Effects of Yam and Diosgenin on Calpain Systems in Skeletal Muscle of Ovariectomized Rats

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SUMMARY

Objective: Yam or diosgenin (extracted from the root of wild yam) is traditionally used for hormone replacement in menopausal women. Calpains are crucially related to the degradation of myofibrillar proteins in skeletal muscle. This study aimed to investigate the effects of yam and diosgenin on the calpain isoform expression in ovariectomized rats, a model of menopausal status.

Materials and Methods: Female rats were divided into: (1) controls; (2) ovariectomized rats; (3) ovariectomized rats receiving yam (250, 750, 1,500 mg/kg/day); (4) ovariectomized rats receiving diosgenin (10, 50, 100 mg/kg/day). Yam and diosgenin were administered for 8 weeks. The expression of μ- and m-calpain in skeletal muscles was determined by reverse transcriptase-polymerase chain reaction.

Results: The μ-calpain/β-actin and m-calpain/β-actin ratios in the control group (0.9 and 1.09, respectively) were significantly higher than those in the ovariectomized group (0.58 and 0.72, respectively). In the yam group, the expression of μ- and m-calpain was lowest in the ovariectomized group receiving no supplementation and lower in the 250 mg group compared with the 750 and 1,500 mg groups (for 0, 250, 750 and 1,500 mg dosage groups, μ-calpain, 0.58, 0.88, 1.24 and 1.13, respectively; m-calpain, 0.72, 1.02, 1.38 and 1.47, respectively). In contrast, there were no significant differences in the expression of μ- and m-calpain mRNAs among the different diosgenin dosage groups (for 0, 10, 50 and 100 mg of diosgenin, μ-calpain, 0.58, 0.56, 0.62 and 0.58, respectively; m-calpain, 0.72, 0.58, 0.71 and 0.54, respectively). Decreased expression of μ- or m-calpain was observed in the ovariectomized group compared with the normal controls.

Conclusion: Yam, but not its extract (diosgenin), is associated with the regulation of calpain isoforms in ovariectomized rats. Adequate yam supplements might improve the muscular calpain-related physiopathology associated with menopausal status. [*Taiwan J Obstet Gynecol* 2008;47(2):180–186]

Key Words: calpain, diosgenin, menopause, ovariectomy, yam

Introduction

Hormone replacement therapy is one of the most commonly prescribed medications for menopausal women. Estrogen replacement therapy can decrease the risk of developing numerous disorders, including osteoporosis, hot flashes and Alzheimer's disease. Artificial synthetic estrogen, the current and most effective agent for hormone replacement therapy, is widely used. However, fears of side effects from estrogen therapy have motivated many patients to seek alternative modalities for symptomatic relief. Requests for complementary/alternative therapies for hormone replacement have thus dramatically increased. A currently popular alternative treatment is wild yam (*Dioscorea villosa*). Wild yam or diosgenin (extracted from the root of wild yam) has been applied in hormone replacement therapy [1].

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Wild yam and its extracts have been used to minimize postmenopausal symptoms. At present, many menopausal women take traditional herbal medicines, such as yam or diosgenin, to substitute for the female hormone (estrogen). Yam contains steroidal saponins, which can influence endogenous steroidogenesis [2]. Diosgenin, a type of steroidal saponin, is synthetically derived from the root of wild yam using an analytical separation assay procedure. Diosgenin can be converted into human progesterone, aldosterone, cortisol and estrogen through a series of enzymatic steps [3]. However, the effect of wild yam or diosgenin on menopausal women remains obscure, as information about the efficacy and safety of natural medicine on menopausal women is still lacking.

The calcium-dependent proteinase (calpain) system is found in every vertebrate cell; contraction of the skeletal muscle is associated with the calcium-dependent protease calpain system [4,5]. Calpains are calcium-modulated proteases which respond to calcium (Ca^{2+}) signals by removing limited portions of protein substrates, thereby irreversibly modifying their functions. Calpain is a cytoplasmic cysteine protease activated by calcium ions [6]. There are several major ubiquitous calpain isoforms, including μ-calpain and m-calpain. Both μ-calpain and m-calpain require micromolar and millimolar calcium concentrations for activation [6,7]. The μ-calpain is accompanied by limited autoproteolysis; m-calpain can be activated by μ-calpain [8]. Although the physiologic function of calpain isoforms remains obscure, they take part in a variety of calcium-regulated cellular processes such as signal transduction, cell proliferation, cell cycle progression, differentiation, apoptosis, membrane fusion, and platelet activation [9–12].

