panel) in growth medium. pRJ treatment at high concentrations (500 and 1000 μg/mL) for 48 and 72 hours increased the proliferation rate of the satellite cells compared with controls and RJ treatment (Figure 2A). Next, to examine the effect of RJ/pRJ on the differentiation of the satellite cells of the aged mice in vitro, we cultured the satellite cells in differentiation medium for 5 days and immunohistochemically stained them for eMyHC (Figure 2B). The pRJ-treated group had more eMyHc immunoreactive areas than did the controls (Figure 2B, upper panel). The mean maximum diameter of the myotubes was greater in the pRJ-treated group than in the controls (Figure 2, lower panel). These results suggested that pRJ promoted the differentiation of the satellite cells of the aged mice. We next examined an intracellular signaling pathway of IGF-1 by Western blot analysis. pRJ treatment increased the intensity of the band of IGF-1R compared with controls (Figure 2C). One of the downstream signaling pathways of IGF-1R is Akt, and pRJ treatment increased the intensity of the band of phosphorylated Akt, which is an activated form of Akt, compared with controls (Figure 2C). Similar to pRJ, the increased activation of Akt was also observed in satellite cells treated with 10 nM insulin (Figure 2C). These results suggested that pRJ increased the proliferation rate, promoted differentiation, and activated the Akt-signaling pathway in the satellite cells from the aged mice compared with the controls in vitro.

RJ-/pRJ-Treated Mice Had Greater Numbers of Satellite Cells, Muscle Weight, and Grip Strength Than Did Controls

To examine the effects of RJ/pRJ treatment on aged mice in vivo, we divided 21-month-old mice into five groups and treated them with five kinds of diets for 3 months, respectively: normal diet (controls), diet mixed with 1% weight RJ (1% RJ), diet with 5% weight RJ (5% RJ), diet with 1% weight pRJ (1% pRJ), and diet with 5% pRJ (5% pRJ). Three mice in the controls, one mouse in the 1% RJ group, and one mouse in the 1% pRJ group died of natural causes during the treatment period. These mice were excluded from the analysis. During the intervention period, the body weight increased similarly in RJ/pRJ-treated groups and control groups (Figure 3A) (p value > .73; effect size ≤ 0.01). The amount of daily diet intake was not different between the groups (Figure 3B). Comparison of the hind-limb muscle weight per body weight between 2-, 8-, and 24-month-old mice showed progressive loss of muscle weight with aging, suggesting the progression of sarcopenia with aging (Figure 3C). The combined weights of the hind-limb muscles of one leg, named one-legged muscle, per body weight in 5% RJ, 1% pRJ, and 5% pRJ groups were greater than those of controls (Figure 3D). The selected muscles included the TA, triceps surae, quadriceps, biceps femoris, gluteus maximus, and iliopsoas muscles. To examine the effect of PJ/pRJ on the numbers of satellite cells in vivo, we counted the cells. The numbers of satellite cells in the hind-limb muscles in the 5% RJ- and 5% pRJ-treated groups were significantly greater than those of the controls (Figure 3E), whereas the numbers of the satellite cells per muscle weight were not different among the groups (per gram; Figure 3F). These results suggested that PJ/pRJ treatment increased the total numbers of satellite cells.

To examine the effect of pRJ on the muscle strength, we performed the wire hang test and measured the maximum duration that the mice could hang on the inverted wire mesh. Consistent with the effect of RJ/pRJ on the muscle mass, the 5% RJ- and 5% pRJ-treated groups hung for longer duration than did the controls, suggesting that RJ/pRJ improved the grip strength of the skeletal muscles (Figure 3G). To examine the effect of RJ/pRJ on muscle fatigue, we measured how many times the mice could hang from the wire mesh. The 5% RJ- and 5% pRJ-treated groups could hang more times than the controls, suggesting that RJ/pRJ improved the fatigue of the skeletal muscles (Figure 3H). Furthermore, within the controls, comparison of the before and after treatment period showed decreased hanging duration and times after the treatment period than before, suggesting the progression of age-related atrophy in muscle function. In contrast, no significant changes were observed within the RJ/pRJ groups between before and after the treatment period. These data suggested that RJ/pRJ treatment prevented the progression of atrophy in muscle weight and function in the aged mice.

