Disuse muscle atrophy-improving effect of ninjin'yoeito in a mouse model

Risa Takemoto *, Takehiro Sejima, Li-Kun Han, Seiwa Michihara, Ryuji Takahashi

Kampo Research Laboratories, Kracie Pharma Ltd., 3-1 Kanebo machi, Takaoka, Toyama 933-0856, Japan

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ABSTRACT

Disuse syndrome indicates psychosomatic hypofunction caused by excess rest and motionless and muscle atrophy is termed disuse muscle atrophy. Disuse muscle atrophy-induced muscle weakness and hypoactivity further induces muscle atrophy, leading to a vicious cycle, and this is considered a factor causing secondary sarcopenia and subsequently frailty. Since frailty finally leads to a bedridden state requiring nursing, in facing a super-aging society, intervention for a risk factor of frailty, disuse muscle atrophy, is important. However, the main treatment of disuse muscle atrophy is physical therapy and there are fewer effective preventive and therapeutic drugs. The objective of this study was to search for Kampo medicine with a disuse muscle atrophy-improving effect. Ninjin'yoeito is classified as a qi-blood sohozai (dual supplement) in Chinese herbal medicine, and it has an action supplementing the spleen related to muscle. In addition, improvement of muscle mass and muscle weakness by ninjin'yoeito in a clinical study has been reported. In this study, the effect of ninjin'yoeito on disuse muscle atrophy was investigated.

A disuse muscle atrophy model was prepared using male ICR mice. After surgery applying a ring for tail suspension, a 1-week recovery period was set. Ninjin'yoeito was administered by mixing it in the diet for 1 week after the recovery period, followed by tail suspension for 14 days. Ninjin'yoeito administration was continued until autopsy including the hindlimb suspension period. The mice were euthanized and autopsied immediately after completion of tail suspension, and the hindlimb muscles were collected. The food and water intakes during the hindlimb unloaded period, wet weight of the collected muscle, and muscle synthesis and muscle degradation-related factors in blood and muscle were evaluated.

Ingestion of ninjin'yoeito inhibited tail suspension-induced reduction of the soleus muscle wet weight. In addition, an increase in the blood level of a muscle synthesis-related factor, IGF-1, and promotion of phosphorylation of mTOR and 4E-BP1 in the soleus muscle were observed.

It was suggested that ninjin'yoeito has a disuse muscle atrophy-improving action. Promotion of the muscle synthesis pathway was considered the action mechanism of this.

1. Introduction

Disuse syndrome is the collective name of secondary disorders occurring in the organs throughout the body due to continuation of a disease-induced resting condition with reduced activity and movement. In the elderly, even mild invasion and short-term rest readily cause disuse syndrome and muscle atrophy is termed disuse muscle atrophy. Muscle weakness and hypoactivity due to disuse muscle atrophy further induce muscle atrophy, leading to a vicious cycle, and this is considered a cause of secondary sarcopenia and subsequent frailty. Since frailty finally leads to a bedridden state requiring nursing, in facing a super-aging society, intervention for a risk factor of frailty, disuse muscle atrophy, is important. However, the main treatment of disuse muscle atrophy is physical therapy and there are fewer effective preventive and therapeutic drugs.

Ninjin'yoeito (NYT) is a prescription of Chinese herbal medicine comprised of 12 crude drugs: ninjin, toki, shakuyaku, jio, byakujutsu, bukuryo, keihi, ogi, chinpi, onji, gomishi, and kanzo (Table 1). This is an ethical drug used to treat decreased physical strength after illness, tiredness and fatigue, anorexia, night sweats, cold extremities, and anemia. It has been reported that NYT increased muscular strength and muscle mass in frail patients and patients in rehabilitation in a clinical study (Morinaga et al., 2020; Sakisaka et al., 2018), so that the drug is expected to be effective for disuse muscle atrophy. Thus, in this study, the effect of NYT on disuse muscle atrophy was investigated.

* Corresponding author.

E-mail address: takemoto_risa@kracie.co.jp (R. Takemoto).