Estrogen and estrogen receptors (ERs) can influence the expression of calpain isoforms in menopausal women [13]. Estrogen influences the development and function of the nervous system through ER-dependent changes in gene expression and by rapidly influencing diverse intracellular signaling pathways [14]. Calpain expression in the pituitary gland may be upregulated by estrogen at the transcription level [15]. Estrogen influences the calpain isoform activities in skeletal and cardiac muscles [16]. In reviewing the MEDLINE database, we found that no investigators have demonstrated the effect of yam or diosgenin administration on the calpain presentation of skeletal muscle in ovariectomized rats or menopausal women. The objective of this study was to determine whether the addition of yam or diosgenin modulates the calpain isoform (μ-calpain, m-calpain) expression in ovariectomized rats. To the best of our knowledge, this is the first such study in the aspect of yam or diosgenin.

Materials and Methods

Animals

Seven-week-old female Sprague-Dawley rats ($n = 56$; birth weight, 300–350 g) were divided into four groups: (1) control rats ($n = 7$); (2) ovariectomized rats ($n = 7$); (3) ovariectomized rats receiving yam (250, 750, 1,500 mg/kg/day; total, $n = 21$; individual dosage group, $n = 7$); (4) ovariectomized rats receiving diosgenin, which is extracted from the root of wild yam (10, 50, 100 mg/kg/day; total, $n = 21$; each subgroup, $n = 7$). Wild Chinese yam (obtained from a traditional herb market) or diosgenin (Sigma Chemical Co., St Louis, MO, USA) was administered to ovariectomized rats for 8 weeks. All groups of rats were of the same age. The ambient temperature was maintained between 22°C–24°C and the animals were kept under an artificial 12-hour light–dark cycle. The rats were provided with standard laboratory chow and water *ad libitum*. All protocols were approved by the Institutional Animal Care and Use Committee of China Medical University and Chang Shan Medical University in Taichung, Taiwan.

RNA extraction and reverse transcription reaction

All rats were sacrificed after 8 weeks of feeding. Tissues were removed immediately and washed with cold phosphate buffer. The tissue was cut into portions and stored separately at −70°C for later analysis. The expression of calpain isoforms (μ-calpain, m-calpain) in skeletal muscles was determined by reverse transcriptase-polymerase chain reaction using β-actin as an internal standard. Total RNA was extracted from the rat skeletal muscles using Trizol (Invitrogen Corp., Carlsbad, CA, USA). One microgram of each total RNA was reverse-transcribed into cDNA with the RNA polymerase chain reaction (PCR) kit (Invitrogen Corp., Carlsbad, CA, USA) using oligo (dT) primer. The reaction was carried out at 50°C for 60 minutes. The reverse transcriptase was inactivated by incubation at 98°C for 5 minutes and kept at 4°C.

PCR conditions

The reverse transcription reaction products were measured by PCR analysis, using mRNA-encoding rat β-actin as an internal standard. All PCR reactions were performed as follows: 1 μL of cDNA was used for amplification with 1 U of Taq DNA polymerase (Qiagen, Hilden, Germany), 5 μL of PCR buffer, 5 μL of each 10 mM dNTPs, and 1 pmol of each specific upstream and downstream primers. The individual primer sequences were designed as follows: μ-calpain, 5′-GGTCAGCCTGTGCACTTGAAGCG and 3′-TTGTGGGGCTCGAAGGTGGAGGG; m-calpain, 5′-CACAACCCGAGCCAGGGAGCG

and 3′-TTGTGGGGCTCGAAGGTGGAGGG; and calpain 10, 5′-AACCCAGCGAGGTGTGTGTGGCTGTT and 3′-GCAGTGTTGCTGTAGGGTGATACGGATG. PCR amplification was performed under the following conditions: 94°C for 5 minutes, then 30 cycles at 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, with a final elongation at 72°C for 7 minutes. The PCR products were analyzed by electrophoresis in 2% agarose gel containing 0.5 μg/mL ethidium bromide.

