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Original Article

The Protective Effects of Zanthoxylum bungeanum Maxim Pharmacopuncture on Disuse Muscle Atrophy in Rat Gastrocnemius Muscle

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ABSTRACT

Background: This study aimed to investigate the protective effects of Zanthoxylum bungeanum Maxim pharmacopuncture on disuse muscle atrophy in the gastrocnemius muscle of rats.
Methods: Thirty male 250 g Sprague-Dawley rats were distributed randomly into 3 groups. The left hindlimb immobilization was performed with casting tape for 2 weeks, and no treatment was given to the right hindlimb. Rats received pharmacopuncture and were injected daily on the BL57 with either 2 mL of Zanthoxylum bungeanum Maxim aqueous extract (ZM-W group), 1 mL pharmacopuncture of Zanthoxylum bungeanum Maxim ethanol extract (ZM-E group), or 2 mL normal saline (control group). After 2 weeks of immobilization, the weight of the whole gastrocnemius muscle was measured, and the morphology of both the left and the right gastrocnemius muscles were assessed by Hematoxylin and Eosin staining. To investigate the immobilization-induced muscular apoptosis, the immunohistochemical analysis of BAX and Bcl-2 was carried out.
Results: ZM-W and ZM-E significantly inhibited the reduction in weight of the left gastrocnemius muscle, the reduction in the left myofibrils, and the cross-sectional area of gastrocnemius, as compared with the control. Moreover, the ZM-W and ZM-E groups showed significantly reduced immunoreactivity for BAX, and increased immunoreactivity of Bcl-2 in left gastrocnemius muscle compared with the control group.
Conclusion: These results suggest that Zanthoxylum bungeanum Maxim pharmacopuncture has protective effects against immobilization-induced muscle atrophy by regulating the activity of apoptosis-associated BAX / Bcl-2 proteins in the gastrocnemius muscle.

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Introduction

Muscle atrophy is characterized by a shortening of muscle fibers and a decrease in muscle mass and strength, which can result in loss of function [1]. Long periods of being immobile or longer limb fixation can lead to atrophy of skeletal muscle fibers, resulting in weaker muscles and a poor quality of life. Such atrophy is caused by several cellular mechanisms that regulate protein synthesis and muscle degradation [2]. One of the main causes of disuse muscle atrophy is oxidative stress where there is an increased production of reactive oxygen species (ROS) [3]. For example, exposure of skeletal muscular fibers to hydrogen peroxide activates proteolytic enzymes that cause protein degradation, whilst inhibiting cellular mechanisms that promote protein synthesis [4]. In the case of excessive generation of ROS, DNA fragmentation, protein oxidation, and lipid peroxidation promote cell apoptosis [5,6]. Expression of apoptosis-related proteins, BAX and Bcl-2 is generally considered an indicator of the induction of the pro-apoptotic cascade [7]. Therefore, ROS an important signaling intermediate causing long-term disuse-induced atrophy, and inhibition of excessive production of ROS is a therapeutic strategy to prevent disuse muscle atrophy [8].

The fruit, peel, and leaves of Zanthoxylum piperitum are used as spices and medicines whose uses include cold and detox medicines and insect repellents [9]. Zanthoxylum bungeanum Maxim (ZM) is a medicine derived from the mature peel of Zanthoxylum piperitum as denoted by “Sinnong-bonchogyeong.” It has also been widely used for its diuretic, anti-inflammatory, anthelmintic, and other properties [10]. Recent studies on ZM have suggested that the methanol extracts from the plants exhibit antioxidative

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activities [11] supporting previous studies that have reported that plant extracts have antioxidant effects [12-14]. Several studies have suggested that antioxidant medicines may be an attractive treatment option for disuse muscle atrophy [15,16], but studies on disuse muscle atrophy using ZM have not yet been reported.

This study was carried out to investigate the effect of ZM pharmacopuncture on insusceptible muscle atrophy using a rat model of disuse muscle atrophy, which was generated by casting the left ankle joint of the rat. The ZM pharmacopuncture was injected into the left side of BL57 mice and changes in body weight and muscle weight were analyzed every 2-weeks. Changes in muscle fiber morphology were examined microscopically and the changes in apoptosis-related proteins (BAX and Bcl-2) were examined using immunohistochemistry.