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2. Materials and methods

1. Experimental animals and maintenance environment: Five-week-old male ICR mice were purchased from Japan SLC Inc. The animals were maintained at 23 ± 2°C room temperature and 55 ± 10% humidity in a 12-h cycle of light on at 8:00 and light off at 20:00. The mice were given free access to food and water during the experimental period. After adaptation to the lighting conditions for 1 week, the healthy animals were used in the following experiments. A wire mounting was performed following the method reported by Ferreira et al. (Ferreira et al., 2011) in 48 mice. At 1 week after surgery, the animals were randomly divided into 4 groups: non-hindlimb suspension group (Normal; n = 12), hindlimb suspension group (HU; n = 12), hindlimb suspension +3% NYT group (HU + NYT3%; n = 12), and hindlimb suspension +5% NYT group (HU + NYT5%; n = 12), and administration was initiated. A hindlimb-suspension was initiated 1 week after initiation of administration. The duration of hindlimb suspension was set to 2 weeks, and a hindlimb-suspension performed as described previously (Ferreira et al., 2011). During the maintenance period with hindlimb suspension, the forelimbs maintained contact with the cage's plastic floor, allowing the animals to move, groom themselves, and obtain food and water freely. After completion of the experiment, blood was collected from the inferior vena cava under isoflurane anesthesia, the muscle was excised from the hindlimbs and the wet weight was measured (Fig. 1). Collected blood was centrifuged at 3000 rpm for 30 min and the supernatant was stored at −40°C until the use as a serum. The soleus muscle specimen was frozen in liquid nitrogen and was stored at −40°C until the use. This experiment was performed after approval by the Animal experiment committee of Kracie Pharmaceutical, Ltd. Kampo Research Laboratories following regulations on animal experimentation specified by the Research Laboratories and ‘Basic guidelines for the conduct of animal experiments in implementing agencies under the jurisdiction the Ministry of Health, Labour and Welfare’.

2. NYT extracts: NYT extract powder (Lot. No. 16101305) were obtained from the second Takatsuki plant of Kracie Pharmaceutical, Ltd.

3. Experimental diet: Laboratory chow pellet (MF, Oriental Yeast Co., Ltd.) was used as a control diet. 3%, 5% NYT extract powder was added into laboratory chow pellet. In this experiment, MF containing 3%, 5% NYT extract was prepared by Oriental Yeast Co., Ltd.

4. Serum IGF-1 concentration measurement: Serum IGF-1 was measured using Mouse/Rat IGF-1/IGF-1 Quantikine ELISA kit (MG-100; R&D Systems, Inc.) following the procedure of the kit.

Table 1
Composition (daily dose) of Kampo Formula Ninjiryoeto (NYT).

| Ingredients | Contents (g) |
|-------------|-------------|
| Poria Sclerotium | 4.0 |
| Japanese Angelica Root | 4.0 |
| Rehmannia Root | 4.0 |
| Atractylodes Rhizome | 4.0 |
| Ginseng | 3.0 |
| Cinnamon Bark | 2.5 |
| Citrus Unshiu Peel | 2.0 |
| Polygala Root | 2.0 |
| Peony Root | 2.0 |
| Astragalus Root | 1.5 |
| Schisandra Fruit | 1.0 |
| Glycyrrhiza | 1.0 |

* Approximate 6700 mg of dried water extract of NYT was prepared in GMP-standardized factory of Kracie Pharma, Ltd. (Japan) on the basis of above described composition.

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Table 2
The composition of blocking buffer and antibody diluent solution.

| Blocking buffer | 1st antibody | 2nd antibody |
|-----------------|--------------|--------------|
| p-4EBP1(65)     | 0.5% Skim    | 5% BSA       | 0.5% Skim    |
| total 4EBP1     | 5% BP1       | 5% BSA       | 0.5% Skim    |
| p-p70 S6 K      | 0.5% Skim    | Can Get      | Can Get      |
| total p70 S6 K  | 0.5% Skim    | Can Get      | Can Get      |
| p-mTOR          | 0.5% Skim    | 5% BSA       | Can Get      |
| mTOR            | 0.5% Skim    | 5% BSA       | Can Get      |

0.5% Skim: 0.5% skim milk (ECL Prime™ Blocking Agent, GE Healthcare) in TBST.
5%BSA: 5% BSA (Albumin, from Bovine Serum, Fraction V pH 7.0, FUJIFILM Wako Pure Chemical.) in TBST.
Can Get: Can Get Signal Immunoreaction Enhancer solution, Toyobo.
BP1: 5% Blocking-One P (Blocking One-P, Nacalai tesque, INC.) in TBST.

