



Original Article

The Effects of Dokhwalgisaeng-tang against Disuse Muscle Atrophy in Gastrocnemius of Rats

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ABSTRACT

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Keywords:

BAX, Bcl-2, apoptosis, Dokhwalgisaeng-tang, gastrocnemius muscle, muscle atrophy

Background: The purpose of this study was to examine the effect of Dokhwalgisaeng-tang on immobilization-induced muscle atrophy.

Methods: Twenty young male Sprague-Dawley rats were divided into 2 groups. The rats in Dokhwalgisaeng-tang group were orally administered Dokhwalgisaeng-tang water extract, and the rats in the control group were given saline only. Hind limb immobilization was performed with casting tape to keep the left ankle joint in a fully extended position. No intervention was performed on the right leg which was used as an intact region. After 2 weeks of immobilization, all animals were sacrificed, and the gastrocnemius muscle was dissected from both legs and weighed. The morphology of the right and the left gastrocnemius muscle in both the Dokhwalgisaeng-tang and the control group was assessed by hematoxylin and eosin staining. The muscle cross sectional area was examined by image analysis (Axiovision LE software). In addition, immunohistochemical staining was carried out using the free-floating method, and the number of apoptotic related proteins were counted (anti-BAX, anti-Bcl-2).

Results: Dokhwalgisaeng-tang showed a significant protective effect against the reduction of the left gastrocnemius muscle (weight and muscle cross sectional area) compared with the control group. Moreover, the treatment with Dokhwalgisaeng-tang significantly reduced protein expression of BAX and increased protein expression of Bcl-2 in the gastrocnemius muscle compared with the control group.

Conclusion: Dokhwalgisaeng-tang showed protective effects against disuse muscle atrophy, potentially through altered BAX and Bcl-2 protein expression in the gastrocnemius muscle.

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Introduction

Muscle atrophy can be defined as the loss of muscle mass resulting from aging, nerve damage, and severe illness [1]. Immobilization-induced muscle atrophy refers to muscle atrophy caused by muscle disuse or malfunction. It can be caused by the fixation of joints or a splint to protect repeated tissue damage due to bone fracture. Muscle atrophy further limits the range of motion of the joints and impairs muscle strength and function, which in turn affects the duration of patients' rehabilitation [2].

Marzetti et al [3] showed that apoptosis plays an important role in the development of atrophy. The expression of B-cell chronic lymphocytic lymphoma 2 (Bcl-2) and Bcl-2 associated X protein (BAX) indicates the induction of anti- and pro-apoptotic regulators, respectively [4]. The function of mitochondria in the apoptosis process is important. BAX plays a role in promoting

apoptosis by altering the membrane permeability of mitochondria, and Bcl-2 helps in inhibiting apoptosis. The ratio of pro-apoptotic protein BAX, and the anti-apoptotic protein Bcl-2, determines apoptosis [5].

Dokhwalgisaeng-tang (DGT) is the first prescription in the Beijiqianjinyaofang. It has effects on liver-kidney depletion, hypertonicity of the sinews, and bone impediment, and thus it is usually prescribed for back pain and gait due to the lack of liver-kidney fluid-humor depletion [6]. Experimental studies in rats using DGT showed that it improves osteoblast proliferation [7] and alleviates osteoporosis-induced rheumatic damage and pain [8], and nerve regeneration following sciatic nerve crush injury [9]. Moreover, DGT is known to be effective in various musculoskeletal disorders and nerve damage, but its effect on immobilization-induced muscle atrophy has not been studied yet.

Therefore, this study aimed to examine the effect of DGT on

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immobilization-induced muscle atrophy. A rat model of muscle atrophy was used, and muscle weight and changes in the muscle fiber were observed. The expression of apoptotic regulators BAX and Bcl-2, were assessed to determine if there were any differences in the expression of these apoptotic proteins.

Materials and Methods

Animals

In this study, 10-week-old white, male Sprague Dawley rats weighing approximately 250 g were used. The rats were segregated at 23°C-24°C, 40%–60% humidity with 12 h of light and dark and given sterile distilled water and food. Experiments on the rats began 1 week after acclimatization. All the procedures in this study were conducted in accordance with the regulations and policies of the Animal Experiment Ethics Committee of Dong-Eui University. (Approval no.: R2017-023)

Herbal medicine

Herbal medicine was purchased from Nanumberb (Kyungbuk, Korea). DGT was used in accordance with the dose described in Donguibogam [10] (Table 1).

Classification of the study groups

Ten rats were randomly assigned into either the DGT group, which was administered with DGT, or the control group, which was administered with the same volume of saline. Both groups

were free to consume water and feed during the experimental period.