Materials and Methods

Animals

In this study, 10-week old Sprague-Dawley male rats (HanTacSam, Samtako, South Korea) were used, weighing about 250 g (n = 30). The rats were given sterile distilled water and food in the breeding room where temperatures were maintained at 23-24°C, humidity was 40-60%, and light was automatically maintained for 12 hours. The rats were allowed to adapt to the laboratory environment for about a week. All the procedures of this study were conducted according to regulations and policies of the Animal Experiment Ethics Committee of Dong-Eui University (Approval no.: R2017-023).

Material for pharmacopuncture

Zirisan wild herb (Gyeongsangnam-do, South Korea) was used to produce 40 g of ZM for pharmacopuncture.

Classification of the study groups

The rats were divided randomly into groups of 10. There was the ZM-W group (for the treatment of coughs and is made with water), the ZM-E group (ethanol extract), and the control group (injected with normal saline). All rats were free to consume water and feed during the experimental period.

Manufacture of pharmacopuncture

ZM was prepared by placing 20 g of prepared plant shells into 1,000 mL (× 2) round bottom flasks (20 g each flask), with 95% ethanol and 500 mL of water added to each flask. They were left for about 3 hours then boiled for 3 hours in a heating mantle (MS-DMS637, aesthetics, Korea), where reflux cooling extraction (Ethanol-approximately 78°C, distilled water-about 102°C) was performed. After centrifugation (J2-MC, Becman Coupler, USA), the extract was filtered through a 0.2 μm bottle top filter, and the extract was enriched and frozen, to obtain 1.94 g of ethanol and 3.45 g of water.

The yield rate of ZM was 17.25% using the water extraction method and 9.7% from the ethanol extraction method. There was 0.1 g of extract used from the ethanol extraction which was dissolved in 100 mL of water (1 mg/mL), and 0.1 g of extract used from the extraction with water (1 mg/mL) which was dissolved in 100 mL of water, and 0.9 g of NaCl (KP) was added to compensate for the electrolytes, and adjusted to pH 7.4 using 0.5 M NaOH (KP). The prepared solution for pharmacopuncture was filtered using a 0.2 μm bottle top filter (Corning, USA) into a 20 mL sterilized bottle. Aliquots were made using a 0.2 μm syringe filter (Sartorius, Germany) into a sterilized vial.

Procedure method

The acupuncture point was taken at the rear (BL57, center of Gastrocnemius muscle) of the experimental animal in accordance with the osteoporosis method [17]. The pharmacopuncture was performed with a dose of 0.1 mL, at a depth of about 2 mm into the skin. Acupuncture was performed using a disposable 1.0 mL syringe (26 needles, Jeonglim Medical Industry, Korea). Since the yield ratio of the hydrothermal and ethanol extraction differed approximately by 50%, the concentration of 40 mg/kg for each animal was determined and delivered using approximately 2 mL of the hydrothermal extract (ZM-W) and 1 mL of the ethanol extract (ZM-E). The control group was injected with 2 mL of normal saline. All animals were injected slowly at 10 AM every morning into the left BL57 using a 1 mL or 2 mL syringe.

Inducing disuse muscle atrophy

In order to induce disuse muscle atrophy in rats, the left ankle joints were completely extended and the ankle joints were fixed using casting tape and remained immobile for 2 weeks. The casting tape was replaced once a week. The right ankle was not treated and was free to move.

Measuring weight and changes in muscle weight

The weight of the rats was measured every day before administration of the drug during the experimental period, and on the last day of the experiment the weight was measured just before autopsy. Two weeks after the experiment, the rats were euthanized and the weight was measured by separating the gastrocnemius muscle from the left and the right hind leg of the rats during the autopsy. The weight of muscle was calculated using the weight of the rat and the weight of muscle per 100 g of weight. The gastrocnemius muscle atrophy rate of each experimental group and control group was measured by the following calculation:

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

The muscle tissue was then sliced at a thickness of about 3-5 mm, at the center of the muscle, frozen with a dry ice-isophentan solution at –50°C, and stored at –80°C.

Anatomical observations and measurement of the muscle cross-sectional area

The mid-belly section of the gastrocnemius muscle was kept frozen and the 8 μm thick section of muscle tissue was stained with Hematoxylin and Eosin dye on ice. The muscle cross-sectional area was measured using Axiovision LE software for image analysis, after observing the section 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 cross-sectional area reduction rate was measured by the following calculation:

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

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) which were diluted with Triton X-100 and phosphate-buffered saline and then left to react at 4°C for 12 hours. The tissues were washed with phosphate-buffered saline and left for 1 hour with abidin–biotin immunoperoxidase (ABC Vectastain Kit). To quantify the result of the immune response, the number of immunopositive cells in the same area under a microscope was measured and quantified, and the ratio of BAX/Bcl-2 was quantified.

