number of viable cells was
counted. Thereafter, the cell suspension was seeded onto a
48-well plate, with 1.0 × 10⁵ cells per well. Cells were cultured
for 48h with RPMI 1640 mixed with 1.0 µg/mL concana-
valin A, 10% fetal bovine serum, 100 U/mL penicillin, and
0.1 mg/mL streptomycin. Then, the culture supernatant was
collected, and interleukin (IL)-10 levels were measured using
an enzyme-linked immunosorbent assay (ELISA).

ELISA Tumor necrosis factor (TNF)-α levels in the
serum were measured using a TNF-α ELISA kit (R&D Sys-
tems, Minneapolis, U.S.A.). Interleukin (IL)-10 serum and
culture supernatant levels were measured using a Novex IL-10
ELISA kit (Invitrogen, Carlsbad, CA, U.S.A.).

Quantitative RT-PCR of MuRF1 and Atrogin-1 Gas-
trocnemius was cut to 0.1 cm width and then homogenized
with POLYTRON RT-MR 3100 (Central Science Trade,
Tokyo, Japan). Blood glucose levels were measured every 7d. Mice in which the blood glucose level
did not exceed 300 mg/mL on day 0 in the STZ-induced mice
were excluded from the experiment.

Myocyte Cross-Sectional Area The excised gastroc-
nemius muscle was fixed with 10% formalin, embedded in
paraffin, sectioned at 5 µm, and stained with hematoxylin and
eosin. A cross-section of the gastrocnemius muscle was traced
using computer analysis software WinROOF (Mitani Corpora-
tion, Ohtsu, Japan), and the cross-sectional area of each fiber
was measured. Cross-sectional areas of 100 muscle fibers
from each mouse were randomly measured, and the average
value was used in the analysis.

Table 1. Crude Drug Contents of JTT

| Crude drug name     | Origin                                      | One-day dose (g) |
|---------------------|---------------------------------------------|------------------|
| Angelicae Radix     | The dried root of Angelica acutiloba (SIEBOLD & ZUCC.) KITAG. | 3                |
| Astragali Radix     | The dried root of Astragalus propinquus SCHISCHKIN | 3                |
| Atractylodis Lanceae Rhizoma | The dried rhizome of Atractylodes lancea DE CANDOLLE | 3                |
| Cinnamomi Cortex    | The dried bark of Cinnamomum cassia (LINNE) J. PRESL | 3                |
| Cnidii Rhizoma      | The dried rhizome of Cnidium officinale MAXINO | 3                |
| Ginseng Radix       | The dried root of Panax ginseng C. A. MAYER | 1.5              |
| Glycyrrhizae Radix  | The dried root and stolon of Glycyrrhiza uralensis FISHER | 3                |
| Poria               | The dried sclerotium of Wolfsparia cocos RYVARDEN ET GILBERTSON | 3                |
| Processi Aconiti Radix | The dried tuberous root of Aconitum carmichaelii DEBEAUX prepared by autoclaving | 3                |
| Rehmanniae Radix    | The dried root of Rehmannia glutinosa (GAERTNER) A. DE CANDOLLE | 3                |

Fig. 1. Effect of JTT on the Level of Blood Glucose

Blood glucose levels were measured at −4, 0, 7, 14, 21, 28, 35 d after JTT admin-
istration. The results were expressed as mean ± standard deviation (S.D.) (n = 5).
The data was evaluated by two-way ANOVA, and the comparison among drug-
treatment groups in each day evaluated by Dunnett’s multiple comparison test.

**p < 0.01 vs. control group.

Man Universal PCR Master Mix were from Applied Biosys-
tems (Foster City, CA, U.S.A.).

Statistical Analysis The transition of blood glucose
was analyzed by two-way ANOVA, and other experimental
results were analyzed by one-way ANOVA, followed by mul-
tiple comparison test using Dunnet’ i-test. These statistical
analyses were conducted using Stat Flex program (View Flex,
Tokyo, Japan). A p-value of <0.05 was considered statistically
significant.

