동의생리병리학회지 제 28권 4호
Korean J. Oriental Physiology & Pathology 28(4):411~416, 2014

ISSN(Print) 1738-7698
ISSN(Online) 2288-2529

Effect of Simple Formulas of Muscle Section in Donguibogam on Myogenic Regulatory Factors and IGF-1 Expression in C2C12 Cells

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Simple formulas (單方) of muscle section in Donguibogam (東醫寶鑑) have long been prescribed for strengthening muscle and/or prevention of age-related muscle loss. However, biological activity and mechanisms by which they influence myoblast differentiation have not been studied. Therefore, in this study, we evaluated the effects of 14 simple formulas on myoblast differentiation in C2C12 myoblast cells under non-cytotoxic (0.5 ㎎/㎖) conditions. C2C12 cells were treated with water extracts of simple formulas for 72 h, and RT-PCR was performed to determine the gene expression levels of myogenic regulatory factors (MRFs), including myoD, myogenin, MRF4, myf5, and insulin like growth factor-1 (IGF-1). Treatment with Colocasiae Rhizoma (CR), Pini Semen (PS), and Sesami Semen (SS) resulted in a significant increase in expression of myogenin in C2C12 cells. Treatment with Allii Macrostemi Bulbus (AM), Colocasiae Rhizoma (CR), and Pini Semen (PS) also resulted in increased expression of MRF4 in C2C12 cells. In addition, enhanced expression of IGF-1 was observed in treatment with Eucommiae cortex (EC), Dioscoreae Rhizoma (DR), Colocasiae Rhizoma (CR), Pini Semen (PS), and Sesami Semen (SS) in C2C12 cells. These results indicate that simple formulas of muscle section in Donguibogam could potentially enhance myoblast differentiation at least in part via increasing expression of myogenin, and/or MRF4 and/or IGF-1.

Key words : C2C12, differentiation, myogenic regulatory factor, insulin like growth factor-1, Donguibogam

Introduction

Sarcopenia is defined as a low level of muscle mass resulting from age-related muscle wasting. After the age of 50, humans lose muscle mass at a rate of 1-2% per year, which has an impact on muscle strength and function[1]. These alterations lead to reduced metabolic performance, disability, increased risk of fall-related injury, and frailty[1,2]. Currently, sarcopenia affects more than 50 million people, and is recognized as a serious geriatric health problem[2]. Thus, prevention of sarcopenia has high clinical relevance, not only for improvement of the quality of life, but also to reduce cost of aged care services.

Despite a huge influx of its underlying pathophysiology, impaired differentiation capacity of myoblasts is generally known to be one of the main mechanisms of sarcopenia[3-5]. Myoblasts are mononucleated myogenic cells, located between the sarcolemma and basement membrane of muscle fibers. They are normally quiescent in adult muscle fibers, but have mitotic potential for differentiation into new fibers. Myoblast differentiation is essential for formation of muscular fibers or replacement of damaged or degenerated muscle fibers[4,6,7]. Interference with myoblast differentiation ability could ultimately result in loss of skeletal muscle mass[3,4]. In vivo studies have provided evidence for a direct role of impaired differentiation capacity in sarcopenia. For example, studies of old age have reported that the differentiation capacity of myoblasts is decreased, which is an important factor in the onset of sarcopenia[3].

Myoblast differentiation is a complex process controlled by myogenic regulatory factors (MRFs). There are four MRFs (myoD, myogenin, MRF4, and myf5), which are basic helix-loop-helix (bHLH) proteins that act like transcription activators due to their DNA binding activity on muscle-specific genes. During differentiation, these MRFs can bind to the promoter region of muscle-specific genes and cause them to be expressed into mRNA, which is then used as a substrate for protein synthesis[8,9]. Other, non-muscle specific transcription factors, such as insulin like growth factor-1 (IGF-1) are also important in the differentiation process[10,11]. IGF-1, a small peptide growth factor with a structure similar to that of

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· Received : 2014/06/19 · Revised : 2014/08/02 · Accepted : 2014/08/12

insulin, is found in the circulation (produced in the liver) and locally in muscle. Expression of IGF-1 stimulates both differentiation and hypertrophy of myoblasts. IGF-1 actions, including the hypertrophic processes, are mediated by Akt, a serine/threonine kinase, a downstream target of IGF-1 signaling[12,13]. Suppression of these factors' expression plays a central role in impaired differentiation capacity of myoblasts; therefore, it is an attractive therapeutic target for sarcopenia characterized by impaired differentiation[14,15].