Fig. 1. Experimental schedule for the hindlimb suspension (HU) model.
Fig. 2. Influence of NYT on the body weight and food intake in mice maintained with hindlimb suspension. 
A: The body weight was measured at autopsy after disuse for 2 weeks. B: The food intake was measured during the disuse period. Data are expressed as mean ± SEM. 
**p < 0.01, *p < 0.05 vs. HU group; HU, Hindlimb-Unloading; NYT, ninjin’yoeito.

Fig. 3. Influence of NYT on the muscle weight. 
The muscle was excised after disuse for 2 weeks and the wet weight was measured. A. The soleus muscle weight. B. The gastrocnemius muscle weight. C. The plantaris muscle weight. Data are expressed as mean ± SEM. **p < 0.01, *p < 0.05 vs. HU group.
5. Serum insulin level measurement: The serum insulin levels were determined using a Morinaga mouse/rat insulin ELISA kit (4917–065, Morinaga Institute of Biological Science, Inc.)

6. Western blot: Collected muscle specimens were re-frozen in liquid nitrogen and finely crushed using sufficiently cooled metal, followed by addition of 100 μL of lysis Buffer (100 mM Tris-HCl, 150 mM
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NaCl, 1% triton X-100, 0.1% SDS, 1 mM EDTA) containing 1% Protease inhibitor (03969–21; Nakarai Tesque) and 1% phosphatase inhibitor (07575–51; Nakarai Tesque). Two ceramic beads (diameter: 3 mm) were added into a microtube and the specimen was sufficiently homogenized at 25 rpm/s for 10 min using Tissue Lyser LT (QIAGEN). The beads were then removed, the homogenate was centrifuged at 12,000 rpm, 4 °C for 20 min, and the supernatant was collected as a protein extract. The protein extract was stored at −40 °C until use for protein quantitation and analysis.

Western blotting was performed using Mini-PROTEAN 3 cell (Bio-Rad). The protein extract of each specimen was thawed on ice, diluted to 50% or higher with Laemmli Sample Buffer (161–0747; BIORAD), warmed at 95 °C for 5 min, and fractionated by the protein amount by SDS-PAGE using 4–20% polyacrylamide gel mini-PROTEAN® TGXTM (456–1096; BIORAD) (10 μg/lane). The bands were then blotted to PVDF membrane (MILLIPORE Immobilon®-FL Transfer Membrane IPFL00010, pore size: 0.45 μm) from the polyacrylamide gel. After blotting, the membrane was washed with TBS (TAKARA T903) containing 0.1% tween 20 (TBST) 3 times, blocked with Blocking Buffer (Table 2) for 1 h, washed with TBST for 5 min 4 times, and reacted with primary antibody. The following primary antibodies were used in Western blotting: Anti-GAPDH antibody (G8795, 1:3000; Sigma-Aldrich), anti- phospho-p70 S6 Kinase (Thr389) (108D2) Rabbit mAb (9234, 1:1000; Cell Signaling Technology), anti-p70 S6 Kinase (49D7) Rabbit mAb antibody (2708, 1:1000; Cell Signaling Technology), anti-Phospho-4E-BP1 (Ser65) antibody (9451, 1:500; Cell Signaling Technology), anti-4E-BP1 antibody (9452, 1:1000; Cell Signaling Technology), anti-phospho-mTOR (Ser2448) Antibody (2971, 1:500; Cell Signaling Technology), anti-mTOR Antibody (2972, 1:1000; Cell Signaling Technology). After washing with TBST for 5 min 4 times, the membrane was reacted with secondary antibody. The following secondary antibodies were used in Western blotting: Anti-mouse Ig antibody (7076, 1:3000; Cell Signaling Technology) and Anti-Rabbit Ig Light-Chain Specific: HRP (RS3251, 1:10,000; ECM Biosciences). After washing with TBST for 5 min 4 times, the membrane was photographed using ECL Plus Western Blotting Detection System (GE Healthcare Amersham™ ECLTM Prime Western Blotting Detection Regent Lot. 14,775,048) and LAS3000 Imaging System (Fuji Film Corp., Japan). For reprobe of protein, Restore WB stripping Buffer (SL257374; Thermo) was used.