Preparation of tissue and cell extracts for Western blotting

Rat skeletal muscles were homogenized in ice-cold buffer B (20 mM Tris-HCl [pH 7.5], 0.25 M sucrose, 50 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 200 μg/mL of leupeptin, 5 mM EDTA, 2 mM EGTA, 10 mM benzamidine) using a homogenizer. Tissues were centrifuged at 100,000 × *g* for 30 minutes at 4°C. Supernatants were used as the cytosolic fraction. To obtain membrane fractions, the pellets were washed and extracted with buffer B containing 1% Triton X-100. After a 30-minute incubation at 4°C, the samples were centrifuged at 100,000 × *g* for 30 minutes, yielding the solubilized membrane fraction. Protein concentrations of individual samples were determined by the Bradford method [17].

Western blot analyses of μ-calpain and m-calpain

Effects of ovariectomization and yam and diosgenin administration upon the m- and μ-calpain in skeletal muscle of female Sprague-Dawley rats were determined by Western blot analysis. Protein samples were subjected to 10% sodium dodecyl-polyacrylamide gel electrophoresis according to the Laemmli method [18]. The separated proteins were electrophoretically transferred to a 0.22 μm nitrocellulose membrane (Hybond-C Super or Hybond-ECL; Amersham Life Science, Buckinghamshire, UK) at 4°C overnight using a Bio-Rad transfer blot apparatus (30 mA; Bio-Rad, CA, USA). Nonspecific sites were blocked with 5% skim milk in TTBS (50 mM Tris-HCl [pH 7.5], 0.15 M NaCl, 0.05% Tween-20) for 1 hour at room temperature. The membranes were incubated for 2 hours at room temperature and were sequentially diluted to 1:2,500 with anti-rabbit μ- or m-calpain. The membranes were washed and incubated for 1 hour with a 1:2,000 dilution of secondary (alkaline phosphatase-conjugated goat anti-rabbit IgG). After three washings, antibody-reactive bands were visualized by an enhanced chemiluminescence detection system (Amersham Life Science, Buckinghamshire, UK). The blots were scanned using a GeneGnome bio imaging system (Syngene, Frederick, MD, USA).

Statistical analysis

All data are presented as mean ± standard deviation. SAS version 8.1 software (SAS Institute Inc., Cary, NC, USA) with unpaired Student's *t* test was utilized for statistical analysis. A *p* value of less than 0.05 was considered statistically significant.

Results

We observed that menopause status (ovariectomization) was related to decreased expression of calpain isoforms. The μ- and m-calpain/β-actin ratios in the control group were significantly higher than those in the ovariectomized groups. The expressions of μ-calpain in the control and ovariectomized groups were 0.9 ± 0.13 and 0.58 ± 0.11, respectively ($p < 0.05$; Figure 1A). The expressions of m-calpain in the control and ovariectomized groups were 1.09 ± 0.1 and 0.72 ± 0.08, respectively ($p < 0.05$; Figure 1B).

In ovariectomized rats receiving the yam, administration of high doses of yam appeared to increase the expression of calpain isoforms. The expressions of μ- and m-calpain were lowest in the ovariectomized group receiving no yam supplementation, and lower in the group receiving 250 mg, compared with those receiving 750 and 1,500 mg. The levels of μ-calpain in the 0, 250, 750, 1,500 mg yam groups were 0.58 ± 0.11, 0.88 ± 0.11, 1.24 ± 0.41, 1.13 ± 0.28, respectively ($p < 0.05$; Figure 2A). The levels of m-calpain in the 0, 250, 750, 1,500 mg yam groups were 0.72 ± 0.08, 1.02 ± 0.12, 1.38 ± 0.37, 1.47 ± 0.21, respectively ($p < 0.05$; Figure 2B).

In contrast, the administration of yam extract (diosgenin) did not seem to influence the expression of calpain isoforms in ovariectomized rats. There were no significant differences in the expressions of μ- and m-calpains among the different diosgenin dosage groups. The levels of μ-calpain in the 0, 10, 50, and 100 mg diosgenin groups were 0.58 ± 0.11, 0.56 ± 0.08, 0.62 ± 0.14, 0.58 ± 0.25, respectively (Figure 3A). The levels of m-calpains in 0, 10, 50, and 100 mg diosgenin groups were 0.72 ± 0.08, 0.58 ± 0.16, 0.71 ± 0.22, 0.54 ± 0.18, respectively (Figure 3B).

