Food and Nutrition Sciences, 2015, 6, 287-298
Published Online February 2015 in SciRes. http://www.scirp.org/journal/fns
http://dx.doi.org/10.4236/fns.2015.62029

Mechanisms Underlying the Antifatigue Effects of the Mycelium Extract of *Cordyceps* (*Paecilomyces hepiali*, CBG-CS-2) in Mice in the Forced Swimming Test

Soo-Wan Chae[1,2], Fusako Mitsunaga[3,4], Su-Jin Jung[2], Ki-Chan Ha[5], Hong-Sig Sin[6], Seung-Hwan Jang[6], Shin Nakamura[3,4*]

[1]Department of Pharmacology, Chonbuk National University Medical School, Jeonju-si, Republic of Korea
[2]Clinical Trial Center for Functional Foods (CTCF2), Chonbuk National University Hospital, Jeonju-si, Republic of Korea
[3]Intelligence and Technology Lab Inc., Kaizu, Japan
[4]NPO Primate Agora, Inuyama, Japan
[5]Healthcare Claims & Management Inc., Jeonju-si, Republic of Korea
[6]Chebigen Inc., Jeonju-si, Republic of Korea
Email: *snakamura@itechlab.co.jp

Received 8 December 2014; accepted 4 February 2015; published 15 February 2015

Abstract

***Cordyceps* (CS) is used as an alternative medicine and functional food. We examined *in vivo* mechanisms underlying the antifatigue effects of the cultured mycelium extract of CS (CS extract) in forced swimming mice, a fatigue model that is induced by muscle exercise. Animals orally administered with CS extract significantly extended the loaded forced-swimming time, indicating its antifatigue effects. CS extract modulated the increased levels of blood IL-6 that was induced by forced swimming. CS extract protected the forced swimming-induced increase in NKp46 expression of splenic NK cells, suggesting regulation of fatigue-elicited hyper-reactivity by activated NK cells. By DNA microarray analysis of the quadriceps femoris muscle, it was uncovered that CS extract prevented the forced swimming-mediated upregulation of the expression of 5 genes associating with muscular inflammation (*Ccl6*, *Ccl8*, and *Wfdc17*) and muscle regeneration (*Sfrp4* and *Nfil3*), whereas it regulated the downregulation in the expression of *Svs5* participating in actin binding. CS extract exhibits the antifatigue effects through preventing IL-6 accumulation in blood, regulating NK cell activation in the spleen, and alleviating altered expression of genes related to inflammation, regeneration, and actin binding in the local muscle. Thus, CS extract is an effective functional food for preventing fatigue.**

*Corresponding author.

How to cite this paper: Chae, S.-W., *et al.* (2015) Mechanisms Underlying the Antifatigue Effects of the Mycelium Extract of *Cordyceps* (*Paecilomyces hepiali*, CBG-CS-2) in Mice in the Forced Swimming Test. *Food and Nutrition Sciences*, **6**, 287-298.
http://dx.doi.org/10.4236/fns.2015.62029

Keywords

DNA Microarray, IL-6, NK Cell Activation, Muscle Inflammation

1. Introduction

Fatigue, which is a complex phenomenon of decreased efficiency following continuous work or exercise, may be divided into mental and physical fatigue [1]. It mainly manifests as a physical decrease in muscle tone and exercise tolerance due to an accumulation of metabolites [2] [3]. Currently, work and life stresses are escalating with the increasing pace of life. Thus, fatigue is common and may significantly affect daily routines. Because treatments for fatigue in modern medicine are limited, the antifatigue effects of potential alternatives, including traditional herbs or medicinal or functional foods that prevent diseases and promote health, have been investigated [4] [5].

Cordyceps (*Cordyceps sinensis*, CS) has been used as a medicine in China for over 300 years. It was featured in the book "Ben-Cao-Bei-Yao" that was edited by Wang Ang in 1694, which marked a turning point for medicinal herbs. CS has immunomodulatory, antitumor, antimetastatic, antioxidant, anti-inflammatory, insecticidal, antimicrobial, hypolipidemic, hypoglycemic, antiaging, neuroprotective, and renoprotective effects [6]-[8]. CS-derived natural products are comprised of complex components, including cordycepin and cordycepic acid, which are thought to be the main active ingredients, although this is debatable [9] [10]. CS mycelium culture techniques were developed to yield alternative CS products with a constant composition [11] [12]. Recently, CS culture products have been used as functional foods [13]-[15].

Previous studies have suggested that CS possesses antifatigue effects, but information on the detailed mechanisms underlying this effect is lacking [15]-[17]. The forced swimming test is commonly used in preclinical examinations to assess the antifatigue abilities of functional foods [18] [19]. DNA microarrays are a powerful functional genomic method used to investigate global gene expression events in target tissues or cells. DNA microarray results provide us precious information on the molecular mechanisms underlying the effects of functional foods. Here, we used the forced swimming test and functional genomics with DNA microarray in order to elucidate the detailed *in vivo* mechanisms underlying the antifatigue effects of the CS culture extract.

The current study provides invaluable information about the unique mechanisms underlying the antifatigue actions of the CS extract that involve preventing interleukin (IL)-6 accumulation in the blood, alleviating NK cell activation in the spleen, and modulating the changes in the expression of several genes in local muscle.

2. Material and Methods

2.1. Preparation of the Extract of the Cultured CS Mycelium (*Paecilomyces hepiali*, CBG-CS-2)

A pure strain of *Paecilomyces hepiali* from the collection at the Chebigen, Inc. (Jeonju, S. Korea) was initially grown on potato-dextrose agar medium slants in Petri dishes, which was followed by transfer into a seed culture medium that was composed of 4% dextrose, 1% yeast extract, and 1% peptone (DYP). The seed culture was grown in a 250-mL flask containing a nutritive medium of 100 mL of 2% potato-dextrose broth at 25˚C in a shaking incubator (150 rpm for 5 - 7 days). The seed culture was then transferred into a stirred tank fermenter containing DYP medium with a 10% (v/v) working volume and then fermented at 25˚C at 150 rpm for 5 days. The fermented product was of the initial volume. The extractants were freeze-dried and used as the extract of the cultured CS mycelium (*Paecilomyces hepiali*, CBG-CS-2) in this study.