Statistical analyses

For comparison of the left and the right legs in each group, the Student *t* test was used. For comparison of the 3 groups, the Student *t* test was performed after using ANOVA to test the difference between the group means. Statistical analysis was performed using SPSS version 12.0 (SPSS, Chicago, IL, USA). The level of significance was reached when $p < 0.05$. The values displayed were the average ± SE.

Results

Changes in body weight

The weights of the animals increased gradually over time for the control, ZM-W, and ZM-E groups. The mean weight of rats on the experimental day was 249.5 ± 1.2 g in the control group, 20.7 ± 1.9 g in the ZM-W group, and 247.8 ± 1.5 g in the ZM-E group. The last weight measured at 2 weeks was 361.0 ± 3.1 g in the control group, 348.5 ± 5.4 g in the ZM-W group, and 350.9 ± 8.6 g in the ZM-E group. The ZM-W and ZM-E groups were slightly lower compared to the control group, but this was not statistically significantly different ($p < 0.05$; Fig. 1).

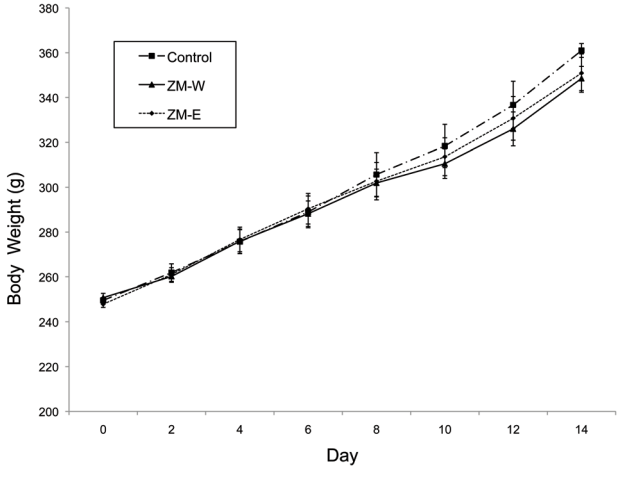

Fig. 1. Change in body weight. Control, ZM-W and ZM-E groups displayed gradual increases for 2 weeks. The final difference in weight between groups was not significant. Data are presented as mean ± SD.
$p < 0.05$ compared with body weight of control group by one-way analysis of variance.
ZM-W group: Group treated with 2 mL pharmacopuncture of ZM by water extract.
ZM-E group: Group treated with 1 mL pharmacopuncture of ZM by ethanol extract.
Control group: Group treated with 2 mL normal saline.
ZM, Zanthoxylum bungeanum Maxim.

Changes in gastrocnemius muscle weight

Two weeks after the experiment, the weight of both gastrocnemius muscles in the rats was measured. In the control group as 516.3 ± 6.4 mg/100 g in the in the right group, 402.5 ± 5.6 mg/100 g in the left group, which was statistically significantly reduced ($p < 0.01$; Table 1). The left gastrocnemius muscle weight decreased by 22.0 ± 0.9% compared with the right side. In the ZM-W group, the weight of the right gastrocnemius muscle was measured as 520.6 ± 6.1 mg/100 g and the left side was measured to be 419.0 ± 5.4 mg/100 g, which was lower than the control group ($p < 0.01$; Table 1). The reduction of the left gastrocnemius muscle weight on the right side was 19.5 ± 0.7%. In the case of the ZM-E group, the weight of the right gastrocnemius muscle was measured to be 522.1 ± 6.0 mg/100 g and the left was 423.5 ± 6.1 mg/100 g, which was lower than the control group ($p < 0.01$; Table 1). The reduction of the left muscle weight on the right side was 18.8 ± 1.1%.

A comparison of the muscle decrease between the control group, ZM-W and ZM-E groups showed that the muscle contraction was statistically significantly less advanced in ZM groups than the control group ($p < 0.05$; Table 1).

Anatomical changes in the muscle cross-sectional area

The gastrocnemius muscle cross section showed that the size of the muscle fibers in the left gastrocnemius muscle was reduced compared with the right side in the control group, and a number of nucleus aggregations around the muscle fiber were observed.

In the case of the ZM-W and ZM-E groups, the size of muscle fibers in the left gastrocnemius muscle were decreased similar to that of the control group, but the decrease was relatively less than that of the control group and the aggregation of the nucleus was decreased. There was no difference between the ZM-W and the ZM-E group (Fig. 2).