In recent years, understanding traditional medical herbs has become an important area of clinical applications[16,17]. Donguibogam (東醫寶鑑), edited by Heo Jun (許俊), is a Korean encyclopedia of traditional medical knowledge and treatments. This book explains the causes and symptoms of various diseases and simple formulas are placed (單方) at the end of each section[18,19]. A simple formula is a prescription consisting of a single or three to four kinds of medicinal herbs. They are easily found, do not require advanced medical knowledge, and, above all, the people must have welcomed the supply of simple economic formulas[19,20]. There are 37 simple formulas of muscle section in Oehyeongpyeon (外形篇), which has long been prescribed for strengthening muscle and/or prevention of age-related muscle loss[21]. However, biological activity and mechanisms for its influence on myoblast differentiation have not been studied. Therefore, we selected 14 simple formulas commonly used in the clinical field (excluding all animal sources), and evaluated their effects on MRFs and IGF-1 expression using C2C12, a mouse myoblast cell line(Table 1).

Table 1. water extract of simple formulas

	Herbs (Abbreviation)	yield
1	乾地黃 Rehmanniae Radix Siccus (RR)	56.65
2	韭薤 Allii Macrostemi Bulbus (AM)	5.6
3	大豆 Glycinis Semen (GS)	19
4	大麥 Hordei Semen (HS)	7.5
5	杜冲 Eucommiae cortex (EC)	13.85
6	蔓菁子 Brassicae Semen (BS)	13
7	木瓜 Chaenomelis Fructus (CF)	36.8
8	覆盆子 Rubi Fructus (RF)	20.1
9	薯蕷 Dioscoreae Rhizoma (DR)	28
10	五茄皮 Acanthopanacis Cortex (AC)	8.80
11	芋 Colocasiae Rhizoma (CR)	12.5
12	何首烏 Polygoni Multiflori Radix (PM)	26.35
13	海松子 Pini Semen (PS)	7.5
14	胡麻 Sesami Semen (SS)	8.7

Materials and Methods

1. Preparation of water extract of simple formulas

Fourteen simple formulas were purchased from Human Herb (Gyeongsan, Korea). The identity of the purchased material was verified by H.M. Shin (College of Oriental Medicine, Dongguk University, Gyeongju, Korea), and a voucher specimen was deposited in the College of Oriental Medicine, Dongguk University. Medicinal herbs were extracted with 200 ㎖ of water at 80℃ for 3 h. The extract was then filtered, evaporated on a rotary vacuum evaporator, and lyophilized. The dried material obtained was stored at -20℃ until use.

1) Cell culture

The C2C12 mouse myoblast cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) and used for all studies. C2C12 mouse myoblasts were cultured in high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% streptomycin/penicillin at 37℃ in a 5% CO_2 humidified atmosphere. At 80% confluence, growth medium was replaced with 2% horse serum to induce differentiation. To study the effect on MRFs and IGF-1 expression, fourteen simple formulas were added to the culture dishes directly after induction of differentiation. Medium was changed daily and then, fourteen simple formulas were added to fresh medium.

2) Cytotoxicity assay

Cytotoxicity was assessed using a colorimetric assay system (XTT Cell Proliferation Kit) based on the metabolization of XTT to formazan by mitochondrial dehydrogenase in living cells. Briefly, attached C2C12 cells were exposed to simple formulas for 24 hours, and then treated with 50 ㎕ of XTT solution. After incubation for 4 h, the amount of formazan was determined by measuring absorbance at 450 nm (using a 650 nm reference filter) on a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

3) RT-PCR (Reverse Transcription‐Polymerase Chain Reaction)

Total RNA was extracted from C2C12 cells using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA), according to the manufacturer's instructions. The quantity of RNA was measured using a UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan), and 1 ㎍ of total RNA was used for cDNA synthesis. PCR was performed using the selective primers(Table 2). PCR was carried out over 27 amplification cycles consisting of denaturation at 95℃ for 10 s, annealing at 58℃ for 30 s, and elongation at 72℃ for 1 min. Final PCR products were separated on 1% agarose gels and visualized by ethidium bromide staining. Transcription amounts were normalized against the GAPDH.

4) Statistical analysis

All results are presented as the mean ± SD of experiments performed at least in triplicate. Data were

analyzed by one-way ANOVA followed by Duncan's multiple range test using GraphPad Prism 4.0 software. Statistical significance was accepted for p values of < 0.05.