Discussion

Artificial estrogen remains the most effective agent for hormone replacement therapy and prevention of menopausal symptoms, hot flashes and osteoporosis, and for decreasing the risk of colorectal cancer. However, estrogen supplement may increase the risk of some disorders or diseases including breast cancer, heart

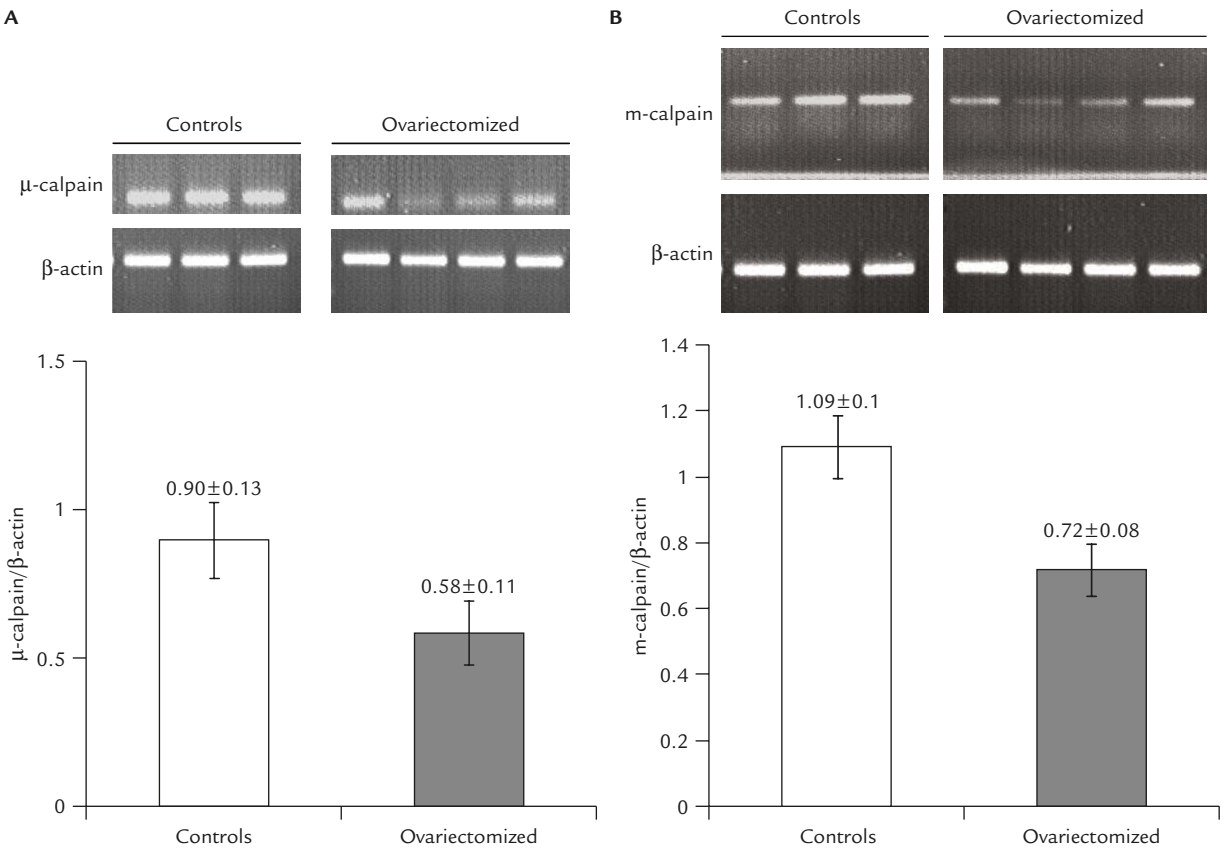

Figure 1. Expression of (A) μ-calpain and (B) m-calpain mRNAs in skeletal muscle of normal controls and ovariectomized rats without supplementation.

disease, stroke, and pulmonary embolism, which compromise its popular application [19]. Over the past decade, people have been seeking natural alternatives to treat menopausal symptoms. Currently, some herbal medicines such as wild yam or its extract have been widely used to minimize postmenopausal symptoms.