The main components of the mycelium culture extract were analyzed to be 30% cordyceps polysaccharide, 7.3% cordycepic acid, 1.3% adenosine, and 0.01% cordycepin. Remaining 61.39% was composed of starch as excipient, hydroxypropyl methylcellulose for binder, and small amounts of amino acids, vitamins, and minerals.

2.2. Animals and the Administration of the CS Mycelium Culture Extract

All of the procedures were conducted according to the Institutional Animal Care and Committee Guide of Intel-

ligence and Technology Lab. Male BALB/c mice (9 weeks old; Japan SLC, Inc., Hamamatsu, Japan) were used in the present study. Mice were housed 4 - 5 per cage under specific pathogen-free conditions (controlled temperature of 18˚C to 28˚C and humidity of 30% to 80%) and fed standard laboratory food (FR-2; Funabashi farm, Inc., Funabashi, Japan) *ad libitum*.

The CS culture extract (*Paecilomyces hepiali*, CBG-CS-2) was prepared by Chebigen, Inc. The CS extract solution that was dissolved in distilled water for injections was orally administered to the mice for 28 days at doses of 120 or 400 mg/kg/day. Mice were divided into the following 8 groups: 1, no swimming control group (NS-cont; n = 10, without swimming); 2 and 3, swimming and 0-mg/kg CS groups (SM-CS0; n = 10 each, with and without loaded swimming); 4 and 5, swimming and 120-mg/kg CS groups (SM-CS120; n = 10 each, with and without loaded swimming); 6 and 7, swimming and 400-mg/kg CS groups (SM-CS400; n = 10 each, with and without loaded swimming); and 8, no swimming and 400-mg/kg CS group (NS-CS400). Before swimming with or without a load, the mice were sprayed with a 5% sodium dodecyl sulfate solution to remove water repellents on the hair surface in order to prevent an air bubble from forming that would cause the mice to float. At 28 days, all of the mice were anesthetized with halothane and subjected to blood collection, which was followed by sacrifice in order to obtain the tissue samples.

2.3. Loaded Swimming Test

One to two hours after the final administration of the CS extract, a lead sheath that weighed 5% of the mouse's body weight was tied to the root of the mouse's tail prior to the mouse being placed in a water container. The swimming time (time between being placed in the water and sinking underwater for >10 s) was measured in a round container with a water depth of 20 cm and a diameter of 19 cm at a temperature of 25˚C ± 1˚C.

2.4. Sampling of the Blood and Tissues from the Forced Swimming Mice

One to two hours after the final feed, the nonloaded mice were placed in the water container and left to swim for 30 min at a temperature of 25˚C ± 1˚C. After a break of 1 h after the forced swimming, all of the mice were anesthetized with isoflurane for blood collection, which was followed by sacrifice in order to harvest the spleen and quadriceps femoris muscle.

2.5. Plasma Inteleukine-6 (IL-6) Assay

Plasma samples were prepared from the collected blood by centrifugation, and the plasma levels of IL-6 were assayed by using ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA) according to the kit instructions. Optical density was measured at 450 nm with reference at 630 nm by a Well Reader SK601 (Seikagaku Corporation, Tokyo, Japan).

2.6. Flow Cytometric Analysis of Splenic NK Cells

Splenic cells were prepared from each group of mice by the centrifugation of mesh-homogenized spleens in phosphate-buffered saline (PBS), which was followed by incubation with antimouse antibodies for fluorescein isothiocyanate (FITC)-labeled CD335/NKp46 (eBioscience, Inc., San Diego, CA, USA) for 30 min in the dark. These cells were fixed in PBS containing 2% formaldehyde and 0.02% NaN_3 on ice for 10 min. The fixed cells were then resuspended in 1X Intracellular-Permeabilization Buffer (Life Technologies Corporation, Grand Island, NY, USA) and incubated at room temperature for 3 min. The FITC-stained splenic cells were subjected to single-color flow cytometry with an EPICS XL-MCL (Beckman Coulter, Inc., Brea, CA, USA). Based on the forward and side scatters, the gate was set to include lymphocytes.

2.7. RNA Preparation

RNA was prepared as described previously [20]. Briefly, total RNA was extracted from the harvested quadriceps femoris muscle with Isogen (NipponGene Co., Ltd., Tokyo, Japan) and DNase-treated in the aqueous phase with the RNase-Free DNase Set (QIAGEN, Inc., Valencia, CA, USA). The extracted RNA was further purified with an RNeasy MinElute Cleanup Kit (QIAGEN, Inc.), and the quantity and purity were evaluated photometri-

cally at 260 nm, 280 nm, and 320 nm with an Ultrospec 2000 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

2.8. DNA Microarray Analysis

Total RNA (200 ng) of the pooled quadriceps femoris muscles from each group was used to generate cDNA and Cy3-labeled cRNA with a Low Input Quick-Amp Labeling Kit (Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer's instructions. The labeled cRNA was photometrically examined to determine the quantity and dye-incorporation ratio with an Ultrospec 2000, and it was hybridized to a Mouse Gene Expression 4 × 44K v2 Microarray (Agilent Technologies, Inc.). The array was scanned with GenePix 6000B (Molecular Devices, LLC, Sunnyvale, CA, USA), and the obtained image was processed with GenePix Pro 6.0 Software (Molecular Devices, LLC). The features were manually examined, and spots of poor quality were flagged and filtered out.

The signal data for the features (spots of array) were then imported into GeneSpring 12.6 (Agilent Technologies, Inc.), and further analyses were performed with the software. The signal data from the arrays were normalized with the 75th percentile method [21], and baseline transformation was performed with the median of the control samples. Quality control was performed in order to filter out signal data with standard errors over 0.2.

To analyze the differentially expressed genes, the fold change (FC) vs. the control samples was calculated, and genes with a FC greater than 1.5 were extracted for the NS-cont and SM-CS0 groups. To analyze the normalizing effects of CS, the genes that were shifted toward control levels in the SM-CS120 and SM-CS400 groups compared to the SM-CS0 group were selected with criteria that were set to a 50% upregulation or downregulation that was induced by forced swimming. A gene ontology analysis of the selected genes was performed with GeneSpring 12.6 software, and gene sets with a corrected p value < 0.1 were considered significant.