Table 2. Primers used for RT-PCR

	Primer name	Sequence (5' to 3')
1	myoD Fw.	AGGACACGACTGCTTTCTTC
2	myoD Rv.	GCACCGCAGTAGGGAAGTGT
3	myogenin Fw	GGCTTTTAAGTGGGGCTGTC
4	myogenin Rv.	CCCAAGATCCACTGCAAATG
5	MRF4 Fw	GAGAGGAACACGTTCTGGCTCC
6	MRF4 Rv.	TGCTGGAGGCTGAGGCATCC
7	myf5 Fw	TGTATCCCCTCACCAGAGGAT
8	myf5 Rv.	GGCTGTAATAGTTCTCCACCTGTT
9	IGF-1 Fw	CAGAGCAGATAGAGCCTGCG
10	IGF-1 Rv.	TGAAGTAAAAGCCCCTCGGT

Results

1. Effect of simple formulas on C2C12 cell viability

XTT assay was performed for measurement of the effect of simple formulas on cell viability in C2C12 cells. As shown Fig 1., treatment with simple formulas at a concentration of 0.5 ㎎/㎖ did not result in a significant decrease of cell viability. Therefore, the concentration 0.5 ㎎/㎖ was used for follow-up experiments.

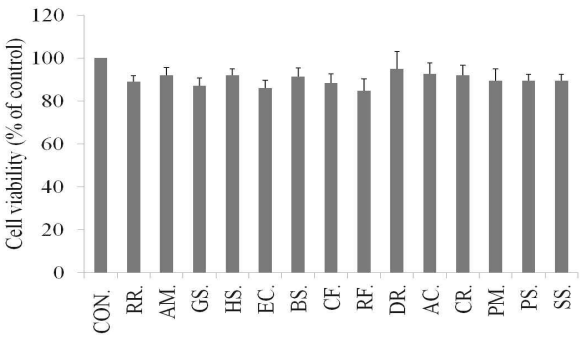

Fig. 1. Effect of simple formulas on C2C12 cell viability. C2C12 cells were treated with simple formulas at a concentration of 0.5 ㎎/㎖ for 24 h. Cell viability was evaluated using a colorimetric assay based on the XTT assay. Data represent the mean± S.D. of three independent experiments.

2. Effect of simple formulas on myoD mRNA expression

Myoblasts undergo differentiation, myoD underpins myoblast determination and onset of fusion[22,23]. RT-PCR was performed to determine the effect of simple formulas on expression of myoD mRNA. As shown in Fig. 2., treatment of myoblasts with HS, BS, and DR resulted in moderate but not significantly increased expression of myoD mRNA in C2C12 cells(Fig. 2).

3. Effect of simple formulas on myogenin mRNA expression

Myogenin, a major MRF, controls the final differentiation step and induces myotube formation[23,23]. RT-PCR was performed to determine the effect of simple formulas on expression of myogenin mRNA. As shown in Fig. 3., treatment of myoblasts with CR, PS, and SS resulted in significantly increased expression of myogenin mRNA(Fig. 3).

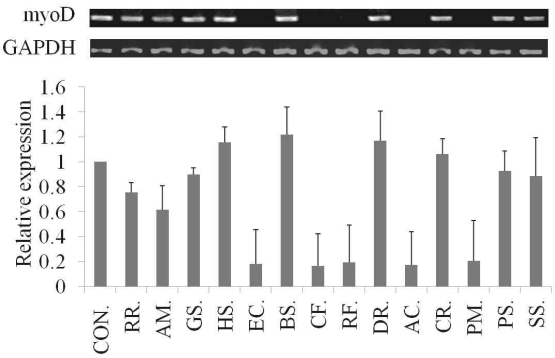

Fig. 2. Effect of simple formulas on myoD mRNA expression. C2C12 cells were grown to 80% confluence and then switched to differentiation media, and were then exposed to simple formulas for 72 h. Total RNA was extracted and RT-PCR was performed to determine the gene expression levels of myoD. Data represent the mean ± SD of three independent experiments. *p < 0.05 versus normal control.