Herbal medicine preparation and administration

About 185 g of the 5-pack amount of herbal medicine was placed in 2 L distilled water. The mixture was heated for 2 hours in a hot water bath and filtered using an herbal nonwoven fabric. The filtrate was concentrated under reduced pressure using a 1 L rotary evaporator, and was lyophilized producing 56.4 g (30.5%). For 2 weeks, each group was orally administered daily at 10am. On the basis that a 70 kg adult has 3 packs per day, about 158.6 mg per 100 g body weight of the rat, was orally administered in 2 mL drinking water.

Inducing disuse muscle atrophy

To induce disuse muscle atrophy, the left ankle joint was fully extended and immobilized for 2 weeks using a casting tape. The casting tape was changed once a week. The right ankle was untreated and had free movement.

Measuring weight and changes in muscle weight

The weight of the rats was measured daily before drug administration during the study period, and before the autopsy on the last day of the experiment. Two weeks later, the rats were euthanized, and the gastrocnemius muscle was removed during autopsy and weighed. The weight of the rats was measured, and the muscle weight per 100 g of body weight was calculated. The

Table 1. Herbal Compositions of Dokhwalsaeng-tang.

Herb name	Scientific Name	Amounts (g)
Angelicae Pubescentis Radix	<i>Heracleum hemsleyanum</i> Michx	3
Angelicae Gigantis Radix	<i>Angelica acutiloba</i> Kitag.	3
Paeoniae Radix Alba	<i>Paeonia obovata</i> Max.	3
Taxilli Ramulus	<i>Loranthus chinensis</i> Danser	3
Rehmanniae Radix Preparata	<i>Rehmannia glutinosa</i> for. <i>hueichingensis</i> Hsia	2
Cnidii Rhizoma	<i>Cnidium officinale</i> Makino	2
Ginseng Radix Alba	<i>Panax ginseng</i> C. A. Mey.	2
Hoelen	<i>Poria coco</i> Wolf	2
Achyranthis Radix	<i>Achyranthes fauriei</i> H.Lev. et Vaniot	2
Eucommiae Cortex	<i>Eucommia ulmoides</i> Oliv.	2
Gentianae Macrophyllae Radix	<i>Gentiana crassicaulis</i> Duthie ex Burk.	2
Asari Herba Cum Radix	<i>Asarum sieboldii</i> var. <i>seoulense</i> Nakai	2
Saposhnikoviae Radix	<i>Ledebouriella divaricata</i> Hiroe	2
Cinnamomi Cortex Spissus	<i>Cinnamomum wilsonii</i> Gamble	2
Glycyrrhizae Radix et Rhizoma	<i>Glycyrrhiza glabra</i> L.	1
Zingiberis Rhizoma Crudus	<i>Zingiber officinale</i> Roscoe	4
Total amount		37

gastrocnemius muscle atrophy rate of each experimental group was measured by the following equation:

$$\text{Muscle atrophy rate (\%)} = \frac{\text{Right muscle weight} - \text{Left muscle weight}}{\text{Right muscle weight}} \times 100$$

Samples of muscle tissue were obtained (3–5 mm thick) from the center of the muscle, and frozen at -50°C in a dry ice–isophentan solution and stored at -80°C .

Anatomical observation and measurement of the muscle cross-sectional area

An 8 μm frozen section was taken from the mid-belly of the gastrocnemius muscle from the frozen muscle tissue samples, and the Hematoxylin and Eosin (H&E) dye method was performed. The muscle cross-sectional area was measured using Axiovision LE software for image analysis after observing under a microscope. The average muscle cross-sectional area of each experimental group was measured by observing at least 30 myofibrils of each muscle tissue. The sectional area reduction rate was measured by the following equation:

$$\begin{aligned} &\text{The sectional area reduction rate (\%)} \\ &= \frac{\text{Right muscle cross sectional area} - \text{Left muscle cross sectional area}}{\text{Right muscle cross-sectional area}} \\ &\times 100 \end{aligned}$$

Immunohistochemical staining

Immunohistochemical staining was performed using the free-floating method. The primary antibodies were anti-BAX (ab7977, 1:200 dilution, rabbit polyclonal; Abcam) and anti-Bcl-2 (sc-783, 1:200 dilution, rabbit polyclonal; Santacruz). They were diluted with phosphate-buffered saline (PBS) and Triton X-100 and then reacted at 4°C for 12 h. The tissues were washed with PBS and reacted for 1 h each with the abidin–biotin immunoperoxidase method (ABC Vectastain Kit).

To quantify the result of the immune response, the number of immunopositive cells within the same fixed area under a microscope was measured and quantified.

Statistical analyses

All measured data were verified at $p < 0.05$ and $p < 0.01$ using Student's t test. All values are expressed as the mean \pm standard error.

Results

Changes in body weight

Both the control and the DGT group showed a tendency to gain weight over time. The mean weight at the start of the experiment was 247.4 ± 2.6 g in the control group and 244.8 ± 2.4 g in the DGT group. The final weight gain at 2 weeks was 343.9 ± 9.6 g in the control group and 350.0 ± 13.3 g in the DGT group. The DGT group showed slightly increased body weight gain compared with the control group, but this was not statistically significantly different (Fig. 1).