2.9. Real Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

We performed quantitative RT-PCR to confirm the DNA microarray results for the following genes: chemokine (C-C motif) ligand 6 (*Ccl*6); chemokine (C-C motif) ligand 8 (*Ccl*8); WAP four-disulfide core domain 17 (*Wfdc*17); secreted frizzled-related protein 4 (*Sfrp*4); nuclear factor interleukin 3 (*Nfil*3); SRY-box containing gene 7 (*Sox*7); SRY-box containing gene 18 (*Sox*18); and seminal vesicle secretory protein 5 (*Svs*5). cDNA was synthesized from the isolated total RNA with PrimeScript Reverse Transcriptase (Takara Bio Inc., Otsu, Japan) with RNase Inhibitor (Takara Bio Inc.), dNTP Mixture (Takara Bio Inc.), and Oligo dT Primer (Takara Bio Inc.). The real time PCR was performed with a Mx3000P QPCR System (Agilent Technologies, Inc.) with a SYBR Premix Ex Taq kit (Takara Bio Inc.) with specific primers for the genes. In each run, a standard curve was generated by a serially diluted known GAPDH amplicon, as described previously [20], to calculate the cDNA copy number of the genes. The quantity of the mRNA of interest was expressed as its ratio against that of a suitable reference gene, low-density lipoprotein receptor-related protein 10 (*Lrp*10) [22]. The sequences of the primers (F: forward 5' to 3', R: reverse 5' to 3') are available upon request.

2.10. Statistics

The results of the loaded swimming test and the IL-6 assay are expressed as the mean ± standard deviation. An F-test was used to examine the variance, and the significance of the differences of each group compared to the control group without CS was determined with a Welch's t-test or Student's t-test according to the results of the F-test. P values less than 0.05 were considered significant.

3. Results

3.1. Effects of CS Extract on the Loaded Swimming Time of Mice

The mice groups were treated with 0 (SM-CS0), 120 (SM-CS120), and 400 (SM-CS400) mg/kg of the CS extract and were subjected to the loaded swimming test. As shown in **Figure 1**, the loaded swimming times of the SM-CS120 and SM-CS400 were longer than those of the SM-CS0. The increment rates of the low- and high-dose groups showed slight (16.6% for SM-CS120) and significant (57.2% for SM-CS400) increases, respectively, compared to the SM-CS0 group. These results showed that the CS extract potentiated the forced swimming abil-

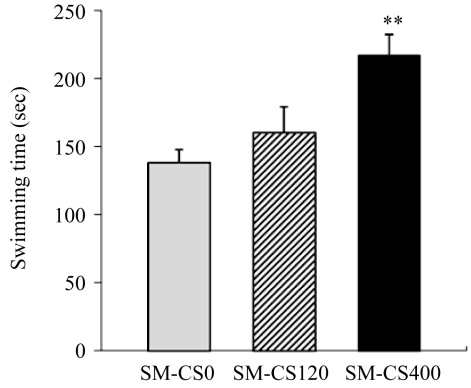

Figure 1. Effects of the *Cordyceps* (CS) extract on the loaded swimming time. SM-CS0: 0 mg/kg CS (control); SM-CS120: 120 mg/kg CS; SM-CS400: 400 mg/kg CS administration. The data were expressed as the means ± SD (n = 10). **p < 0.01 compared with control.

ity in mice, suggesting an antifatigue effect of the CS extract.

3.2. Effects of the CS Extract on Plasma IL-6 Levels

Figure 2 shows the plasma IL-6 levels in the groups of control mice (NS-cont), forced swimming mice (SM-CS0), and forced swimming mice that were treated with 120 mg/kg (SM-CS120) or 400 mg/kg (SM-CS400) of CS extract. In the mice subjected to forced swimming, plasma IL-6 levels were markedly increased compared to the NS-cont group (1.3 ± 0.2 pg/mL) and the SM-cont group (4.5 ± 0.5 pg/mL). The CS extract prevented the forced swimming-mediated increase in plasma IL-6 levels in the SM-CS120 group (3.1 ± 0.3 pg/mL) and the SM-CS400 group (2.8 ± 0.5 pg/mL).

3.3. Effects of the CS Extract on Splenic NK Cell Activation

In order to examine the effects of the CS extract on NK cell activation, flow cytometric analyses were performed with splenic cells and a FITC-labeled antibody to NKp46, which is an activated NK cell marker. The flow cytometric results are shown in **Figure 3**. The cell population ratios of NKp46-positive NK cells were 6.3% and 8.2% in the NS-cont and SM-CS0 groups, respectively, suggesting NK cell activation by forced swimming. Interestingly, in both the CS extract-administered groups, SM-CS120 and SM-CS400, the cell population ratios of the NKp46-positive cells were 6.5% (SM-CS120) and 6.4% (SM-CS400). The CS extract prevented the forced swimming-mediated NK cell activation in the spleen).

3.4. Effects of the CS Extract on Gene Expression in the Quadriceps Femoris Muscle

3.4.1. DNA Microarray Analysis

DNA microarray analyses were performed with RNA from the quadriceps femoris muscles of the following 4 mice groups: NS-cont, SM-CS0, SM-CS120, and SM-CS400. The genes that had expression levels in the SM-CS0 group that were increased more than 2-fold or decreased less than 0.5-fold compared to those of the NS-cont group were selected from the DNA microarray results and were defined as upregulated or downregulated by the forced swimming. There were 59 upregulated genes and 12 downregulated genes (data not shown).