Changes in the muscle cross-sectional area size

The cross-sectional area of muscle fibers was measured at 4,527.7 ± 37.2 μm^2 in the control group, compared with 3,635.5 ± 47.8

Table 1. Change of Gastrocnemius Muscle Weight and Atrophy Ratio.

Group	Right (intact)	Left (disuse)	Atrophy ratio (%)
Control	516.3 ± 6.4	402.5 ± 5.6*	22.0 ± 0.9
ZM-W	520.6 ± 6.1	419.0 ± 5.4*†	19.5 ± 0.7†
ZM-E	522.1 ± 6.0	423.5 ± 6.1*†	18.8 ± 1.1†

Data are presented as mean ± SE.
* $p < 0.01$ compared with right gastrocnemius muscle by student t test.
† $p < 0.05$ compared with left gastrocnemius muscle of control group by analysis of variance.
ZM-W group: Group treated with 2 mL pharmacopuncture of ZM by water extract.
ZM-E group: Group treated with 1 mL pharmacopuncture of ZM by ethanol extract.
Control group: Group treated with 2 mL normal saline.
ZM, Zanthoxylum bungeanum Maxim.

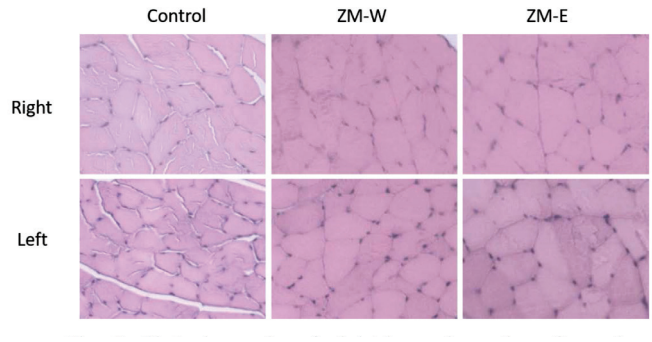

Fig. 2. Photomicrographs of skeletal muscle sections for each group. The cast immobilization decreased myofibers of the left gastrocnemius muscle in control, ZM-W and ZM-E groups. The accumulation of nuclei around the myofibers was observed, suggesting the presence of inflammatory cells. However, the reduction of the left myofibrils in the ZM-W and ZM-E groups was reduced less than in the control group (magnification ×200).
ZM-W group: Group treated with 2 mL pharmacopuncture of ZM by water extract.
ZM-E group: Group treated with 1 mL pharmacopuncture of ZM by ethanol extract.
Control group: Group treated with 2 mL normal saline.
ZM, Zanthoxylum bungeanum Maxim.

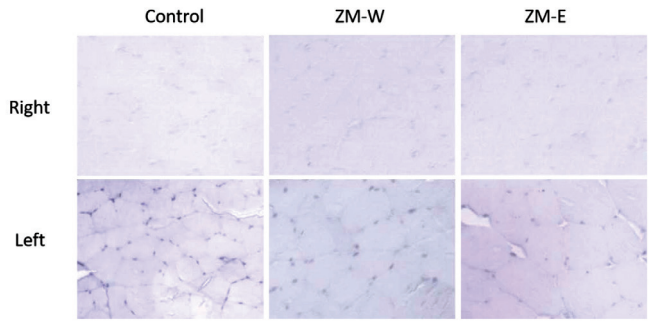

Fig. 3. Change in expression of the pro-apoptotic protein BAX. In the control group, the immunoreactivities of the BAX protein in left gastrocnemius muscle was increased significantly to compared with the right muscle. In the ZM-W and the ZM-E groups, the immunoreactivities of BAX also increased in the left gastrocnemius muscle to compared with the right muscle. However, the expressions of BAX on the left gastrocnemius of the ZM-W and the ZM-E groups were less than that of the control group (magnification, x400).
ZM-W group: Group treated with 2 mL pharmacopuncture of ZM by water extract.
ZM-E group: Group treated with 1 mL pharmacopuncture of ZM by ethanol extract.
Control group: Group treated with 2 mL normal saline.
ZM, Zanthoxylum bungeanum Maxim.

Table 2. Change in Cross Sectional Area of Gastrocnemius Muscle.