Changes in gastrocnemius muscle weight

In the control group, the weight of the right the gastrocnemius muscle was 507.2 ± 10.0 mg/100 g whilst the weight of the left gastrocnemius muscle was 386.7 ± 10.2 mg/100 g. There was a significant decrease in the left gastrocnemius muscle weight ($p < 0.01$, Fig. 2). The rate of muscle atrophy in the left gastrocnemius muscle was $23.7 \pm 1.7\%$.

In the DGT group, the weight of the right gastrocnemius muscle was 510.6 ± 10.6 mg/100 g and that of the left gastrocnemius muscle was 420.3 ± 9.5 mg/100 g. As in the control group, a significant weight reduction in the left gastrocnemius muscle was confirmed in the DGT group ($p < 0.01$, Fig. 2). The rate of muscle atrophy was $17.4 \pm 2.4\%$. Nevertheless, there was a significantly lower weight loss and a lower rate of muscle atrophy in the DGT group compared with the control group ($p < 0.05$, Fig. 2).

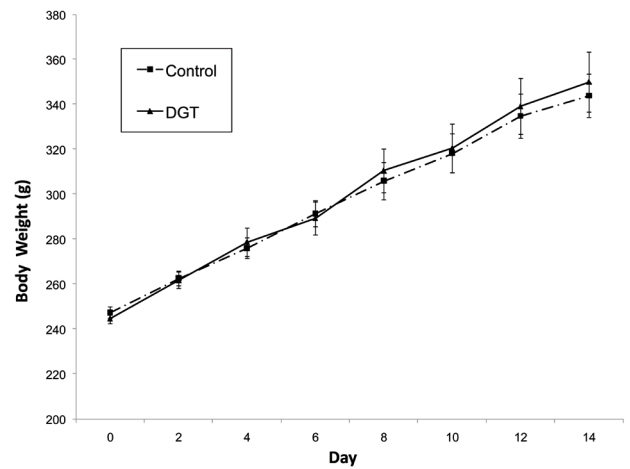


Fig. 1. Changes in body weight.

Both the control and the DGT group displayed gradual increases for 2 weeks. The final difference in weight between groups was not significant.

Data are presented as mean \pm SE.

DGT group: DGT treated group. Control group: saline treated group.

DGT, Dokhwalgisaeng-tang.

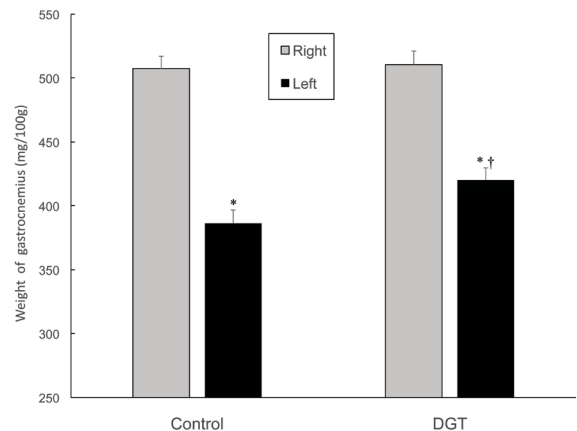


Fig. 2. Changes in the gastrocnemius muscle weight.

The mean weight of the left gastrocnemius muscle in the DGT group was heavier than that of the control group. The muscle atrophy ratio of the left gastrocnemius muscle in the DGT group was also significantly less than that of the control group.

* $p < 0.01$ compared with the right gastrocnemius muscle.

† $p < 0.05$ compared with the control group.

DGT group: DGT treated group. Control group: saline treated group.

DGT, Dokhwalgisaeng-tang.

Anatomical changes in the muscle cross-sectional area

The size of the left gastrocnemius muscle fiber in the control group decreased in comparison with the right muscle. Many nucleus aggregations were observed around the myofibers (Fig. 3). In the DGT group, the size of the myofiber decreased similarly to that in the control group, but the decrease was relatively small and the degree of aggregation of the nuclei was reduced.