Pretreatment with 120 or 400 mg/kg of the CS extract prevented the forced swimming-induced alterations in gene expression. **Table 1** lists the ontology results of the informatics analysis with GeneSpring of the genes that were upregulated by forced swimming and that were prevented from exhibiting upregulation by the forced swimming test when treated with CS extract. This included 4 genes that are involved in immunity/inflammation (*Ccl*6, *Ccl*8, *Wfdc*17, and *Ly*86), 2 genes related to cancer (*Plac*8 and *Sbsn*), and 2 genes associated with muscle regeneration/inflammation (*Sfrp*4 and *Nfil*3). **Table 2** lists the ontology results of the informatics analysis with GeneSpring of the genes that were downregulated by forced swimming and that were prevented from exhibiting

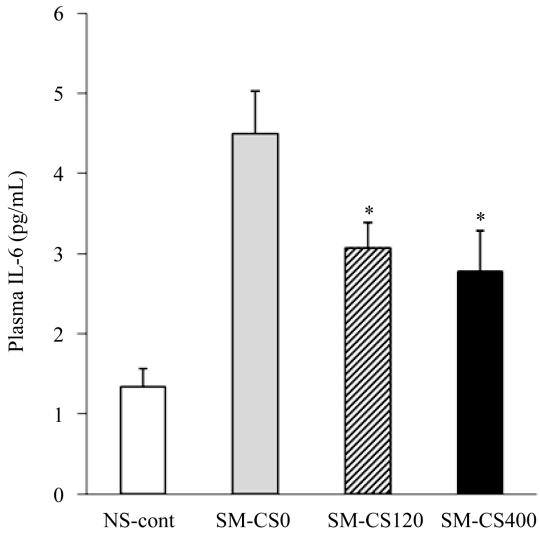

Figure 2. Effects of the Cordyceps (CS) extract on the plasma IL-6 levels in forced swimming mice. NS-cont: 0 mg/kg CS without swimming (no swimming control); SM-CS0: 0 mg/kg CS (forced swimming control); SM-CS120: 120 mg/kg CS; SM-CS400: 400 mg/kg CS administration. The data were expressed as the means ± SD (n = 10). *p < 0.05 compared with forced swimming control.

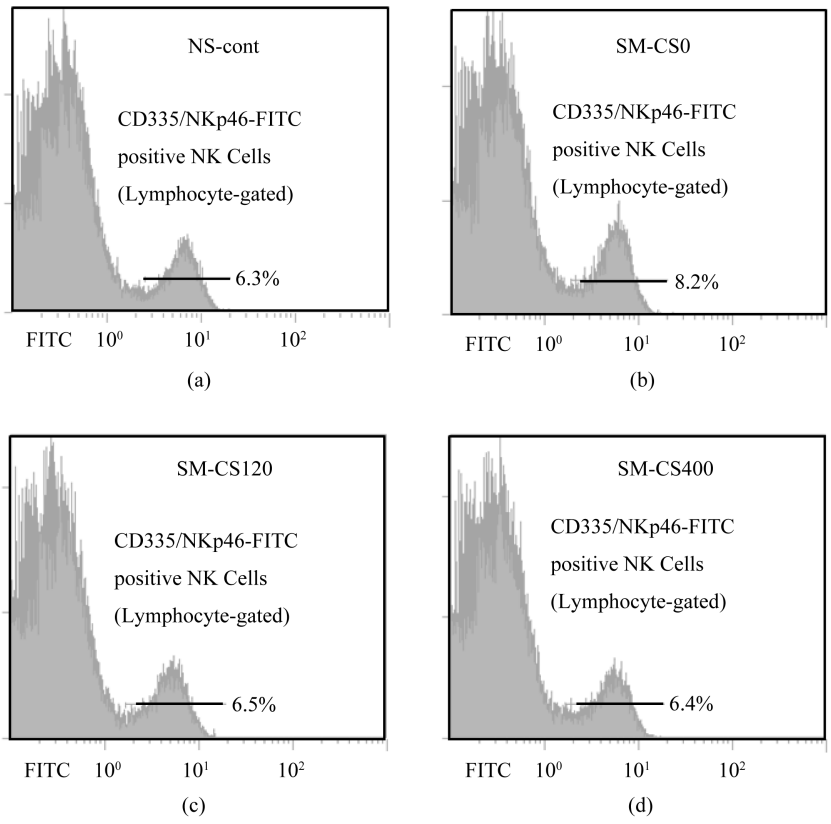

Figure 3. Effects of the Condyceps (CS) extract on the activation of splenic NK cells in forced swimming mice. The single-color (FITC) flow cytometric analysis on CD335/NKp46 positive splenic cells gated as lymphocytes by forward and side scatters. (a) NS-cont: 0 mg/kg CS without swimming (no swimming control); (b) SM-CS0: 0 mg/kg CS (forced swimming control); (c) SM-CS120: 120 mg/kg CS; (d) SM-CS400: 400 mg/kg CS administration. The number in each panel indicates the percentage of CD335/NKp46 positive cells in the lymphocyte-gated cells.

Table 1. List of genes that their expressions were upregulated (>2-fold) by forced swimming and alleviated with oral administration of *Cordyceps* (CS) extract.

Gene symbol	Gene name	Fold change[†]	
		SM-CS120[‡]	SM-CS400[§]
Ccl6	Chemokine (C-C motif) ligand 6	0.66	0.68
Ccl8	Chemokine (C-C motif) ligand 8	0.26	0.27
Wfdc17	WAP four-disulfide core domain 17	0.33	0.45
Ly86	Lymphocyte antigen 86	0.54	0.80
Plac8	Placenta-specific 8	0.41	0.42
Sbsn	Suprabasin	0.55	0.73
Sfrp4	Secreted frizzled-related protein 4	0.58	0.72
Nfil3	Nuclear factor	0.86	0.72

[†]Fold change was expressed as ratio against SM-CS0 (forced-swimming control); [‡]SM-CS120: 120 mg/kg oral administration of *Cordyceps* extract; [§]SM-CS400: 400 mg/kg oral administration of *Cordyceps* extract.

Table 2. List of genes that their expressions were downregulated (>2-fold) by forced swimming and alleviated with oral administration of *Cordyceps* (CS) extract.