7. Western band analysis and Calculation of values: The fluorescence intensity around the Western band was obtained by subtracting the background in individuals based on Western blot images of each factor. The phosphorylation amount/protein level and protein level/GAPDH ratios were calculated. Samples from the same individual were poured on each membrane. The individual’s value for each membrane was calculated, regarding the individual’s phosphorylation amount/protein level and protein level/GAPDH ratios as 1, and corrected.

8. Statistics: All data are expressed as the mean with SEM. Significance was determined by one-way analysis of variance followed by the Tukey-Kramer test. P-values of less than 0.05 were considered significant.

3. Results

1. Influence of NYT on the body weight and food intake in mice maintained with hindlimb suspension

The body weight after 2-week hindlimb suspension (autopsy day) is shown in Fig. 2A and food intake during the hindlimb suspension period

Fig. 6. Influence of NYT on soleus muscle 4E-BP phosphorylation and expression levels. Protein was extracted from the soleus muscle excised after disuse for 2 weeks and 4E-BP1 phosphorylation and expression levels was detected by WB. Data are expressed as mean ± SEM. *p < 0.05 vs. HU group.
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2. Influence of NYT on the muscle weight

The muscle weight was corrected with the body weight. The soleus muscle weight significantly decreased in HU compared with that in Normal, and increased both in HU + NYT3% and HU + NYT5% (Fig. 3A). However, there was no obvious difference in the gastrocnemius and plantaris muscles among the Normal group (gastrocnemius muscle: 417.89 ± 9.46 mg/100 g body weight; plantaris muscle: 54.24 ± 2.21 mg/100 g body weight), HU group (gastrocnemius muscle: 413.94 ± 16.49 mg/100 g body weight; plantaris muscle: 53.82 ± 2.65 mg/100 g body weight), HU + NYT3% group (gastrocnemius muscle: 423.62 ± 12.31 mg/100 g body weight; plantaris muscle: 58.45 ± 1.74 mg/100 g body weight), or HU + NYT5% group (gastrocnemius muscle: 414.13 ± 10.48 mg/100 g body weight; plantaris muscle: 56.47 ± 2.29 mg/100 g body weight) (Fig. 3B, C).

3. Influence of NYT on the serum IGF-1 and insulin concentrations

The plasma IGF-1 concentration decreased in HU compared with that in Normal and significantly recovered in HU + NYT5% (Fig. 4A). No significant difference was noted in the blood insulin concentration among the groups (Fig. 4B).

4. Influence of NYT on soleus muscle synthesis

Phosphorylation of mTOR involved in muscle synthesis was investigated. No difference was noted between Normal and HU, but phosphorylation significantly increased in HU + NYT5% (Fig. 5A). Thus, phosphorylation of 4E-BP1 and p70S6K located downstream of mTOR was investigated. Hyper-phosphorylation of 4E-BP1 decreased in HU compared with that in Normal, tended to increase in HU + NYT3%, and significantly increased in HU + 5% (Fig. 6A). On the other hand, phosphorylation of p70S6K tended to increase in HU + NYT5% although it was not significant (Fig. 7A).

4. Discussion

Skeletal muscles are constantly degraded and synthesized, and the muscle mass is maintained by the balance between these (Ilha et al., 2018). When a decrease in the mechanical load on skeletal muscle continues, the skeletal muscle mass and function decline and this is termed disuse muscle atrophy. Disuse muscle atrophy is treated mainly with muscular training and there is no effective drug therapy.