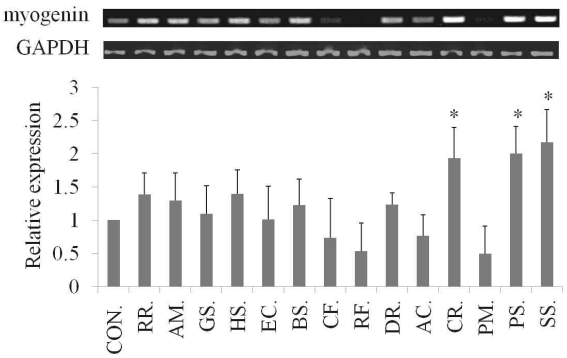

Fig. 3. Effect of simple formulas on myogenin mRNA expression. C2C12 cells were grown to 80% confluence and then switched to differentiation media, and were then exposed to simple formulas for 72 h. Total RNA was extracted and RT-PCR was performed to determine the gene expression levels of myogenin. Data represent the mean ± SD of three independent experiments. *p < 0.05 versus normal control.

4. Effect of simple formulas on MRF4 mRNA expression

Among four MRFs, MRF4 may be involved at the latest stages of differentiation[22,23]. Simple formulas were further investigated to determine whether they affect the MRF4 expression levels. As shown in Fig. 4., treatment of myoblasts with AM, CR, and PS resulted in significantly increased expression of MRF4 mRNA(Fig. 4).

5. Effect of simple formulas on myf5 mRNA expression

Next, we analyzed the effect of simple formulas on expression of myf5, which is the first MRF to be expressed[23]. RT-PCR was performed to determine the effect of simple

formulas on expression of myf5 mRNA. As shown in Fig. 5., treatment of myoblasts with HS and BS resulted in moderate but not significantly increased expression of myf5 mRNA in C2C12 cells(Fig. 5).

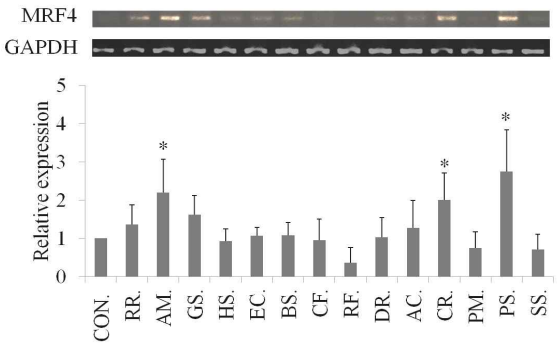

Fig. 4. Effect of simple formulas on MRF4 mRNA expression. C2C12 cells were grown to 80% confluence and then switched to differentiation media, and were then exposed to simple formulas for 72 h. Total RNA was extracted and RT-PCR was performed to determine the gene expression levels of MRF4. Data represent the mean ± SD of three independent experiments. *p < 0.05 versus normal control.

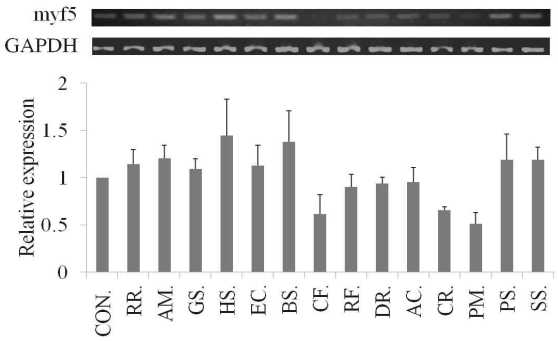

Fig. 5. Effect of simple formulas on myf5 mRNA expression. C2C12 cells were grown to 80% confluence and then switched to differentiation media, and were then exposed to simple formulas for 72 h. Total RNA was extracted and RT-PCR was performed to determine the gene expression levels of myf5. Data represent the mean ± SD of three independent experiments. *p < 0.05 versus normal control.

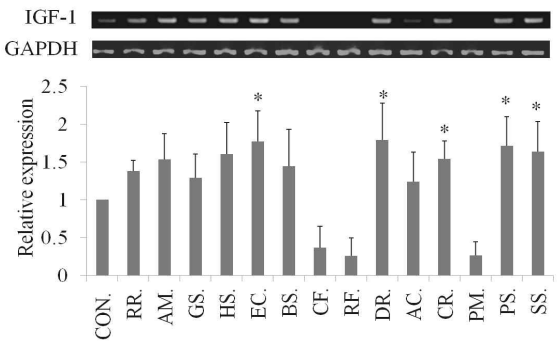

Fig. 6. Effect of simple formulas on IGF-1 mRNA expression. C2C12 cells were grown to 80% confluence and then switched to differentiation media, and were then exposed to simple formulas for 72 h. Total RNA was extracted and RT-PCR was performed to determine the gene expression levels of IGF-1. Data represent the mean ± SD of three independent experiments. *p < 0.05 versus normal control.