RESULTS

Blood Glucose Levels We measured blood glucose levels
every 7d from the start of JTT administration to confirm the
influence of JTT on blood glucose. There was no difference
in the blood glucose level in each group at the time of intra-
peritoneal STZ injection 4d before JTT administration (day
−4) (Fig. 1). In the period from the JTT administration start
date (day 0) to the administration end date (day 35), the blood
glucose level was significantly elevated in the STZ induced
group (STZ group) compared with the STZ group at each day (Fig. 1).
Two way ANOVA results indicated that these effects was sig-
nificant about the time (F_6,84 = 104, p < 0.01), drug-treatment
(F_2,84 = 665, p < 0.01) and interaction of the time and drug-

Fig. 1. Effect of JTT on the Level of Blood Glucose
Myocyte Cross-Sectional Area
The cross-sectional area of muscle fibers in the gastrocnemius extracted 35 d after JTT administration was compared among the groups to confirm the effect of JTT on preventing skeletal muscle atrophy. In the STZ group, the gastrocnemius muscle fiber cross-sectional area was significantly reduced compared with the control group \((p < 0.01)\), and this reduction was significantly lower in the JTT group \((p < 0.01)\) (Figs. 2A, B). One way ANOVA result indicated that the effect was significant \((F_{2,12} = 16.9, p < 0.01)\).

Inflammatory Cytokines
TNF-\(\alpha\) levels in serum collected 35 d after the start of JTT administration were measured to confirm the effect of JTT on inflammatory cytokines. In the STZ group, serum TNF-\(\alpha\) levels were significantly increased compared with the control group \((p < 0.01)\), but the decrease was prevented in the STZ + JTT group \((p < 0.05)\) (Fig. 3A). One way ANOVA result indicated that the effect was significant \((F_{2,12} = 15.4, p < 0.01)\). Additionally, cells were harvested from the spleen of these mice and cultured, and IL-10 levels contained in the culture supernatant were measured. Thus, IL-10 levels were significantly decreased in the STZ group compared with the control group \((p < 0.05)\), but this decrease tended to be prevented in the STZ + JTT group \((p = 0.066)\) (Fig. 4). One way ANOVA result indicated that the effect was significant \((F_{2,12} = 4.30, p < 0.05)\).

Anti-inflammatory Cytokines
IL-10 anti-inflammatory cytokine levels after JTT administration were measured. In the STZ group, serum IL-10 levels were significantly decreased compared with the control group \((p < 0.01)\), but the decrease was prevented in the STZ + JTT group \((p < 0.05)\) (Fig. 3B). One way ANOVA result indicated that the effect was significant \((F_{2,12} = 15.4, p < 0.01)\). Additionally, cells were harvested from the spleen of these mice and cultured, and IL-10 levels contained in the culture supernatant were measured. Thus, IL-10 levels were significantly decreased in the STZ group compared with the control group \((p < 0.05)\), but this decrease tended to be prevented in the STZ + JTT group \((p = 0.066)\) (Fig. 4). One way ANOVA result indicated that the effect was significant \((F_{2,12} = 4.30, p < 0.05)\).

Ubiquitin Ligase mRNA Expression
Total RNA was extracted from the gastrocnemius muscle that was excised from the mouse lower limb, and ubiquitin ligase mRNA expression levels (atrogin-1, MuRF1) were measured. In the STZ group, MuRF1 and Atrogin1 expression levels were significantly increased \((F_{2,12} = 10.7, p < 0.01)\).

**Fig. 2. Myocyte Cross-Sectional Area of Gastrocnemius**
Gastrocnemius muscle was removed from mice 35 d after the start of JTT administration. After that, stained with Haematoxylin and Eosin. (A) Myocyte cross-sections of gastrocnemius, (B) the summary data of myocyte cross sectional area. These data were expressed as mean \pm S.D. \((n = 5)\), and evaluated by one way ANOVA, and followed by Dunnett’s multiple comparison test. **\(p < 0.01\) between the groups. (Color figure can be accessed in the online version.)

**Fig. 3. The Levels of Cytokines in the Serum of Mice**
Blood was collected from mice at 35 d after the start of JTT administration. And, (A) the levels of TNF-\(\alpha\) and (B) the level of IL-10 in the serum of mice. These data were expressed as mean \pm S.D. \((n = 5)\), and evaluated by one way ANOVA, and followed by Dunnett’s multiple comparison test. **\(p < 0.01\), *\(p < 0.05\) between the groups.
increased compared with the control group \( (p < 0.01) \), but this increase in both MuRF1 and Atrogin1 levels was significantly reduced in the STZ + JTT group \( (p < 0.05) \) (Figs. 5A, B). One way ANOVA result indicated that these effects were significant (MuRF1: \( F_{2,12} = 8.81, p < 0.01 \), atrogin-1: \( F_{2,12} = 7.92, p < 0.01 \)).