RJ/pRJ Treatment Accelerated the Regeneration of Injured Skeletal Muscles

We next examined the effect of RJ/pRJ treatment on the regenerating capacity of the skeletal muscles in aged mice in vivo by injuring the TA muscles with cardiotoxin injection and observing their regeneration. We isolated the muscles 5 days after the cardiotoxin injection and subjected them to staining. Hematoxylin and eosin staining showed greater amounts of muscle fibers in the RJ/pRJ groups than in the controls (Figure 4A, upper panels). To confirm the regenerating capacity of the skeletal muscles, we immunohistochemically stained the muscles for eMyHc, which is a marker of immature myotubes including regenerating muscles but not of mature muscles (Figure 4A, middle line panels). Quantification of the eMyHc immunoreactive area showed greater immunoreactive areas in the RJ/pRJ groups than in the controls (Figure 4B). These results suggested that RJ/pRJ treatment accelerated the regeneration of the injured skeletal muscles.

RJ/pRJ Treatment Increased Serum IGF-1 Levels

Because RJ has been suggested to have an anti-inflammatory effect, we examined the effect of RJ/pRJ treatment on serum proinflammatory mediator levels in the aged mice.
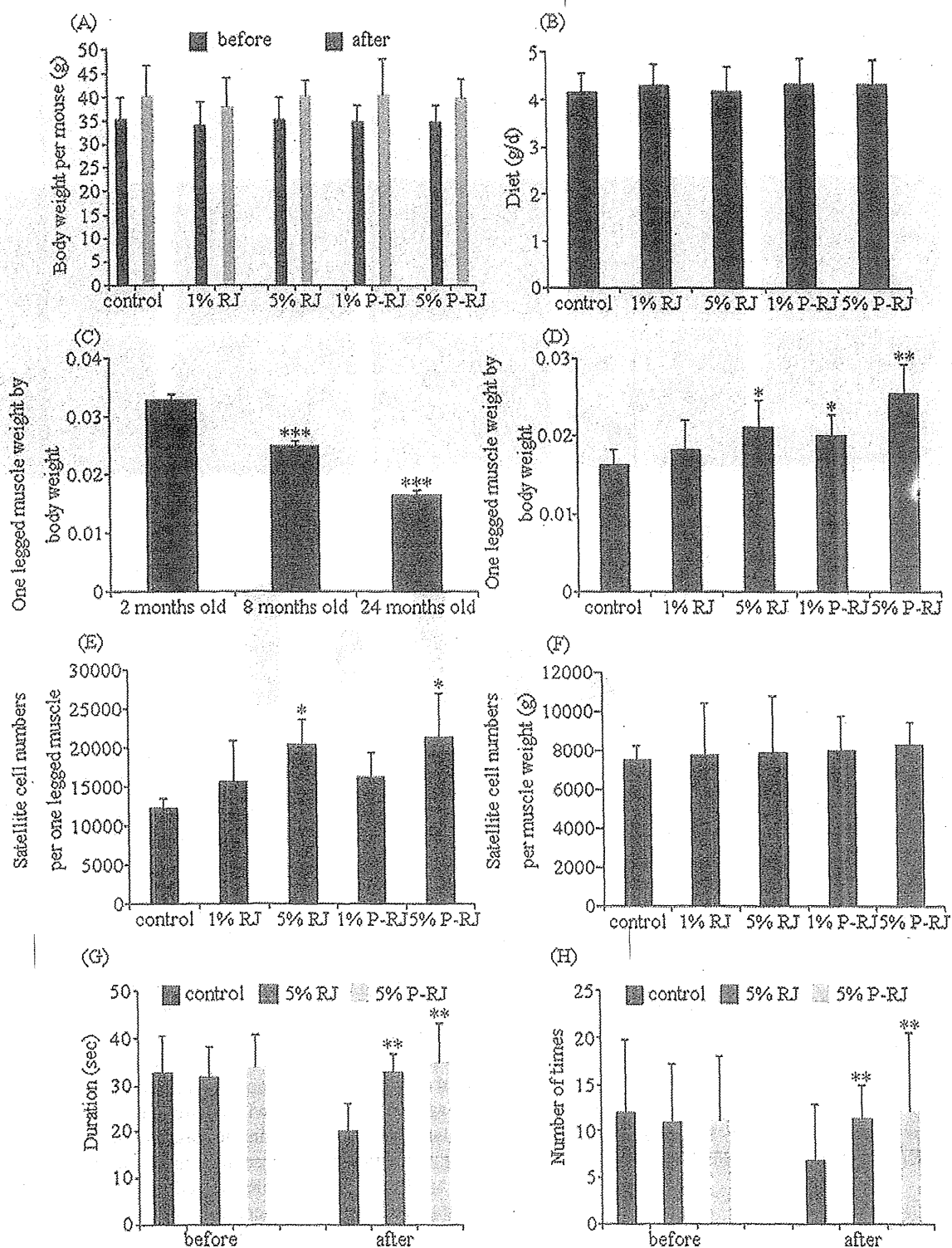
Figure 3. Effects of RJ/pRJ treatment on aged mice in vivo. Twenty-one-month-old mice were treated with a diet mixed with 1% weight RJ (1% RJ), diet with 5% weight RJ (5% RJ), diet with 1% weight pRJ (1% pRJ), or diet with 5% pRJ (5% pRJ) for following 3 months. (A) Control, RJ, or pRJ treatment did not show changed body weight. (B) RJ or pRJ did not change the amount of the daily diet intake. (C) The progressive loss of muscle weight with aging. (D) RJ- and pRJ-treated groups had greater hind-limb muscle weights per body weight than did controls. (E) RJ- and pRJ-treated groups had greater numbers of satellite cells in the hind-limb muscles than did controls. (F) RJ or pRJ treatment did not change the numbers of satellite cells per muscle weight (g). (G) Five% RJ- and pRJ-treated mice hung for longer durations than did controls. (H) Five% RJ- and pRJ-treated mice hung more times than did controls. Columns are mean ± SD, $n \geq 5$ in each group. $*p < .05$, $**p < .01$, and $***p \leq .0001$ compared with control.
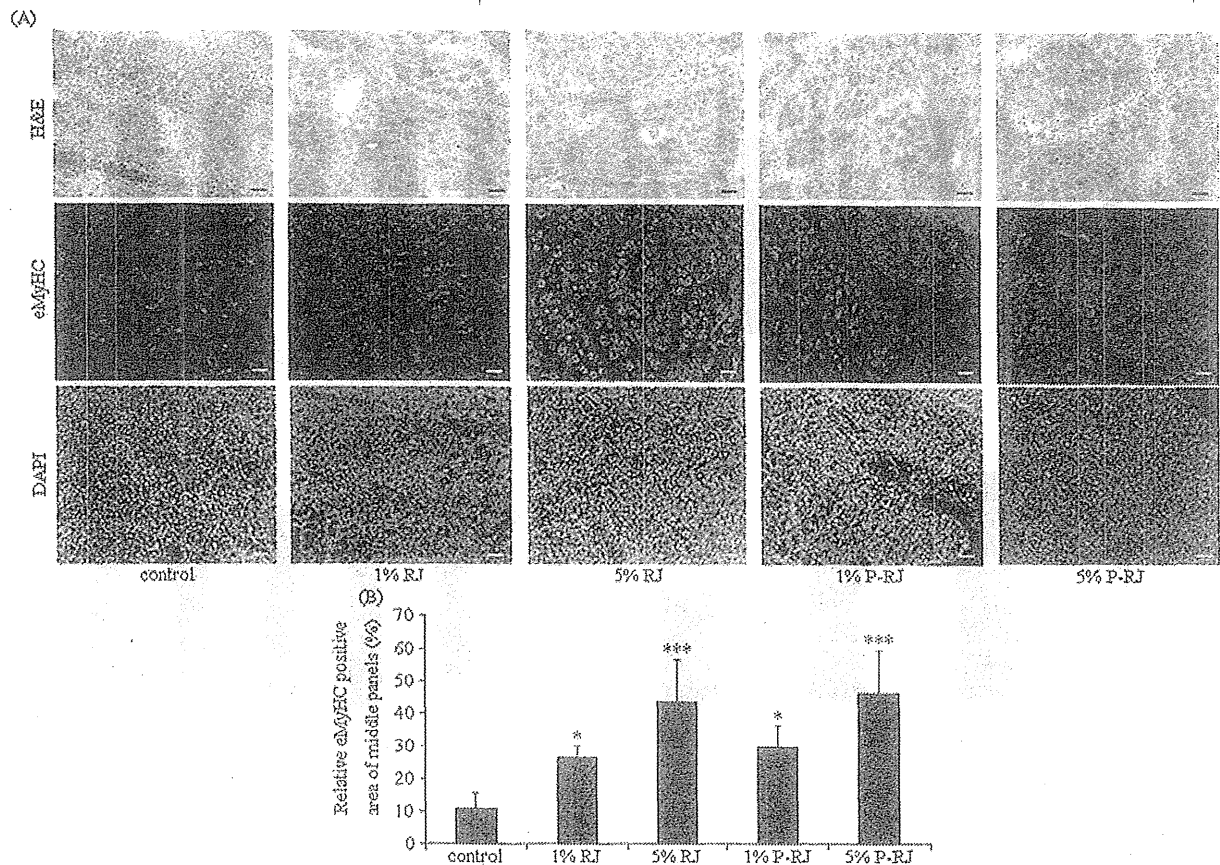
(A)