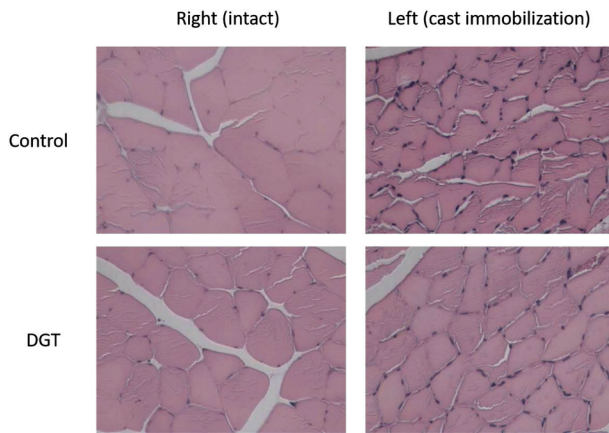


Fig. 3. Anatomical changes in the muscle cross-sectional area. Photomicrographs of skeletal muscle suggest that the cast immobilization decreased myofibers of the gastrocnemius muscle in both the control and the DGT group. The accumulation of nuclei around the myofibers was observed, suggesting the presence of inflammatory cells. However, the reduction of left myofibrils in the DGT group was less than that of the control group (magnification, $\times 200$). DGT group: DGT treated group. Control group: saline treated group. DGT, Dokhwalgisaeng-tang.

Changes in the muscle cross-sectional area size

In the control group, the cross-sectional area of the right gastrocnemius muscle was $4,409.9 \pm 87.7 \mu\text{m}^2$, and that of the left side was $3,622.7 \pm 81.8 \mu\text{m}^2$, showing a significant decrease on the left side ($p < 0.01$, Fig. 4). The cross-sectional area reduction rate was $17.7 \pm 2.0\%$. In the DGT group, the cross-sectional area of the right gastrocnemius muscle was $4,438.4 \pm 111.3 \mu\text{m}^2$, and that of the left side was $3,893.7 \pm 95.6 \mu\text{m}^2$, showing a significant decrease similar to the control group ($p < 0.01$, Fig. 4). The cross-sectional area reduction rate was $12.2 \pm 1.0\%$. In comparison with the control group, the DGT group showed a statistically significantly lower cross-sectional area reduction ($p < 0.01$, Fig. 4).

Changes in apoptosis-related proteins

Pro-apoptotic protein BAX

In the 2 groups, BAX protein expression significantly increased more in the left gastrocnemius muscle than in the right gastrocnemius muscle but decreased more in the DGT group than in the control group (Figure 5). The expression level of BAX was $14.3 \pm 1.8/10^5 \mu\text{m}^2$ in the right and $22.3 \pm 2.4/10^5 \mu\text{m}^2$ in the left gastrocnemius muscle of the control group, and was $13.5 \pm 1.9/10^5 \mu\text{m}^2$ in the right and $16.1 \pm 1.7/10^5 \mu\text{m}^2$ in the left gastrocnemius muscle of the DGT group. This result indicates a significant increase in expression of BAX protein in the left gastrocnemius muscle ($p < 0.01$, Table 2).

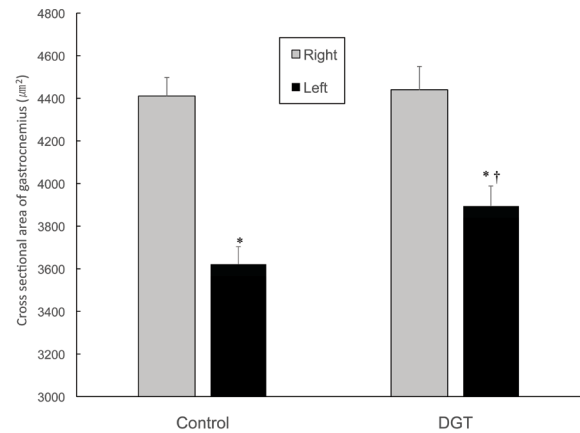


Fig. 4. Changes in the muscle cross-sectional area size. The muscle cross-sectional area size of the left gastrocnemius muscle in the DGT group was larger than that of the control group. The reduction ratio of muscle cross-sectional area size of the left gastrocnemius muscle to right in the DGT group was also significantly lower than that of the control group. * $p < 0.01$ compared with the right gastrocnemius muscle. † $p < 0.01$ compared with the control group. DGT group: DGT treated group. Control group: saline treated group. DGT, Dokhwalgisaeng-tang.

Table 2. Changes of Pro-apoptotic Protein BAX.

Group	Right (intact)	Left (disuse)
Control (counts/ $10^5 \mu\text{m}^2$)	14.3 ± 1.8	$22.3 \pm 2.4^*$
DGT (counts/ $10^5 \mu\text{m}^2$)	13.5 ± 1.9	$16.1 \pm 1.7^{*,\dagger}$

Data are presented as mean \pm SE.

* $p < 0.01$ compared with the right gastrocnemius muscle.

† $p < 0.05$ compared with the control group.

DGT group: DGT treated group. Control group: saline treated group.

BAX, Bcl-2 associated X protein ; DGT, Dokhwalgisaeng-tang.