Group	Right (intact)	Left (disuse)	Atrophy ratio (%)
Control(μm^2)	4,527.7 ± 37.2	3,635.5 ± 47.8*	19.6 ± 1.4
ZM-W(μm^2)	4,566.8 ± 68.8	3,813.6 ± 55.9*†	16.4 ± 1.0†
ZM-E(μm^2)	4,598.9 ± 44.6	3,868.4 ± 61.5*‡	15.9 ± 1.2‡

Data are presented as mean ± SE.
* $p < 0.01$ compared with right gastrocnemius muscle by student t test.
† $p < 0.05$, ‡ $p < 0.01$ compared with left gastrocnemius muscle of control group by analysis of variance.
ZM-W group: Group treated with 2 mL pharmacopuncture of ZM by water extract.
ZM-E group: Group treated with 1 mL pharmacopuncture of ZM by ethanol extract.
Control group: Group treated with 2 mL normal saline.
ZM, Zanthoxylum bungeanum Maxim.

μm^2 in the left group, which was statistically significantly reduced ($p < 0.01$; Table 2). The mean reduction of the left muscle cross-sectional area on the right side was 19.6 ± 1.4%.

In the case of ZM-W group, the cross-sectional area of the right gastrocnemius muscle was measured as 4,566.8 ± 68.8 μm^2. The left side was measured at 3,813.6 ± 55.9 μm^2, which was statistically significantly reduced compared with the control group ($p < 0.01$; Table 2). The mean reduction of the left muscle cross-sectional area on the right side was 16.4 ± 1.0%.

For the ZM-E group, the cross-sectional area of the right gastrocnemius muscle was measured as 4,598.9 ± 44.6 μm^2. The left side was measured to be 3,868.4 ± 61.5 μm^2, which was statistically significantly reduced compared with the control group ($p < 0.01$; Table 2). The mean reduction of the left muscle cross-sectional area on the right side was 15.9 ± 1.2%.

When comparing the reduction of muscle in a cross-section of the left gastrocnemius muscle, muscle atrophy was significantly less advanced in the ZM-W and ZM-E group compared with the control group (ZM-W group $p < 0.05$, ZM-E group $p < 0.01$; Table 2).

Changes in apoptosis-related proteins

Immunohistochemical staining was used to observe the changes in BAX and Bcl-2 protein expression in the gastrocnemius muscle. After measuring the number of positive cells through image analysis, BAX/Bcl-2 ratio was quantified (Table 3).

Pro-apoptotic protein BAX

The expression of BAX protein was significantly increased in the left gastrocnemius muscle compared with the right gastrocnemius muscle in control, ZM-W, and ZM-E groups, but the expression in the left gastrocnemius muscle was decreased in ZM-W and ZM-E groups compared with the control group (Fig. 3).

In the control group, the mean expression in the right gastrocnemius muscle was 12.1 ± 1.0/10^5 μm^2, which was statistically significantly increased in the left gastrocnemius muscle at 30.0 ± 1.6/10^5 μm^2 ($p < 0.01$; Table 3).

The ZM-W group was observed to have quantifiable BAX expression of 12.2 ± 1.2/10^5 μm^2 on the right side, with significantly higher levels (25.1 ± 1.6/10^5 μm^2) on the left side ($p < 0.01$). Similarly, in the ZM-E group, 11.5 ± 1.1/105 μm^2 BAX expression was detected on the right side, with significantly higher levels (24.2 ± 2.0/10^5 μm^2) on the left side ($p < 0.01$; Table 3).

The expression of BAX in the right gastrocnemius muscle did not differ among the control, ZM-W, and ZM-E group. However, the left gastrocnemius muscle showed a statistically significant decrease in the expression of BAX in ZM-W and ZM-E groups compared with the control group ($p < 0.05$; Fig. 3; Table 3).

Anti-apoptotic protein Bcl-2

In the case of Bcl-2 protein, the left gastrocnemius muscle was observed to have an increased immune response in the control, ZM-W, and ZM-E groups compared with the right gastrocnemius muscle. The increased immune response and expression of Bcl-2 in the left gastrocnemius muscle compared with the right, was more evident in the ZM-W and ZM-E groups.

In the case of positive response cells, the control group showed a statistically significant increase in the expression of Bcl-2in the right gastrocnemius muscle, with an average of 16.7 ± 1.3/10^5 μm^2 and 25.7 ± 1.6/105 μm^2 in the left gastrocnemius muscle,

Table 3. Changes in Expression of Pro-apoptotic Protein BAX, Anti-apoptotic Protein Bcl-2 and the BAX/Bcl-2 Ratio in the Left Gastrocnemius Muscle.