Gene symbol	Gene name	Fold change[†]	
		SM-CS120[‡]	SM-CS400[§]
Sox7	SRY-box containing gene 7	1.86	1.13
Sox18	SRY-box containing gene 18	1.47	1.27
Socs1	Suppressor of cytokine signaling 1	1.64	1.87
Svs5	Seminal vesicle secretory protein 5	4.20	3.59

[†]Fold change was expressed as ratio against SM-CS0 (forced-swimming control); [‡]SM-CS120: 120 mg/kg oral administration of *Cordyceps* extract; [§]SM-CS400: 400 mg/kg oral administration of *Cordyceps* extract.

downregulation by the forced swimming test when treated with CS extract, including 3 genes that are involved in muscle development/mechanical stretch (*Sox*7, *Sox*18, and *Socs*1) and one gene related to actin binding in muscles (*Svs*5).

3.4.2. Real-Time RT-PCR Analysis

In order to confirm the DNA microarray results, several genes were selected and subjected to quantitative gene expression analysis with real time RT-PCR. **Figure 4** and **Figure 5** show the RT-PCR results. CS extract prevented the forced swimming-mediated upregulation in the expression of 5 genes (*Ccl*6, *Ccl*8, *Wfdc*17, *Nfil*3, and *Sfrp*4) (**Figure 4**) and the downregulation in the expression of the *Svs*5 gene (**Figure 5**). Little change in the expression of these 6 genes was observed in quadriceps femoris muscles in the NS-CS400 group (no swimming mice group treated with CS extract), suggesting that above mentioned alleviating effect on altered gene expression is specific antifatigue action of the CS extract.

4. Discussion

It is difficult in modern medicine to treat complicated phenomena, such as fatigue and other mild malfunctions, and people have much interest in the potential alternatives of functional food or traditional herbs to prevent these complicated events and promote healthy conditions [4] [5]. Because information on the action mechanisms, especially the *in vivo* molecular mechanisms, of medicinal food is limited, biomedical studies of these alternatives are needed.

Fatigue can be described as a time-dependent exercise-induced reduction in the maximal force-generating capacity of a muscle [2]. Performance in sports, work, and other activities is influenced by fatigue-related factors,

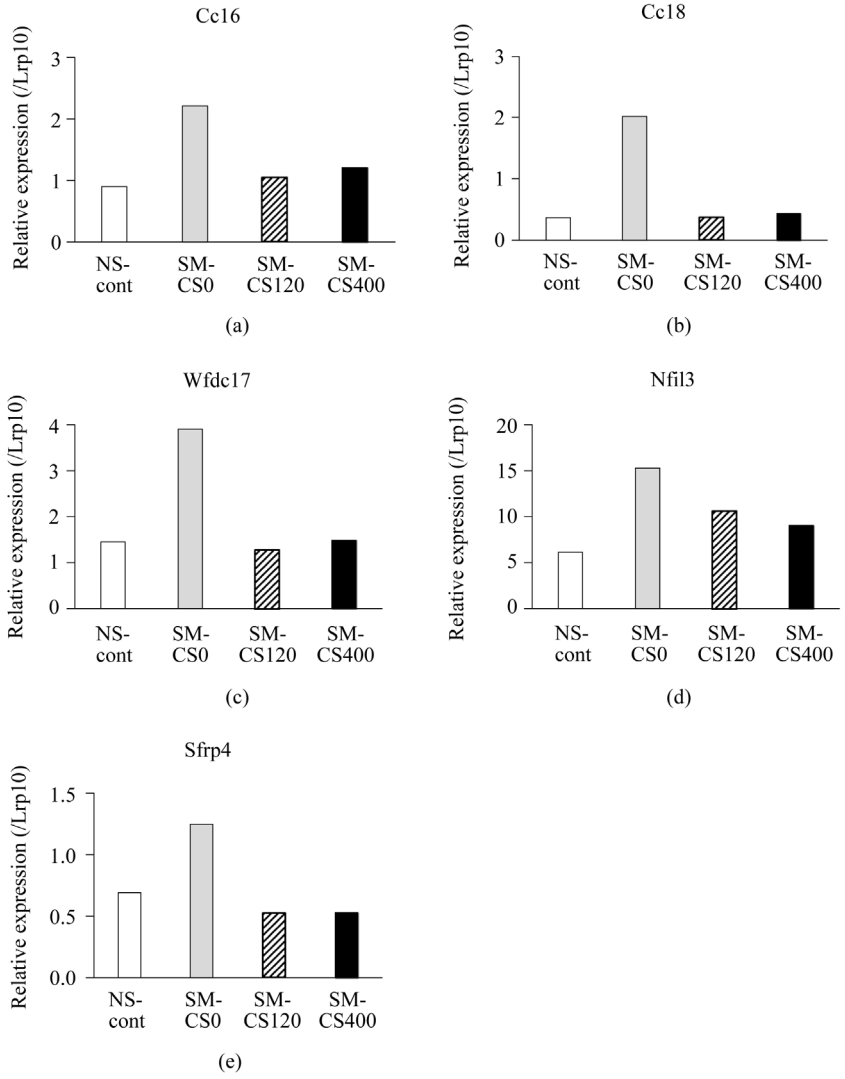

Figure 4. Quantitative gene expression analysis by real-time PCR. Confirmation of alleviating effects of *Cordyceps* (CS) extract on the forced swimming-mediated upregulation of gene expression. NS-cont: 0 mg/kg CS without swimming (no swimming control); SM-CS0: 0 mg/kg CS (forced swimming control); SM-CS120: 120 mg/kg CS; SM-CS400: 400 mg/kg CS administration. (a) *Ccl*6; (b) *Ccl*8; (c) *Wfdc*17; (d) *Nfil*3; (e) *Sfrp*4 genes. The data were expressed as the relative expression against the reference gene (*Lrp*10).

such as decreased muscular power and endurance, decreased motor skill performance, and mental lapses. Fatigue is also related to altered immunity of cell-mediated immune responses that are associated with T-cells and NK cells [23] [24]. *Cordyceps* (*Cordyceps sinensis*, CS) is a traditional Chinese medicine that has multiple effects, and antifatigue actions of its wild and cultured products have been suggested [15]-[17]. In the current study, we confirmed the antifatigue effects of the CS culture extract in forced swimming mice, a fatigue model that is induced by acute muscle exercise.