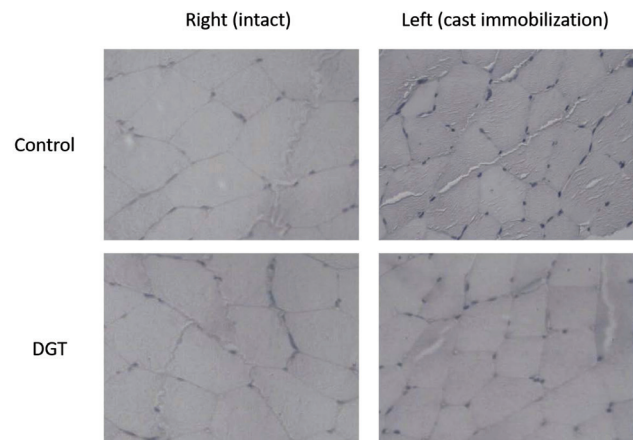


Fig. 5. Changes of pro-apoptotic protein BAX. In control group, the immunoreactivities of BAX protein in the left gastrocnemius muscle were increased significantly compared with the right gastrocnemius muscle. In the DGT group, BAX protein expression also increased in the left gastrocnemius muscle compared with the right muscle. However, BAX expression on the left gastrocnemius muscle in the DGT group was less than the left gastrocnemius muscle of the control group. (magnification, $\times 400$). DGT group: DGT treated group. Control group: saline treated group. BAX, Bcl-2 associated X protein ; DGT, Dokhwalgisaeng-tang.

In comparing the control group and the DGT group, no difference was found in the expression between the 2 groups in the right gastrocnemius muscle. However, the DGT group showed a significant inhibition of BAX protein expression in the left gastrocnemius muscle ($p < 0.05$, Table 2).

Anti-apoptotic protein Bcl-2

Bcl-2 protein expression increased more in the left gastrocnemius muscle than in the right in both the control and the DGT groups. The increase in Bcl-2 immunoreactivity on the left gastrocnemius muscle was more pronounced in the DGT group (Fig. 6). The number of positive cells in the control group was $18.9 \pm 2.2/10^5 \mu\text{m}^2$ per unit area on the right, and $25.0 \pm 1.8/10^5 \mu\text{m}^2$ on the left side in the control group. There was a significant increase in Bcl-2 protein expression in the gastrocnemius muscle with induced disuse muscle atrophy ($p < 0.05$, Table 3). In the DGT group, there was a significantly increased expression of Bcl-2 from $19.7 \pm 2.0/10^5 \mu\text{m}^2$ on the right side, to $30.2 \pm 1.6/10^5 \mu\text{m}^2$ on the left side, similar to the control group ($p < 0.01$, Table 3).

No difference was found in Bcl-2 expression between the right gastrocnemius muscles in both groups. However, the DGT group significantly increased in expression more than the control group in the left gastrocnemius muscle. ($p < 0.05$, Table 3).

Table 3. Changes of Anti-apoptotic Protein Bcl-2.

Group	Right (intact)	Left (disuse)
Control (counts/ $10^5 \mu\text{m}^2$)	18.9 ± 2.2	$25.0 \pm 1.8^*$
DGT (counts/ $10^5 \mu\text{m}^2$)	19.7 ± 2.0	$30.2 \pm 1.6^{*†}$

Data are presented as mean \pm SE.

* $p < 0.01$, † $p < 0.05$ compared with the right gastrocnemius muscle.

† $p < 0.05$ compared with the control group.

DGT group: DGT treated group. Control group: saline treated group.

Bcl-2, B-cell chronic lymphocytic lymphoma 2 ; DGT, Dokhwalgisaeng-tang.

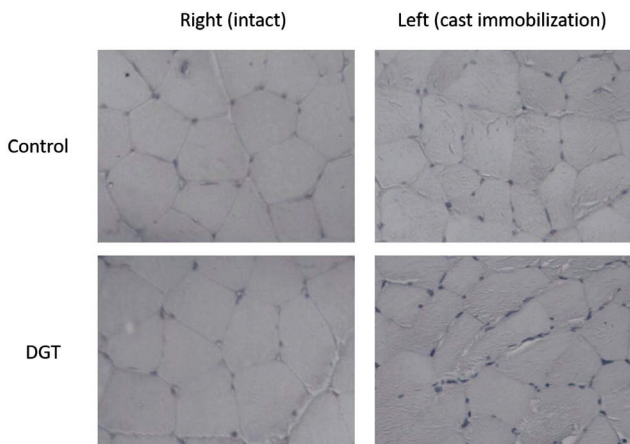


Fig. 6. Changes of anti-apoptotic protein Bcl-2

The immunoreactivity of Bcl-2 protein in the left gastrocnemius muscle was increased significantly compared with the right in both the control and the DGT group. Higher level of expression of Bcl-2 protein were detected in the left gastrocnemius muscle of the DGT group compared with the control group. (magnification, $\times 400$).

DGT group: DGT treated group. Control group: saline treated group.

Bcl-2, B-cell chronic lymphocytic lymphoma 2 ; DGT, Dokhwalgisaeng-tang.