Chinese yam (*Dioscorea*), a staple food in several tropical countries, is a good source of steroid used in the manufacture of birth control pills and other sex hormone preparations [20]. Yam is a common raw material used for drug and health food manufacture. Wild yam has been used for women with hot flashes as well as many other menopausal conditions [21]. Administering yam improves the status of sex hormones, lipids and antioxidants in postmenopausal women. [22]. Wild yam preparations contain diosgenin which can be converted into progesterone [2,21].

Diosgenin, a biologically active compound of yam, has tremendous medical applications. Compared with wild yam, diosgenin appears to be free of some adverse effects. Diosgenin administration in ovariectomized rats resulted in increased adrenal tissues, whereas these areas decreased in diosgenin-treated animals [1,3]. Diosgenin administration might protect the kidney from morphologic changes associated with ovariectomy [3]. These findings suggest that diosgenin affects endocrine function. However, there are insufficient data about these herbal alternative therapies for menopausal women.

The calpain system, a family of calcium-dependent cysteine proteases, has been studied for decades [23]. Calpains represent a superfamily of Ca^{2+}-activated cysteine proteases, which are important mediators of apoptosis and necrosis. These calpain isoforms might act independently through different proteasome pathways and cleave a number of cellular substrates, including kinases, phosphatases, transcription factors and cytoskeletal proteins [24]. Calpain activity has been associated with cleavages that alter regulation of various enzyme activities, remodeling or disassembly of the cell cytoskeleton, and cleavages of hormone receptors [9]. Calpains are involved in a variety of physiologic and pathologic disorders [25]. Alterations in calpain isoform activity might be related to a number of disorders, including stroke, traumatic brain injury, Alzheimer's disease, cataract, limb-girdle muscular dystrophy and gastric cancer [26,27].

The calpain system comprises three molecules: μ-calpain, m-calpain, and calpastatin [28]. The function of calpastatin is to inhibit μ-calpain and m-calpain. Both μ- and m-calpain are heterodimers containing