Group	BAX (counts/10^5 μm^{22})		Bcl-2 (counts/10^5 μm^2)		BAX/Bcl-2 ratio
	Right (intact)	Left (disuse)	Right (intact)	Left (disuse)	
Control	12.1 ± 1.0	30.0 ± 1.6†	16.7 ± 1.3	25.7 ± 1.6*	1.22 ± 0.12
ZM-W	12.2 ± 1.2	25.1 ± 1.6†‡	15.3 ± 1.4	30.9 ± 1.5†‡	0.82 ± 0.05$^{\|\|}$
ZM-E	11.5 ± 1.1	24.2 ± 2.0†‡	16.2 ± 1.2	33.0 ± 1.8†§	0.73 ± 0.05$^{\|\|}$

Data are presented as mean ± SE.
* $p < 0.05$, † $p < 0.01$ compared with right gastrocnemius muscle by student t test.
‡ $p < 0.05$, § $p < 0.01$ compared with left gastrocnemius muscle of control group by analysis of variance.
$^{\|}$ $p < 0.05$ compared with control group by student t test.
ZM-W group: Group treated with 2 mL pharmacopuncture of ZM by water extract.
ZM-E group: Group treated with 1 mL pharmacopuncture of ZM by ethanol extract.
Control group: Group treated with 2 mL normal saline.
ZM, Zanthoxylum bungeanum Maxim.

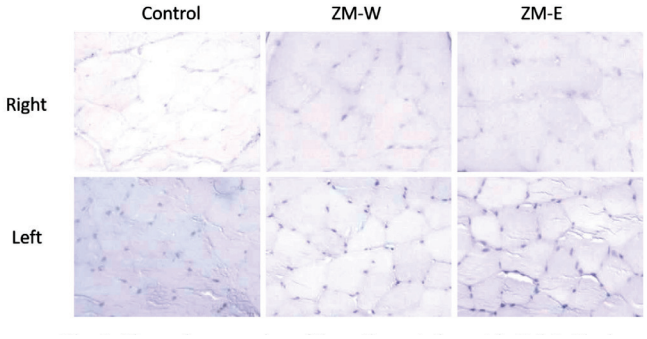

Fig. 4. Change in expression of the anti-apoptotic protein Bcl-2. The immunoreactivities of the Bcl-2 protein in left gastrocnemius muscle increased significantly to compared with the right gastrocnemius muscle in the control, ZM-W and ZM-E groups. The expressions of Bcl-2 on the left gastrocnemius of the ZM-W and the ZM-E groups were observed as more than that of the control group (magnification ×400).
ZM-W group: Group treated with 2 mL pharmacopuncture of ZM by water extract.
ZM-E group: Group treated with 1 mL pharmacopuncture of ZM by ethanol extract.
Control group: Group treated with 2 mL normal saline.
ZM, Zanthoxylum bungeanum Maxim.

respectively ($p < 0.05$). The ZM-W group was observed to have 15.3 ± 1.4/10^5 μm^2 Bcl-2 expression on the right side, and 30.9 ± 1.5/10^5 μm^2 on the left side, and the ZM-E group was observed to have 16.2 ± 1.2/10^5 μm^2 on the right side, and 33.0 ± 1.8/10^5 μm^2 on the left side. The left side showed statistically significant increase in expression of Bcl-2 compared with the right side for each group (ZM-W group $p < 0.01$, ZM-E group $p < 0.01$, control group $p < 0.01$).

The expression of Bcl-2 was not different in the right gastrocnemius muscle between the control, ZM-W, and ZM-E groups, but the expression of Bcl-2 in the ZM-W and ZM-E groups was statistically significantly increased in the left gastrocnemius muscle compared with the control group (ZM-W group $p < 0.05$, ZM-E group $p < 0.01$; Fig. 4; Table 3).

In the left gastrocnemius muscle, the BAX/Bcl-2 ratio for the control group was 1.22, the ZM-W group was 0.82, and the ZM-E group was 0.73. This was statistically significantly different between the ZM-W group and the ZM-E group ($p < 0.05$; Table 3).

Adverse reaction

There were no adverse events in all experiments.

Discussion

When an individual is bedridden for an extended period of time, or does not participate in physical exercise, muscles shrink due to lack of external stimuli such as weight bearing exercise. This may lead to a deterioration in an individual's quality of life due to lack of muscle strength, which may prove to be fatal in the elderly [18]. Disuse muscle atrophy refers to muscle atrophy due to disuse or loss of function [19]. Without long-term use of muscles, systemic maximal oxygen consumption, myocardial contractility, venous return, cardiac output, and blood volume are reduced, resulting in localized atrophy [2].