(B)

Figure 4. RJ/pRJ treatment accelerated the regeneration of the injured skeletal muscles in aged mice. After 3 months of RJ/pRJ treatment, we injected cardiotoxin into the tibialis anterior muscles of the aged mice to injure the muscles and isolated them 5 days later. (A) Hematoxylin and eosin staining (top panels) and immunohistochemical staining for eMyHC (middle panels) or DAPI (bottom panels) of the injured tibialis anterior muscles. Upper lines, scale bar, 50 μm; middle and lower lines, scale bar, 100 μm. (B) The graph shows the data calculated from quantification of the percentage of eMyHc-immunoreactive area per field for each group (10 randomly selected fields at ×200 magnification per sample were quantified). Columns are mean ± SD, $n ≥ 3$ in each group. $*p < .05$, and $***p < .0001$ compared with controls.

We chose IL-1α, IL-1β, IL-6, and tumor necrosis factor-α as proinflammatory mediators, as previously shown (31,32), and measured their levels in serum. The levels of these mediators were not significantly different between RJ-/pRJ-treated groups and controls, but the serum IL-1α concentration tended to be lower in the RJ/pRJ groups than in the controls (Figure 5A). Because IGF-1 plays a central role in stimulating satellite cells, we measured the serum levels of IGF-1. The serum levels of IGF-1 were greater in the 5% RJ- and pRJ-treated groups than in the controls (Figure 5B).

DISCUSSION

In this study, using aged mice, we showed that RJ/pRJ treatment increased the number of satellite cells, the skeletal muscle weight, grip strength, regenerating capacity of injured skeletal muscles, and the serum IGF-1 levels compared with controls in vivo. In vitro, compared with controls, pRJ treatment increased the cell proliferation rate,

promoted differentiation, and activated the Akt-signaling pathway in the satellite cells of the aged mice.

RJ/pRJ treatment increased the number of satellite cells of the aged mice, promoted their differentiation compared with controls, which could be the mechanisms by which the skeletal muscle weight and grip strength were increased, and accelerated the regeneration of injured skeletal muscles in aged mice compared with controls. Because these effects antagonized the loss of muscle mass and strength, the results suggested that RJ/pRJ treatment might improve sarcopenia in aged mice. The RJ-/pRJ-treated groups hung for longer durations than did the controls, but when we compared between before and after the treatment period within the same groups, the hanging duration did not change in the RJ-/pRJ-treated groups, whereas the hanging duration decreased after the same period in controls, suggesting that RJ/pRJ treatment might not improve but rather attenuated the progression of the decrease in grip strength. Therefore, the effects of RJ/pRJ on skeletal muscles might be
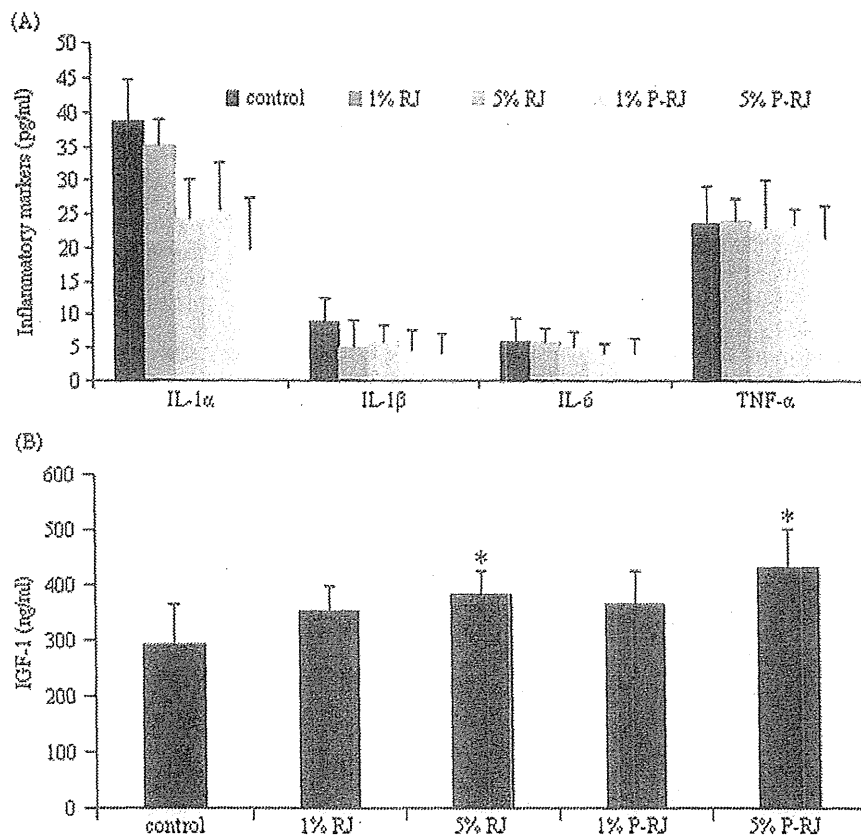
(A)