CS extract significantly extended the loaded swimming times (**Figure 1**), indicating its potent antifatigue effects. Exercise-induced muscle fatigue is known to trigger an inflammatory reaction and to produce IL-6, which is a proinflammatory cytokine and an accurate biomarker of muscle fatigue [3]. The CS extract prevented the muscle fatigue-mediated increase in plasma IL-6 levels at both doses of 120 mg/kg and 400 mg/kg (**Figure 2**). Because IL-6 levels increase in response to muscle contractions [25] [26], and stimulate immune responses to trauma or tissue damage, leading to muscle inflammation, the CS extract demonstrates antifatigue effects in the

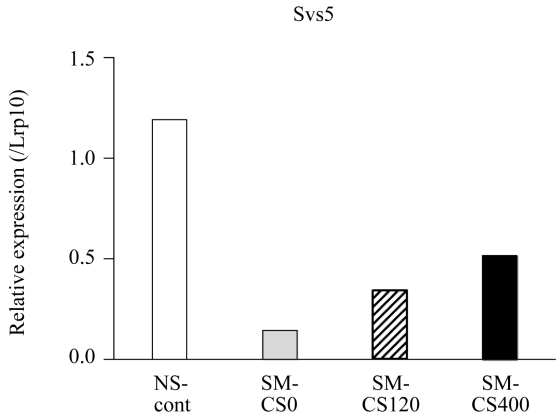

Figure 5. Quantitative gene expression analysis by real-time PCR. Confirmation of alleviating effects of *Cordyceps* (CS) extract on the forced swimming-mediated downregulation of gene expression. NS-cont: 0 mg/kg CS without swimming (no swimming control); SM-CS0: 0 mg/kg CS (forced swimming control); SM-CS120: 120 mg/kg CS; SM-CS400: 400 mg/kg CS administration. The data of *Svs*5 gene were expressed as the relative expression against the reference gene (*Lrp*10).

regulation of IL-6 production by preventing muscle dysfunction or damage.

NK cells are cytotoxic lymphocytes that belong to the innate immune system [27]. NK cells are present in lymphoid organs, including the spleen, and are involved in killing and/or the lysis of cancer and virus-infected cells [28] [29]. NKp46 is a member of the killer/lysis receptors that are expressed in activated NK cells [30] [31]. Previous studies have suggested that exercise or fatigue is associated with NK cells [32] [33]. We observed a muscle fatigue-induced increase in NKp46-positive NK cells, which are activated NK cells, in splenic cells by flow cytometry (**Figure 3**). Interestingly, CS extract suppressed the fatigue-induced increase in activated NK cells at both the low and high doses. These results indicated the protective effects of the CS extract on the hyper-reactivity of NK cells that arise in fatigue. The hyper-reactivity of NK cells is involved in the development of type-1 diabetes [34], vascular inflammation and myocardial infarction [35], and brain infarction [36]. These regulating effects of CS extract on NK cell activation are beneficial in depressing fatigue-related inflammatory dysfunction in immune tissues or organs.

DNA microarray that was followed by quantitative real time PCR provided previously unknown information concerning target genes for the antifatigue actions of the CS extract (**Table 1** and **Table 2**). Among the genes that were upregulated by muscle fatigue in the quadriceps femoris muscle, 3 genes (*Ccl*6, *Ccl*8, and *Wfdc*17) participate in muscle inflammation [37]-[39] and 2 genes (*Nfil*3 and *Sfrp*4) are associated with muscle regeneration [40] [41]. One gene (*Svs*5) that was downregulated by muscle fatigue is involved in muscle actin binding [42] [43]. Characteristically, the CS extract demonstrated an ability to prevent the altered expression of these genes (**Figure 4** and **Figure 5**), which occurs in response to muscle fatigue. Thus, the antifatigue effects of CS extract were exerted by modulating the altered transcriptional events in genes that are associated with muscle damage, regeneration, and/or repair in the local muscle.

5. Conclusion

In conclusion, invaluable information on the molecular mechanisms underlying the *in vivo* antifatigue actions of the extract of the cultured *Cordyceps* mycelium (*Paecilomyces hepiali*, CBG-CS-2) was revealed in this study. The antifatigue effects of the CS extract occurred through the anti-inflammatory modulation of IL-6 accumulation in the blood, NK cell activation in the spleen, and gene expression in muscles.

Conflict of Interests

The authors declare that they have no competing interests.

Acknowledgements

This study was financially supported by a contract with the Ministry of Agriculture, Food and Rural Affairs (MAFRA)/Korea National Food Cluster (FOODPOLIS to S-WC). The authors thank Dr. Dong-Chool Kim, President, TTC Korea Co., Ltd. for his kind support.

References

[1] Gandevia, S.C. (2001) Spinal and Supraspinal Factors in Human Muscle Fatigue. *Physiological Reviews*, **81**, 1725-1789.

[2] Bogdanis, G.C. (2012) Effect of Physical Activity and Inactivity on Muscle Fatigue. *Frontiers in Physiology*, **3**, 142. http://dx.doi.org/10.3389/fphys.2012.00142

[3] Finsterer, J. (2012) Biomarkers of Peripheral Muscle Fatigue during Exercise. *BMC Musculoskeletal Disorders*, **13**, 218. http://dx.doi.org/10.1186/1471-2474-13-218

[4] Ren, J., Zhao, M., Wang, H., Cui, C. and You, L. (2011) Effects of Supplementation with Grass Carp Protein versus Peptide on Swimming Endurance in Mice. *Nutrition*, **27**, 789-795. http://dx.doi.org/10.1016/j.nut.2010.08.020

[5] Shao, J.T., Wang, M.Y. and Zheng, L.B. (2013) Antifatigue Effect of *Gracilaria eucheumoides* in Mice. *Experimental and Therapeutic Medicine*, **6**, 1512-1516.