Disuse muscle atrophy occurs mainly in muscles with several types of muscle fibers and can be evident in the muscles of the lower limbs e.g. the gastrocnemius muscle which has both fast and slow myosin-heavy chains [20]. Muscle atrophy is caused by decreased protein synthesis and increased protein breakdown, decreased muscle mass, reduced cross-sectional area of the muscle, shrinkage of the neuromuscular junction, and regression of the blood vessels which result in decreased muscle weight and function [21]. Once muscle atrophy occurs, it takes a lot of time and effort to restore muscle function to its normal range [22]. Desaphy et al [23] reported that if the lower limbs of experimental animals were fixed for 2 weeks to induce muscle atrophy, the return to normal contraction would be 4 weeks (twice the immobilized period). These results suggest that it is important to prevent muscle atrophy through pre-intervention rather than restoration of a muscle that has undergone atrophy.

Dirks et al [24] and Chen et al [25] have reported that stretch therapy, ultrasound therapy, electrical stimulation, and treadmill exercise can help in restoration of muscle function. However, this treatment option is difficult to apply to inactive patients, and is not a very effective treatment for disuse muscle atrophy.

Disuse muscle atrophy can be induced experimentally through bandaging [26], receiving a nerve block [27], or having the tail suspended [28]. The nerve block method is useful for studying the pathology of surgical injuries where nerve function is altered. Tail hanging is an appropriate and effective method used in the absence

of weight loading, whilst maintaining skeletal mass and weightless atrophy. Bandage fixation, where artificial immobilization of the limb occurs, is the most widely used method in the study of muscular atrophy [26]. In this study, casting tape was used to fix the limb and induce disuse muscle atrophy.

Flowers of ZM have a unique scent and acidity whose berries and young leaves are widely used as spices and traditional medicine [29]. ZM has been widely used as an anti-inflammatory, analgesic, detoxifying, diuretic, antiparasitic, and digestive medicine because it contains limonene, sanshool, phellandrene, citronellal, and flavonoids [30]. In this current study, the effects of the antioxidant components of ZM plant extracts on the levels of nitric oxide synthase (iNOS), cyclooxygenase-2 (Cox-2), and lipopolysaccharide (LPS) were investigated. ZM plant extracts exhibit antioxidative properties through the inhibition of iNOS, Cox-2, and lipid peroxidation [11]. In addition, it has been reported that methanol and butanol extracts of plants showed high antioxidant activity, and the functional antioxidative compound was speculated to be flavonoids [13]. Moreover, the antioxidant activity of the root extracts, stem, and leaf of the Zanthoxylum piperitum was observed to be the most active between 0.05 - 0.25 mg/mL in ethyl acetate and methylene chloride (1:1) [12]. Furthermore, it has been reported that ZM extracts have excellent antioxidant properties, preventing tooth decay [14].

This current study was carried out to investigate the protective effect of pharmacopuncture using ZM (prepared with water or ethanol) injected into the left ankle joint against disuse muscle atrophy.

No statistically significant differences in weight between groups was observed over the course of the study (Fig. 1). In a murine model where the back was bandaged Wall et al [2] reported a statistically significant reduction in gastrocnemius muscle weight after bandaging. In this study, the weight of the left and the right muscles of both the control group and the ZM-E group, after strapping the left ankle joint for 2 weeks, showed a significant decrease in the weight of both the control group and the ZM-W and ZM-E groups. A statistically significant change in the ratio of the proximal axis (according to the method by Shibaguchi et al [31]) was observed when the left muscle mass change was compared to the right, with a reduction of about 22.0% for the control group, 19.5% for the ZM-W group, and 18.8% for the ZM-E group (Table 1). These results indicated that pharmacopuncture using ZM showed significant protection against weight loss in the gastrocnemius muscle caused by disuse muscle atrophy.

Hematoxylin and Eosin staining of the 8 μm section for gastrocnemius muscle in the control group, ZM-W group and ZM-E group showed that the size of the left gastrocnemius muscle fibers were reduced compared with the right, but the ZM-W group and ZM-E group showed relatively smaller reduction compared with the right muscle group in the ZM-W and ZM-E groups. In addition, relatively lower nuclear aggregation was observed around the muscle fibers, which may be indicative of the presence of inflammatory cells in muscle, and proliferation of muscle satellite cells [32] (Fig. 2). Udaka et al [26] reported that the decrease in muscle weight due to the lack of use of muscle was accompanied by a decrease in muscle fiber in the muscle. In this current study, the decrease in muscle fiber size was observed along with a decrease in muscle weight due to ankle fixation for 2 weeks.