Discussion

Muscular atrophy can be classified into denervation or disuse atrophy. Denervation atrophy is induced by blocking neural control of muscles caused by lesions or injuries of the peripheral nerves [11]. Disuse atrophy is caused by a reduction of the stimulus to the muscles due to long-term bed rest and restricted activity [12], which are common problems after clinical fixation for fracture treatment [13]. Atrophy, which induces muscle weight changes, morphological changes, and muscle strength reduction, limits the normal daily life of patients, resulting in poor quality of life and increased mortality in older patients [14].

Muscle atrophy occurs most frequently during the early stages of activity limitation. The reduction of activity increases the proteolysis of skeletal muscle, resulting in decreased synthesis and muscle weight. After 72 h of inaction, the size of the muscle fiber has been shown to be reduced by 14–17% by the proteolytic process, and this intramuscular change is frequent within the first 2 weeks after fixation [15].

After fixation removal, the fixed muscles become ineffective and cannot maintain normal muscle strength, resulting in secondary complications such as joint damage and osteochondrosis [16]. The degree of muscle atrophy maybe greater than that experienced in the fixed period, with longer recovery times [17]. These problems affect the length of patients' rehabilitation.

In Korean medicine, muscle atrophy belongs to the category of wilting disease, which is characterized by the symptom of partial muscle atrophy. Wilting disease has a progressive course, that is, the muscles of the limbs continue to be relaxed and helpless [18].

The current treatments for muscle atrophy that have been studied so far include electrical stimulation such as reported by Morimoto et al [19], Dirks et al [20], and Chen et al [21], regular exercise on a treadmill, and physical therapy to prevent muscle atrophy. However, confirming them as effective treatments for disuse muscle atrophy is difficult because most treatments are limited to active patients. Many studies have been conducted using Korean medicine, Oh [22], Kim [23], and Cho et al [1] but only a few experimental studies have demonstrated the protective effect of apoptosis on disuse muscle atrophy. The results showed that weight change in both groups were not significantly different (Fig. 1). Wall et al [24] reported that the gastrocnemius muscle weight significantly decreased after fixing the bandage to the hind limb of rats. In the current study, the weight of the left gastrocnemius muscle significantly decreased in both the control and the DGT groups after the 2-week left ankle joint fixation. According to Shibaguchi et al [25] measuring the change in muscle weight on the left side of the right gastrocnemius muscle showed statistically significant changes in the rate of muscle atrophy. The control group showed a 23.7% decrease and the DGT group showed a 17.4% decrease (Fig. 2). These results demonstrated that the administration of DGT has a significant protective effect on the weight loss of gastrocnemius muscle induced disuse muscle atrophy.

The size of the left gastrocnemius muscle fibers in both groups decreased in comparison with the right muscle. However, compared with the control group, the DGT group showed less reduction in the size of the left muscle fiber. Moreover, nuclear aggregation around the muscle fibers was relatively low. This nuclear aggregation is a sign of the presence of inflammatory cells in the muscle and the proliferation of muscle satellite cells (Fig. 3) [26]. Udaka et al reported that the decrease in muscle weight due to muscle non-use is accompanied by a decrease in muscle fiber [27]. In this study, a decrease in the size of muscle fibers accompanied by a decrease in gastrocnemius muscle weight

was observed following ankle fixation for 2 weeks. When the actual gastrocnemius muscle cross-sectional area was measured, the reduction rate of the control group was about 17.7% in the left side compared with the right side, and the reduction rate in the DGT group was about 12.1%. A significant decrease was found in both groups, but the DGT group showed a statistically significant lower cross-sectional area reduction than the control group (Fig. 4). These results are consistent with those of Matsumoto et al [28] who showed that the muscle fiber cross-sectional area of the gastrocnemius muscle significantly reduced after the long-term artificial disuse of the muscle. The current study suggests that the administration of DGT inhibits the decrease in the gastrocnemius muscle weight due to the disuse of muscle and has a significant protective effect on the reduction of the cross-sectional area of muscle fiber.