[6] Paterson, R.R. (2008) *Cordyceps*: A Traditional Chinese Medicine and another Fungal Therapeutic Biofactory? *Phytochemistry*, **69**, 1469-1495. http://dx.doi.org/10.1016/j.phytochem.2008.01.027

[7] Zhou, X., Gong, Z., Su, Y., Lin, J. and Tang, K. (2009) *Cordyceps* Fungi: Natural Products, Pharmacological Functions and Developmental Products. *The Journal of Pharmacy and Pharmacology*, **61**, 279-291. http://dx.doi.org/10.1211/jpp.61.03.0002

[8] Shin, S., Kwon, J., Lee, S., Kong, H., Lee, S., Lee, C.K., *et al*. (2010) Immunostimulatory Effects of *Cordyceps militaris* on Macrophages through the Enhanced Production of Cytokines via the Activation of NF-*κ*B. *Immune Network*, **10**, 55-63. http://dx.doi.org/10.4110/in.2010.10.2.55

[9] Li, S.P., Yang, F.Q. and Tsim, K.W.K. (2006) Quality Control of *Cordyceps sinensis*, a Valued Traditional Chinese Medicine. *Journal of Pharmaceutical and Biomedical Analysis*, **41**, 1571-1584. http://dx.doi.org/10.1016/j.jpba.2006.01.046

[10] Yue, K., Ye, M., Zhou, Z., Sun, W. and Lin, X. (2013) The genus *Cordyceps*: A Chemical and Pharmacological Review. *The Journal of Pharmacy and Pharmacology*, **65**, 474-493. http://dx.doi.org/10.1111/j.2042-7158.2012.01601.x

[11] Kim, H.O. and Yun, J.W. (2005) A Comparative Study on the Production of Exopolysaccharides between Two Entomopathogenic Fungi *Cordyceps militaris* and *Cordyceps sinensis* in Submerged Mycelial Cultures. *Journal of Applied Microbiology*, **99**, 728-738. http://dx.doi.org/10.1111/j.1365-2672.2005.02682.x

[12] Xu, C.P., Sinha, J., Bae, J.T., Kim, S.W. and Yun, J.W. (2006) Optimization of Physical Parameters for Exo-Biopolymer Production in Submerged Mycelial Cultures of Two Entomopathogenic Fungi *Paecilomyces japonica* and *Paecilomyces tenuipes*. *Letters in Applied Microbiology*, **42**, 501-506. http://dx.doi.org/10.1111/j.1472-765X.2006.01884.x

[13] Lo, H.C., Hsu, T.H., Tu, S.T. and Lin, K.C. (2006) Anti-Hyperglycemic Activity of Natural and Fermented *Cordyceps sinensis* in Rats with Diabetes Induced by Nicotinamide and Streptozotocin. *The American Journal of Chinese Medicine*, **34**, 819-832. http://dx.doi.org/10.1142/S0192415X06004314

[14] Xiao, J.H., Li, Y., Xiao, Y. and Zhong, J.J. (2013) Advance and Prospect of Studies on Bioactivity and Mechanism of *Cordyceps* Fungi. *Zhongguo Zhong Yao Za Zhi*, **38**, 640-647.

[15] Yan, W., Li, T., Lao, J., Song, B. and Shen, Y. (2013) Anti-Fatigue Property of *Cordyceps guangdongensis* and the Underlying Mechanisms. *Pharmaceutical Biology*, **51**, 614-620. http://dx.doi.org/10.3109/13880209.2012.760103

[16] Koh, J.H., Kim, K.M., Kim, J.M., Song, J.C. and Suh, H.J. (2003) Antifatigue and Antistress Effect of the Hot-Water Fraction from Mycelia of *Cordyceps sinensis*. *Biological and Pharmaceutical Bulletin*, **26**, 691-694. http://dx.doi.org/10.1248/bpb.26.691

[17] Kumar, R., Negi, P.S., Singh, B., Ilavazhagan, G., Bhargava, K. and Sethy, N.K. (2011) *Cordyceps sinensis* Promotes Exercise Endurance Capacity of Rats by Activating Skeletal Muscle Metabolic Regulators. *Journal of Ethnopharmacology*, **136**, 260-266. http://dx.doi.org/10.1016/j.jep.2011.04.040

[18] Jung, K.A., Han, D., Kwon, E.K., Lee, C.H. and Kim, Y.E. (2007) Antifatigue Effect of *Rubus coreanus* Miquel Extract in Mice. *Journal of Medicinal Food*, **10**, 689-693. http://dx.doi.org/10.1089/jmf.2006.006

[19] Zhang, X.L., Ren, F., Huang, W., Ding, R.T., Zhou, Q.S. and Liu, X.W. (2010) Anti-Fatigue Activity of Extracts of Stem Bark from *Acanthopanax senticosus*. *Molecules*, **16**, 28-37. http://dx.doi.org/10.3390/molecules16010028

[20] Jeong, A.R., Nakamura, S. and Mitsunaga, F. (2008) Gene Expression Profile of Th1 and Th2 Cytokines and Their Receptors in Human and Nonhuman Primates. *Journal of Medical Primatology*, **37**, 290-296.

[21] Shippy, R., Fulmer-Smentek, S., Jensen, R.V., Jones, W.D., Wolber, P.K., Johnson, C.D., *et al*. (2006) Using RNA Sample Titrations to Assess Microarray Platform Performance and Normalization Techniques. *Nature Biotechnology*, **24**, 1123-1131. http://dx.doi.org/10.1038/nbt1241

[22] Gabrielsson, B.G., Olofsson, L.E., Sjögren, A., Jernås, M., Elander, A., Lönn, M., *et al*. (2005) Evaluation of Reference Genes for Studies of Gene Expression in Human Adipose Tissue. *Obesity Research*, **13**, 649-652. http://dx.doi.org/10.1038/oby.2005.72

[23] Maes, M., Twisk, F.N., Kubera, M. and Ringel, K. (2012) Evidence for Inflammation and Activation of Cell-Mediated Immunity in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS): Increased Interleukin-1, Tumor Necrosis Factor-α, PMN-Elastase, Lysozyme and Neopterin. *Journal of Affective Disorders*, **136**, 933-939. http://dx.doi.org/10.1016/j.jad.2011.09.004

[24] Curriu, M., Carrillo, J., Massanella, M., Rigau, J., Alegre, J., Puig, J., *et al*. (2013) Screening NK-, B- and T-Cell Phenotype and Function in Patients Suffering from Chronic Fatigue Syndrome. *Journal of Translational Medicine*, **11**, 68. http://dx.doi.org/10.1186/1479-5876-11-68