Experiments comparing the gastrocnemius muscle cross-sectional area showed that control group exhibited a reduction rate of about 19.6% in the left side upon ankle fixing as compared with the right side, and ZM-W and ZM-E groups showed a reduction by about 16.4% and 15.9%, respectively. ZM-W and ZM-E groups showed significantly lower cross-sectional area reduction than the control group as shown in Table 2. These results are consistent with those of the study by Matsumoto et al [20] who reported that the cross-sectional muscle fibers of the gastrocnemius muscle, were significantly reduced after muscle disuse. In addition, pharmacopuncture with ZM not only inhibited the reduction in weight of the gastrocnemius muscle (due to disuse), but also had a significant protective effect on the reduction of the cross-sectional area of muscle fibers.

Skeletal muscle atrophy has multiple causes in which apoptosis plays an important role. It is well known that apoptosis in muscles increases in various pathological situations [33]. In this study, the expression of the pro-apoptotic proteins BAX [34], and the anti-apoptotic protein Bcl-2 was investigated using posterior fixation of the ankle joint as the mechanism of induced muscle atrophy. BAX plays a major role in promoting apoptosis by changing the membrane permeability of mitochondria, and Bcl-2 is an apoptosis inhibitor. In other words, the in situ ratio of BAX and Bcl-2 is an important determinant of cell death [35]. In this current study, immunohistochemistry was performed to observe changes in the expression of BAX and Bcl-2 protein in the gastrocnemius muscle. The expression of BAX was upregulated in the left gastrocnemius muscle compared with the right side in the control group, the ZM-W group, and the ZM-E group. In order to quantify this, image analysis was used and a statistically significant increase was observed. In contrast, BAX immunoreactivity in the left gastrocnemius muscle of the ZM-W, and the ZM-E group was reduced compared with the control group. Quantification of the immune response indicated that the number of immune cells in the left gastrocnemius muscle was statistically significantly inhibited by ZM in the ZM-W and ZM-E groups compared with the control group (Fig. 3; Table 3).

Furthermore, the immune response to Bcl-2 protein was higher in the control group, ZM-W group, and ZM-E group on the right side than on the left side. The expression of Bcl-2 was clearly more evident in the ZM-W and ZM-E groups than in the control group. Measurement and quantification of Bcl-2-positive cells showed that there was no statistically significant difference between the control, ZM-W, and ZM-E groups in the right gastrocnemius muscle, however, in the left gastrocnemius muscle, the number of Bcl-2-posivite cells in the ZM-W, ZM-E groups was statistically significantly higher than in the control group (Fig. 4; Table 3). The BAX/Bcl-2 ratio in the left gastrocnemius muscle was also lower in the ZM-W and ZM-E groups than observed in the control group (Table 3). This suggested that ZM pharmacopuncture reduced the expression of BAX protein which promotes disuse muscle atrophy and further increases the levels of Bcl-2 protein which inhibit apoptosis, thereby inhibiting apoptosis. Hence, it was hypothesized that ZM had a protective effect against disuse muscle atrophy via the inhibition of apoptosis.

In conclusion, the pharmacopuncture using ZM treatment for disuse muscle atrophy, induced by bandage fixation of the left ankle joint for 2 weeks in rats, showed a statistically significant protective effect on weight reduction in the gastrocnemius muscle and muscle fiber reduction. . These mechanisms may have a protective effect inhibiting muscle atrophy by regulating the expression of apoptosis-related proteins BAX and Bcl-2. Kim et al [36], Kim [37], Kim [38] and Gong et al [39] reported that oral inhalation of Danguibohyeol-tang, Daeyoung-jeon, and Dokhwalgisaeng-tang was effective against muscle atrophy. However, in this current study ZM was injected directly into the muscle where atrophy may occur.

In this study, we only investigated the effect of ZM pharmacopuncture on apoptosis in disuse muscle atrophy, but it is necessary to study other mechanisms that may contribute to the

protective effects of ZM. Further studies are therefore needed to determine which components of ZM cause antioxidant activity, and more investigation is needed to examine the changes in muscle types, including other types of muscle fibers in addition to the gastrocnemius muscles which were tested in this study.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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