**DISCUSSION**

The most important finding in this study is that skeletal muscle atrophy was prevented by prophylactic administration of JTT to STZ-induced diabetic mice (Figs. 2A, B). However, there was no improvement in the hyperglycemic condition resulting from JTT administration (Fig. 1), suggesting that JTT improved muscle atrophy regardless of the blood glucose level.

The inflammatory cytokine expression level increases under hyperglycemic conditions.\(^3,4\) Inflammatory cytokines increase ubiquitin ligase expression (MuRF1, atrogin-1) and cause skeletal muscle atrophy.\(^5,6\) In STZ-induced mice, skeletal muscle atrophy occurs through elevated ubiquitin ligase expression, which results from an increase in inflammatory cytokine levels.\(^17,18\) The same phenomenon with STZ injection also occurred in this study. However, prophylactic JTT administration to STZ-induced diabetic mice reduced serum levels of the inflammatory cytokine TNF-\( \alpha \) (Fig. 3A). Additionally, the increase in Atrogin1 and MuRF1 expression was also suppressed (Figs. 5A, B). Thus, we suggest that JTT inhibits skeletal muscle atrophy by suppressing inflammatory cytokine levels and the enhancing the ubiquitin proteasome system.

IL-10 is an anti-inflammatory cytokine that is known to suppress inflammatory cytokine levels and reduce inflammation that is mainly produced from Th2 cells, B cells, and mast cells.\(^{21,22}\) IL-10 levels decrease in diabetic patients, and this is considered to cause worsening of inflammatory diseases.\(^23\) It is also known to improve diabetic symptoms and skeletal muscle atrophy by increasing the IL-10 levels in diabetic mice.\(^{24–26}\) It is expected to be a target factor for the treatment of diabetes and its complications. Additionally, in this study, IL-10 levels decreased with injection of STZ, and this decrease was reversed by prophylactic JTT administration (Fig. 3B). Most IL-10 is produced in the spleen,\(^{27,28}\) and diabetic complications are exacerbated by removal of the spleen.\(^{29,30}\) In this study, cells harvested from spleens isolated from these mice were cultured and IL-10 levels in the culture supernatant were measured. IL-10 expression was shown to decrease in the STZ-induced group, but in mice that received JTT, decrease tended to be prevented (Fig. 4). It is thought that JTT acts on immune cells such as spleen and has an action to promote the release of IL-10. However, as the reason that changes in IL-10 did not become significant in this study, much of IL-10 is produced in the spleen, but IL-10 production also occurs from tissues other than spleen. Therefore, it was thought that serum IL-10 concentration could not be explained only by those derived from spleen cells. JTT may also act on immune tissues other than spleen, such as adipocytes, lymph nodes, and thymus.

Similar to this study, there are several prior studies examining the effect of preventing effect for skeletal muscle atrophy in diabetic mice. For example, flavonoids from Glycyrrhiza species and panaxatriol from Panax species have been re-
ported to suppress inflammatory cytokines and muscle atrophy.\textsuperscript{11–33} Juzen-taiho-to contains root of Glycyrrhiza uralensis and root of Panax ginseng,\textsuperscript{10} and, these compounds may be one of the active ingredients of juzen-taiho. But, the doses in previous studies of these active ingredients are higher compared to the JTT doses in this study. Therefore, it is unlikely that juzen-taiho alleviates muscle atrophy of diabetes by effect of these active ingredient, and juzen-taiho may contain other active ingredients.

Thus, we suggest that JTT acts on immune cells, shows an anti-inflammatory effect and decreases ubiquitin ligase expression. And this suppresses skeletal muscle atrophy. In clinical practice, it is expected that juzen-taiho inhibits inflammation and prevents muscle atrophy in diabetic patients.

**Conflict of Interest**  Mitsuko Miyamura received a research grant from Tsumura Co., Ltd.

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

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