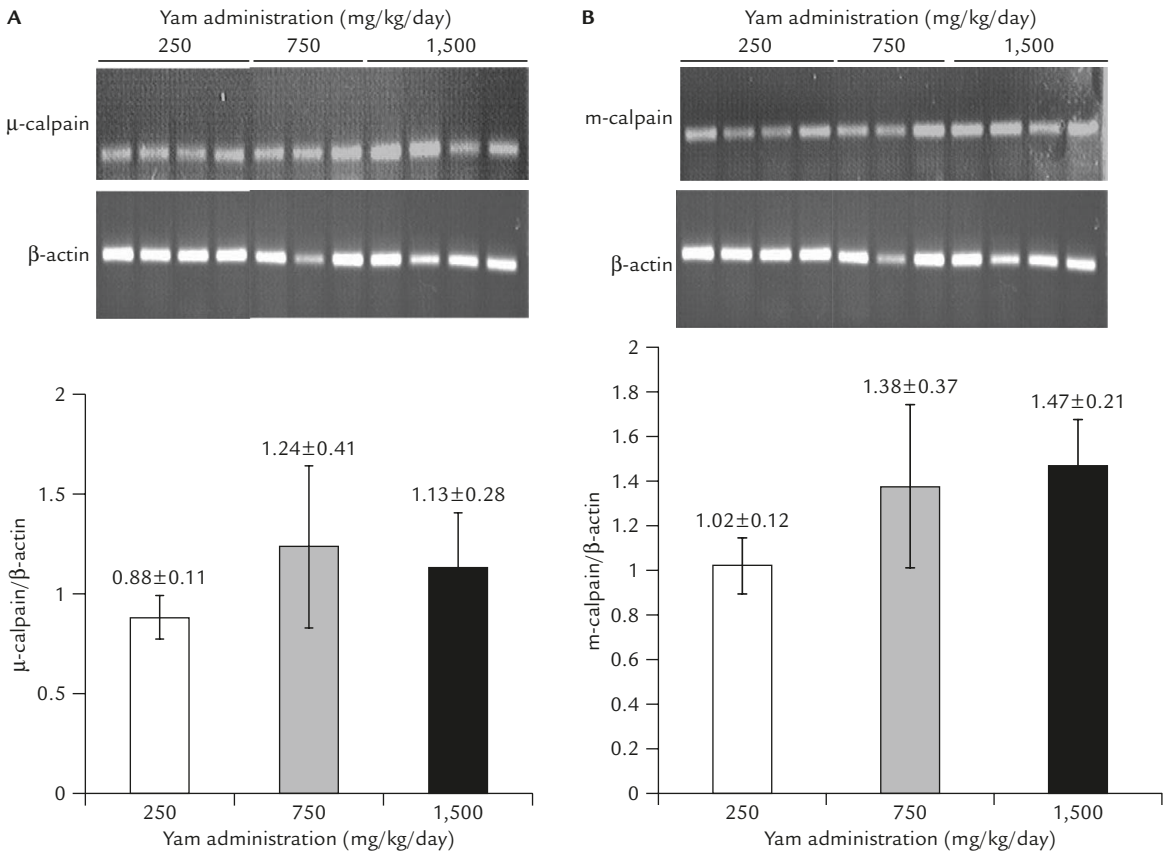

Figure 2. Expression of (A) μ-calpain and (B) m-calpain mRNAs in skeletal muscle of ovariectomized rats after yam administration (250, 750, 1,500 mg/kg/day).

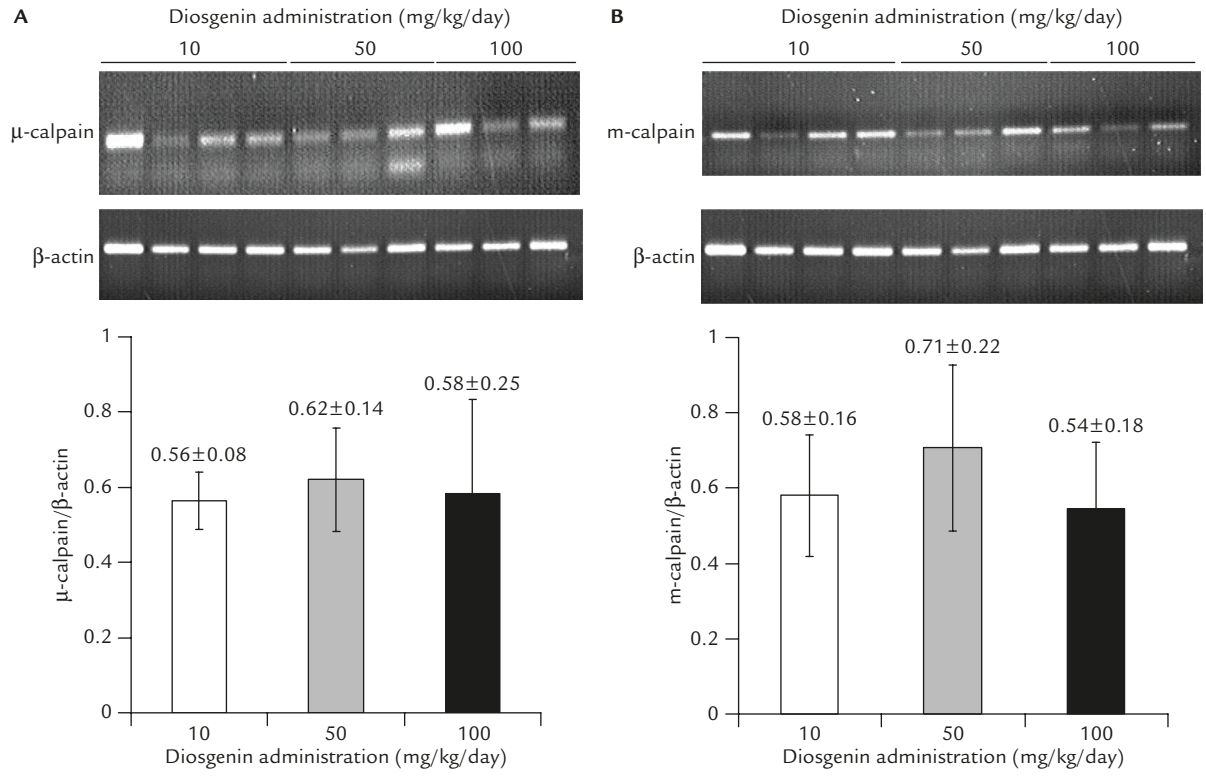

Figure 3. Expression of (A) μ-calpain and (B) m-calpain mRNAs in skeletal muscle of ovariectomized rats after diosgenin administration (10, 50, 100 mg/kg/day).

a 30-kDa small subunit and an 80-kDa large subunit [29]. However, the mechanism regulation of activity of the calpain system *in vivo* is unknown. At the cell membrane, calpain is activated in the presence of Ca^{2+} and phospholipids. Suzuki and Ohno [29] indicated that calpain exists in the cytosol as an inactive enzyme and translocates to membranes in response to increases in the cellular Ca^{2+} level. Calpains usually exist in an inactive form and are activated by calcium and phospholipids [25,30].