Skeletal muscle atrophy is caused by various reasons, with apoptosis known to play an important role. Mitochondria are central to apoptosis regulation and accommodate a number of mediators that regulate apoptosis-related signaling [29]. The permeability of the mitochondria outer membrane is essential for the intracellular release of the pro-apoptotic factor present in the inner membrane. The outer membrane is preserved by the balance of pro- and anti-apoptotic regulators, including Bax and Bcl-2 interacting domain death agonist, and among the Bcl-2 family of proteins and anti-apoptotic proteins, including Bcl-2 and Bcl-2-related gene long isoform. Pro-apoptotic proteins may act in the inner membrane, whilst anti-apoptotic proteins block the permeability of the outer membrane. When the outer membrane of the mitochondria becomes permeable, various proteins that mediate apoptosis are released from the mitochondria into the cytoplasm and cytochrome c, which activates various sub-caspases, ultimately mediating apoptosis [3]. Moreover, apoptosis has been shown to be involved in atrophy induced by hind limb fixation of experimental animals [30]. In the current study, the expression of pro-apoptotic protein BAX, and anti-apoptotic protein Bcl-2 was examined to determine whether there were indicators of apoptosis occurring in the muscle. In the study by Siu et al, the long-term inactivity of muscle fibers in the experimental animals led to a marked increase in BAX protein in the muscle, and a significant increase in the expression of Bcl-2 protein to compensate for this outcome [31]. The immunohistochemical staining for BAX/Bcl-2 proteins revealed that the expression of BAX protein significantly increased in the left gastrocnemius muscle of the control and the DGT group in comparison with the right control group. This result was supported by image analysis. Conversely, in comparing the BAX immunoreactivity in the left gastrocnemius muscle between the 2 experimental groups, the DGT group showed a markedly decreased expression pattern in comparison with the control group. In the DGT group, a significant inhibition in the number of immunoreactive cells to the BAX protein of the left gastrocnemius muscle was observed, compared with the control group (Fig. 5, Table 2). The level of Bcl-2 protein revealed that the immune response on the left side increased in both the control and the DGT groups compared with the right side. The expression of Bcl-2 was more apparent in the DGT group than in the control group. The number of positive cells in the right gastrocnemius muscle was not significantly different from that in the control group. However, in the left gastrocnemius muscle, the number of immunoreactive cells significantly increased in the DGT group in comparison with the control group (Fig. 6, Table 3). This result suggests that DGT reduces the expression of the BAX protein, which promotes apoptosis in disuse muscle atrophy, and increases the Bcl-2 protein, which inhibits apoptosis and thus suppressing it, at the same time. These results suggest that DGT has a protective effect on disuse

muscle atrophy.

In conclusion, DGT showed a significant protective effect on and gastrocnemius muscle atrophy. The mechanism of this protective action seemed to suppress atrophy by controlling the expression of the apoptosis-related proteins BAX and Bcl-2. However, in this study, we observed only the effect of DGT on apoptosis in the development of disuse muscle atrophy. Further studies are needed to investigate other potential developmental mechanisms. Moreover, the change in disuse muscle atrophy should be investigated by observing changes in the muscle fiber type, soleus muscle, and quadriceps muscle, among others, aside from the gastrocnemius muscle observed in this study.

Conflicts of Interest

The authors have no conflicts of interest to declare.

References

- [1] Cho SG [Dissertation]. Beneficial effects of an herbal formula consisting of schisandra chinensis (turcz.) baill, lycium chinense mill and eucommia ulmoides oliv on disuse muscle atrophy: in vitro and in vivo evaluation. Seoul (Korea): Kyunghee University; 2017. [in Korean].
- [2] Kim EJ [Dissertation]. Effects of stretching and ultrasound therapy on motor function and histological recovery in the disuse muscle atrophy model of rats. Naju (Korea): Dongshin University; 2011. [in Korean].
- [3] Marzetti E, Hwang JC, Lees HA, Wohlgemuth SE, Dupont-Versteegden EE, Carter CS, Bernabei R et al. Mitochondrial death effectors: Relevance to sarcopenia and disuse muscle atrophy. *Biochim Biophys Acta* 2010; 1800: 235-244.
- [4] Calvani R, Joseph A, Adihetty PJ, Miccheli A, Bossola M, Leeuwenburgh C et al. Mitochondrial pathways in sarcopenia of aging and disuse muscle atrophy. *Biol Chem* 2013;394:393-414.
- [5] Yu J, Zhou X, He X, Dai M, Zhang Q. Curcumin induces apoptosis involving bax/bcl-2 in human hepatoma SMMC-7721 cells. *Asian Pac J Cancer Prev* 2011;12:1925-1929.
- [6] Son Samak. *Bigeupchungeumyobang*, 16th ed. Beijing(China): People's Medical Publishing House Co.; 1976. 198 p. [in Chinese]
- [7] Baek SE, Jang SB, Yoo JE, Yoo DY. Effects of Dokwhalgisaeng-tang Gamibang (DGG) Water Extract on Dexamethasone-treated Osteoblast. *J Korean Obstet Gynecol* 2016;29:1-14. [in Korean].
- [8] Lee YS, Kim JH, Cho HB, Choi GS. Effect of Dokhwalkisaengtang on the ovariectomized rat model of postmenopausal osteoporosis. *J Korean Obstet Gynecol* 2000;13:104-119. [in Korean].
- [9] Lee SG, Won JK, Yeom SR, Lee SK, Song YS, Kwon YD. The effects of dokhwalkisaeng-tang and jungsongouhyul pharmacopuncture on pain control and nerve regeneration in the crush-induced sciatic nerve injury of the rat. *J Korean Med Rehabil* 2009;19:15-32. [in Korean].
- [10] Heo J. *Donguibogam*, 1st ed. Seoul(Korea): Bubin Publishers Company; 2005. p. 578, 581. [in Korean].
- [11] Yoon BC, Yu BK, Lee MH. Exercise effects on the atrophy of denervated muscles in rat. *Phys Ther Korea* 2000;7:34-48. [in Korean].
- [12] Berg HE, Eiken O, Miklavcic L, Mekjavic IB. Hip, thigh and calf muscle atrophy and bone loss after 5-week bedrest inactivity. *Eur J Appl Physiol* 2007;99:283-289.
- [13] Caron AZ, Drouin G, Desrosiers J, Trens F, Grenier G. A novel hind limb immobilization procedure for studying skeletal muscle atrophy and recovery in mouse. *J Appl Physiol* 2009;106:2049-2059.
- [14] Metter EJ, Talbot LA, Schrager M, Conwit R. Skeletal muscle strength as a predictor of all-cause mortality in healthy men. *J Gerontol* 2002;57:359-365.
- [15] Choe MA. Steroid induced muscle atrophy. *Perspect Nurs Sci* 2005;2:19-36. [in Korean].
- [16] Cho SY. The effects of aqua-exercise on the muscle atrophy of hind limb in rats. *J Korean Phys Ther* 2002;14:1-13. [in Korean].
- [17] Desapay J, Pierno S, Liantonio A, De Luca A, Didonna MP, Frigeri A et al. Recovery of the soleus muscle after short-and long-term disuse induced by hind limb unloading: Effects on the electrical properties and myosin heavy chain profile. *Neurobiol Dis* 2004;18:356-365.
- [18] Jung TS, Kang SS, Choi CW. Clinical study on 1 case of both lower limbs weakness patient diagnosed wei symptom. *Korean J Oriental Physiol Pathol*