[25] Febbraio, M.A. and Pedersen, B.K. (2005) Contraction-Induced Myokine Production and Release: Is Skeletal Muscle an Endocrine Organ? *Exercise & Sport Sciences Reviews*, **33**, 114-119. http://dx.doi.org/10.1097/00003677-200507000-00003

[26] Pedersen, B.K. (2007) IL-6 Signaling in Exercise and Disease. *Biochemical Society Transactions*, **35**, 1295-1297. http://dx.doi.org/10.1042/BST0351295

[27] Moretta, A., Bottino, C., Mingari, M.C., Biassoni, R. and Moretta, L. (2002) What Is a Natural Killer Cell? *Nature Immunology*, **3**, 6-8. http://dx.doi.org/10.1038/ni0102-6

[28] Degli-Esposti, M.A. and Smyth, M.J. (2005) Close Encounters of Different Kinds: Dendritic Cells and NK Cells Take Centre Stage. *Nature Reviews Immunology*, **5**, 112-124. http://dx.doi.org/10.1038/nri1549

[29] Stewart, C.A., Vivier, E. and Colonna, M. (2006) Strategies of Natural Killer Cell Recognition and Signaling. *Current Topics in Microbiology and Immunology*, **298**, 1-21. http://dx.doi.org/10.1007/3-540-27743-9_1

[30] Mandelboim, O. and Porgador, A. (2001) NKp46. *The International Journal of Biochemistry & Cell Biology*, **33**, 1147-1150. http://dx.doi.org/10.1016/S1357-2725(01)00078-4

[31] Vankayalapati, R., Garg, A., Porgador, A., Griffith, D.E., Klucar, P., Safi, H., *et al*. (2005) Role of NK Cell-Activating Receptors and Their Ligands in the Lysis of Mononuclear Phagocytes Infected with an Intracellular Bacterium. *Journal of Immunology*, **175**, 4611-4617. http://dx.doi.org/10.4049/jimmunol.175.7.4611

[32] Jonsdottir, I.H. (2000) Exercise Immunology: Neuroendocrine Regulation of NK-Cells. *International Journal of Sports Medicine*, **21**, 20-23. http://dx.doi.org/10.1055/s-2000-1447

[33] Wang, J.S. and Weng, T.P. (2011) Hypoxic Exercise Training Promotes Antitumour Cytotoxicity of Natural Killer Cells in Young Men. *Clinical Science* (*London*), **121**, 343-353. http://dx.doi.org/10.1042/CS20110032

[34] Gur, C., Porgador, A., Elboim, M., Gazit, R., Mizrahi, S., Stern-Ginossar, N., *et al*. (2010) The Activating Receptor NKp46 Is Essential for the Development of Type 1 Diabetes. *Nature Immunology*, **11**, 121-128. http://dx.doi.org/10.1038/ni.1834

[35] Knorr, M., Münzel, T. and Wenzel, P. (2014) Interplay of NK Cells and Monocytes in Vascular Inflammation and Myocardial Infarction. *Frontiers in Physiology*, **5**, 295. http://dx.doi.org/10.3389/fphys.2014.00295

[36] Gan, Y., Liu, Q., Wu, W., Yin, J.X., Bai, X.F., Shen, R., *et al*. (2014) Ischemic Neurons Recruit Natural Killer Cells that Accelerate Brain Infarction. *Proceedings of the National Academy of Sciences of the United States of America*, **111**, 2704-2709. http://dx.doi.org/10.1073/pnas.1315943111

[37] Porter, J.D., Guo, W., Merriam, A.P., Khanna, S., Cheng, G., Zhou, X., *et al*. (2003) Persistent Over-Expression of Specific CC Class Chemokines Correlates with Macrophage and T-Cell Recruitment in *mdx* Skeletal Muscle. *Neuromuscular Disorders*, **13**, 223-235. http://dx.doi.org/10.1016/s0960-8966(02)00242-0

[38] Henningsen, J., Pedersen, B.K. and Kratchmarova, I. (2011) Quantitative Analysis of the Secretion of the MCP Family of Chemokines by Muscle Cells. *Molecular BioSystems*, **7**, 311-321. http://dx.doi.org/10.1039/c0mb00209g

[39] Ferreira, Z., Seixas, S., Andrés, A.M., Kretzschmar, W.W., Mullikin, J.C., Cherukuri, P.F., *et al*. (2013) Reproduction and Immunity-Driven Natural Selection in the Human WFDC Locus. *Molecular Biology and Evolution*, **30**, 938-950. http://dx.doi.org/10.1093/molbev/mss329

[40] Zhao, P. and Hoffman, E.P. (2004) Embryonic Myogenesis Pathways in Muscle Regeneration. *Developmental Dynamics*, **229**, 380-392. http://dx.doi.org/10.1002/dvdy.10457

[41] Tamboli, R.A., Hajri, T., Jiang, A., Marks-Shulman, P.A., Williams, D.B., Clements, R.H., *et al*. (2011) Reduction in

Inflammatory Gene Expression in Skeletal Muscle from Roux-en-Y Gastric Bypass Patients Randomized to Omentectomy. *PLoS ONE*, **6**, e28577. http://dx.doi.org/10.1371/journal.pone.0028577

[42] Seitz, J. and Aumüller, G. (1990) Biochemical Properties of Secretory Proteins from Rat Seminal Vesicles: Biochemische Eigenschaften sekretorischer Proteine der Ratten-Bläschendrüse. *Andrologia*, **22**, 25-32. http://dx.doi.org/10.1111/j.1439-0272.1990.tb02068.x

[43] Seitz, J., Keppler, C., Aumüller, G., Polzar, B. and Mannherz, H.G. (1992) SVS II—An Androgen-Dependent Actin-Binding Glycoprotein in Rat Semen. *European Journal of Cell Biology*, **57**, 308-316.

Scientific Research Publishing (SCIRP) is one of the largest Open Access journal publishers. It is currently publishing more than 200 open access, online, peer-reviewed journals covering a wide range of academic disciplines. SCIRP serves the worldwide academic communities and contributes to the progress and application of science with its publication.

Other selected journals from SCIRP are listed as below. Submit your manuscript to us via either submit@scirp.org or Online Submission Portal.