Calpain activity may be regulated by binding Ca^{2+} to specific sites on the calpain molecule, with binding to each site eliciting a response (e.g. proteolytic activity, calpastatin binding) specific for that site. The binding of the calpains to phospholipid in a cell membrane might lower the Ca^{2+} concentration, which is required for the autolysis of calpains. Furthermore, the autolysis of calpains converts an inactive proenzyme into an active protease [9]. The calcium-dependent protease calpain causes endothelial dysfunction and vascular inflammation in the microcirculation of the rat [31]. Inhibition of calpain activity in skeletal muscle is related to equilibrium in muscle metabolism [32].

Estrogen and ER can exert protective activity in menopausal women by influencing the expression of calpains and their endogenous inhibitor, calpastatin [13]. Estrogen supplements could, therefore, influence the amount and size of muscle fiber [33]. Hormone deficiency status in ovariectomized rats might result in reduced amount of myosin heavy chain [34]. Furthermore, the reduced myosin heavy chain expression could be reversed with estrogen supplements, which suggests the estrogen-dependent nature of muscle fibers. Estrogen might counteract the calpain isoform, which further influences muscle function in the menopausal women. Estrogen supplementation in ovariectomized rats also influences cardiac calpain activity [16,33].

In contrast, calpain plays an important role in the feedback regulation of estrogen activity and ER function [35,36]. Calpain might intervene in estrogen activity by diminishing irreversibly the amount of cytoplasmic ER capable of translocating into the nucleus [37]. Calpain has been reported to hydrolyze the ERs [35]. Calpain is associated with the regulation of ER function as well as malignant transformation in breast cancer tissues [36].

In this study, we observed decreased levels of μ- or m-calpain mRNA in ovariectomized rats compared with normal controls. Ovarian hormone might be related to expression of calpain isoforms. Administration of large dosages of yam increased the expression of both μ- and m-calpain mRNAs in the skeletal muscle of ovariectomized rats. Adequate dosages of yam supplement might compensate for the deficiency of ovarian hormone as well as influence and regulate the calpain isoform-related pathways in skeletal muscle. These findings suggest the potential of yam administration in improving the physiopathology of muscle in ovariectomized or menopausal women. Ovarian hormone or yam administration plays a role in increasing calpain expression or decreasing calpain degradation, which are associated with muscle function in menopausal women. These findings are in agreement with previous studies, which revealed that estrogen administration improves menopause-related sequences.

In contrast, some have reported insignificant improvement in menopausal symptoms after short-term administration of topical wild yam extract [2]. We also found that diosgenin administration had no significant effect on calpain isoform expression. This research reported on the muscular regulation efficiency of yam compared with wild yam extract (diosgenin). The results suggest that the extraction and separation procedure results in the loss of some related compounds that are essential for calpain isoform regulation. The information is important, because compromised calpain isoform activity might pose potential muscular dysfunction in menopausal females. However, the underlying mechanisms and physiologic consequences of this activity are yet to be determined.

In conclusion, ovarian hormone or yam administration appears to importantly determine the expression of calpain isoforms. Yam but not its extract (diosgenin) can regulate calpain isoforms in ovariectomized rats. The results suggest that yam supplements might improve the muscular and calpain-related physiopathology of menopausal status. The efficacy of yam administration is dose-dependent. This research has highlighted the correlation between yam or diosgenin supplements and calpain expression in menopause. However, the underlying mechanism and related issues merit further study. Recruitment of larger cohorts for human survey may be required for further clarification. Although the real role of calpain isoforms has not been clarified, it deserves more attention in terms of the related molecular mechanisms. After the resolution of these issues, yam supplementation might become an alternative consideration for the menopausal population.

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