NYT has been reported to increase the muscle mass and grip strength in frail patients (Morinaga et al., 2020; Sakisaka et al., 2018), being expected to be effective for disuse muscle atrophy. In the disuse muscle atrophy model used in this study, the hindlimbs are made weightless by fixing the tail and the model is considered similar to a long-term bedridden or resting state in which the muscle mass decreases based on the kinematic use of muscle or dynamics of metabolic substances (Haida, 1990). Thus, we pre-investigated the effects of NYT on muscle atrophy using this animal model. Many studies have reported that tail suspension significantly atrophies slow muscle fiber-dominant muscles (slow muscles) compared to fast muscle fiber-dominant muscles (fast muscles) (Po, 2001; Thomason and Booth, 1990). In this study, NYT treatment prevented the loss of soleus muscle mass in mice during 14 days of hindlimb suspension. However, there was no obvious difference in the gastrocnemius and plantaris muscles among the Normal group,
HU group, HU + NYT3% group, or HU + NYT5% group. Furthermore, there were no significant changes in the body weight, food intake, water intake, or muscle weight after oral NYT administration to healthy rodents (data not shown). In this study, we first paid attention to blood IGF-1 and insulin as its mechanism. IGF-1 and insulin are famous as a factor inducing muscle hypertrophy. Regarding IGF-1, in 2001, Rommel et al., 2001 reported the presence of an intracellular signaling molecule system involved in promotion of muscle constituent protein synthesis (Rommel et al., 2001). When IGF-1 acts on the IGF-1 receptor, Akt is activated, and muscle synthesis is promoted via mammalian target of rapamycin (mTOR). Thus, we measured the serum IGF level and observed that NYT recovered the IGF concentration decreased by hindlimb suspension. To clarify the mechanism of muscle atrophy inhibition by NYT in muscle protein, first, phosphorylation of mTOR was investigated and it was clarified that ingestion of 5% NYT mixed in feed influenced mTOR phosphorylation. Thus, the phosphorylation levels of 4E-BP1 and p70S6K located downstream of mTOR and involved in muscle synthesis (Vary et al., 2000) were investigated. NYT mixed in feed at 5% slightly promoted phosphorylation of p70S6K and markedly promoted phosphorylation of 4E-BP1. On the other hand, 3% NYT mixed in feed did not influence the phosphorylation level of p70S6K and slightly promoted 4E-BP1 phosphorylation, suggesting that a mechanism promoting mTOR-dependent/–independent 4E-BP1 phosphorylation and slightly promoting p70S6K phosphorylation acted in 5% mixture feed. On the other hand, 3% mixture feed was not involved in activation of mTOR, being likely to promote 4E-BP1 phosphorylation independently from mTOR. The food intake was not increased by NYT, suggesting that nutrition was not simply increased and the ingredients unique to NYT contributed to improvement of muscle atrophy. It was reported that schisandraceae fructus extracts, ginseng, astragalus polysaccharide and their components have an effect on muscle or normalizing insulin resistance and carbohydrate metabolism (Geng et al., 2017; Go et al., 2017; Kim et al., 2018; Takamura et al., 2017). We would like to consider that detailed examination in the future in vitro. Since it has been reported that NYT recovered muscle atrophy in not only the disuse model investigated in this study but also cancer-bearing mice (Ohsawa et al., 2018), Klotho mice (Takahashi et al., 2018), cigarette exposure model (Miyamoto et al., 2020), and broad muscle atrophy models, it is expected to exhibit an effect on muscle atrophy induced by various causes in clinical cases.

Author statement

Ninjin'yoeto (NYT) is a prescription of Chinese herbal medicine comprised of 12 crude drugs. This study investigated the effects of NYT on disuse muscle atrophy was investigated. We believe that our study makes a significant contribution to the literature because we found that ingestion of NYT increased blood level of a muscle synthesis-related factor, IGF-1, promoted phosphorylation of mTOR and 4E-BP1 in the soleus muscle, and inhibited tail suspension-induced reduction of the soleus muscle wet weight. Further, we believe that this paper will be of interest to the readership of your journal because this study is the first to find that NYT has a disuse muscle atrophy-improving action, and promotion of the muscle synthesis pathway was considered the action mechanism of this. The present findings provide some insights into the mechanisms underlying the beneficial effects of NYT on muscle atrophy.

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