6. Effect of simple formulas on IGF-1 mRNA expression

Next, we analyzed the effect of simple formulas on expression of IGF-1, another well-known mediator of differentiation produced in skeletal muscle. RT-PCR was performed to determine the effect of simple formulas on expression of IGF-1 mRNA. As shown in Fig. 6., treatment of myoblasts with EC, DR, CR, PS, and SS resulted in significantly increased expression of IGF-1 mRNA in C2C12 cells(Fig. 6).

Discussion

Sarcopenia, age-related muscle loss, results from impaired myoblast differentiation capacity, which is essential for formation or replacement of muscle fibers. It is caused by down-regulation of MRFs expression, such as myoD, myogenin, MRF4, and myf5[3-5,24]. Thus, stimulator of MRFs may be useful for enhancement of myoblast differentiation and potentially preventing sarcopenia. Simple formulas of muscle section in Donguibogam have long been used in traditional Korean medicine for strengthening muscle and/or prevention of age-related muscle loss[21].

However, no focused in vitro studies were conducted for investigation of the molecular mechanisms of simple formulas on skeletal myoblast differentiation. The current study was conducted in order to investigate the effect of simple formulas on MRFs and IGF-1 mRNA expression using C2C12 mouse myoblast cell lines. First, we observed that addition of several simple formula extracts to the differentiation medium of C2C12 cells resulted in enhanced MRFs mRNA expression. Thus, CR, PS, and SS extracts (0.5 ㎎/㎖) induced a significant increase in expression of myogenin mRNA from C2C12 cells, while AM, CR, and PS exhibited significant enhancement effects on MRF4 expression. Second, another novel aspect of our work is represented by the finding that EC, DR, CR, PS, and SS extracts (0.5 ㎎/㎖) activate expression of IGF-1.

Previous study demonstrated that MRFs (myoD, myogenin, MRF4, and myf5) expression is enhanced, which enables more rapid differentiation of myoblasts and complete myotube formation[11,25]. The specific role of the different MRFs in skeletal muscle differentiation has not yet been completely defined due to the cross regulatory loops between them[8]. However, it appears that myogenin is a major muscle transcription factor that promotes myotube formation and controls the final differentiation step[8,22,23]. In vivo, the role of this gene during differentiation has also been determined. A myogenin-null mutation blocks myoblast differentiation, resulting in a dramatic decrease in muscle fiber formation[26]. In

this study, treatment of C2C12 cells with CR, PS, and SS resulted in increased expression of myogenin. Function of MRF4 is similar to that of myogenin, with a few differences. MRF4 is involved at the latest stages of differentiation[22,23]. We found that treatment with AM, CR, and PS also resulted in increased expression of MRF4 in C2C12 cells.

Among MRFs, myogenin and MRF4 were considered to be the late MRFs, while myoD and myf5 were considered to be the early MRFs. MyoD and myf5 are expressed during the early stage differentiation and play an important role in cell cycle regulation[8,22,23]. In this study, all simple formulas had moderate or no significant effect on expression of myoD and myf5. These findings suggested that stimulation of myogenin and MRF4 expression may be a possible molecular target for use of simple formulas in treatment of impaired differentiation capacity of sarcopenia.

IGF-1 is another regulatory factor in myoblast differentiation, and its mechanism and pathway has been studied extensively. IGF-I-mediated PI3K/Akt signal pathway activation in skeletal muscle induces myoblast differentiation and hypertrophy[13]. In recent years, creatine treatment increased differentiation of C2C12 cell via over-expression of IGF-1, providing additional evidence of the relationship between IGF-1 and myoblast differentiation[25]. In this study, we found that EC, DR, CR, PS, and SS extracts are a positive stimulus for expression of IGF-l in C2C12 cells. From these results, we suggest that EC, DR, CR, PS, and SS could increase differentiation capacity through an IGF-1 mediated pathway in myoblasts.

In summary, we have suggested that the biological action of simple formulas may be related to enhanced expression of myogenin, MRF4, and IGF-1. The possibility that different action mechanisms or other factors may be involved in their activities could not be excluded. However, this finding implies that several simple formula extracts induce an increase of myogenin, MRF4, and IGF-1 expression, which may be a potential strategy for prevention of impaired myoblast differentiation related diseases such as sarcopenia. Based on the results observed in this study, it is expected that further advances in identification of active compounds of simple formulas will provide more solid promise as drugs for treatment of sarcopenia.

Acknowledgements

This study was supported by the Korean Health Technology R&D Project (#B120010), the Korean Ministry of Health & Welfare.

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