- 2012;26:784-787. [in Korean].
- [19] Morimoto A, Winaga H, Sakurai H, Ohmichi M, Yoshimoto T, Ohmichi Y et al. Treadmill running and static stretching improve long-lasting hyperalgesia, joint limitation, and muscle atrophy induced by cast immobilization in rats. *Neurosci Lett* 2012;534:295-300.
- [20] Dirks ML, Wall BT, Snijders T, Ottenbros CL, Verdijk LB, Loon L. Neuromuscular electrical stimulation prevents muscle disuse atrophy during leg immobilization in humans. *Acta Physiol (Oxf)* 2014;210:628-641.
- [21] Chen G, Mou C, Yang Y, Wang S, Zhao Z. Exercise training has beneficial anti-atrophy effects by inhibiting oxidative stress-induced MuRF1 upregulation in rats with diabetes. *Life Sci* 2011;89:44-49.
- [22] Oh YS [Dissertation]. Experimental study of apitoxin and exercise on disuse atrophy in rat skeletal muscles. Seoul (Korea): Dongguk University; 1999. [in Korean]
- [23] Kim BH. The protective effects of Dangguibohyultang(dangguibuxuetang) against disuse muscle atrophy in rats. *J Korean Med Rehabil* 2017;27:1-9. [in Korean].
- [24] Wall BT, Dirks ML, van Loon LJ. Skeletal muscle atrophy during short-term disuse: Implications for age-related sarcopenia. *Ageing Res Rev* 2013;12:898-906.
- [25] Shibaguchi T, Yamaguchi Y, Miyaji N, Yoshihara T, Naito H, Goto K et al. Astaxanthin intake attenuates muscle atrophy caused by immobilization in rats. *Physiol Rep* 2016;4:1-8.
- [26] Yen Y, Tu P, Chen C, Lin Y, Hsieh S, Chen C. Role of acid-sensing ion channel 3 in sub-acute-phase inflammation. *Mol Pain* 2009;5:1-16.
- [27] Udaka J, Terui T, Ohtsuki I, Marumo K, Ishiwata S, Kurihara S et al. Depressed contractile performance and reduced fatigue resistance in single skinned fibers of soleus muscle after long-term disuse in rats. *J Appl Physiol* 2011;111:1080-1087.
- [28] Matsumoto Y, Nakano J, Oga S, Kataoka H, Honda Y, Sakamoto J et al. The non-thermal effects of pulsed ultrasound irradiation on the development of disuse muscle atrophy in rat gastrocnemius muscle. *Ultrasound Med Biol* 2014;40:1578-1586.
- [29] Chipuk JE, Green DR. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? *Trends Cell Biol* 2008;18:157-164.
- [30] Dupont-Versteegden EE. Apoptosis in muscle atrophy: Relevance to sarcopenia. *Exp Gerontol* 2005;40:473-481.
- [31] Siu PM, Pistilli EE, Alway SE. Apoptotic responses to hind limb suspension in gastrocnemius muscles from young adult and aged rats. *Am J Physiol Regul Integr Comp Physiol* 2005;289:1015-1026.