(B)

Figure 5. RJ/pRJ treatment increased the serum IGF-1 levels but did not affect the serum proinflammatory mediator levels. ELISA determined serum levels of proinflammatory mediators and IGF-1 in aged mice treated with RJ/pRJ for 3 months. (A) RJ/pRJ treatment did not change the serum levels of the proinflammatory mediators. (B) Five% RJ and 5% pRJ treatment significantly increased the serum IGF-1 levels. Columns are mean ± SD, n = 5 in each group. *p < .05 compared with controls.

attenuating the atrophy rather than improving the muscle mass and strength in aged mice.

pRJ increased the number of satellite cells of the aged mice in vivo and in vitro, whereas RJ increased the number of the satellite cells in vivo but not in vitro. The presence of protease treatment in pRJ and its absence in RJ might explain this discrepancy. Protease is present in vivo, which indicates that all the RJ is treated with protease after their intake in vivo, whereas protease is not present in vitro.

Because IGF-1 has favorable effects on satellite cells, the skeletal muscles, and sarcopenia, the increased serum levels of IGF-1 after RJ/pRJ treatment might be one of the mechanisms of the effects of RJ/pRJ treatment. However, the increases in the levels of IGF-1 after RJ/pRJ treatment were moderate. Therefore, RJ/pRJ treatment may have other mechanisms besides increasing IGF-1. Previous studies indicated that nutrition plays a central role in the regulation of the IGF-1 levels (33). The serum IGF-1 levels decline in an age-dependent manner and are a reliable index of protein-energy malnutrition in elderly patients (34–36). Increased serum levels of IGF-1 after RJ/pRJ treatment may suggest that RJ/pRJ treatment improved the malnutrition in the aged animals. Many nutritional components in RJ/pRJ

such as vitamins, minerals (Table 1), and amino acids might have contributed to preventing sarcopenia. Because this is a single study, we could not evaluate the contribution of each component to the prevention of sarcopenia. However, the results suggested that whole RJ/pRJ improved sarcopenia in aged mice.

Akt-signaling pathway plays a central role in muscle protein synthesis and in inhibiting muscle proteolysis. Akt activation prevents muscle atrophy including sarcopenia (37). Moreover, the activation of Akt in myoblasts increased their cell proliferation rate and rescued them from cell death (22). In vitro, pRJ activated the Akt-signaling pathway in satellite cells of the aged mice. Because pRJ contains a wide variety of components (9), it is not clear which component(s) activated Akt. However, the activation of Akt, possibly by IGF-1, suggests that one of the mechanisms of the effects of RJ/pRJ was via Akt. Furthermore, because RJ and pRJ are natural products, some natural factors such as seasonal or environmental factors may affect the percentage or quality of ingredients in RJ/pRJ. Further studies are required to identify the mechanisms of action of RJ/pRJ.

Some studies reported that IGF-1 deficiency extended life spans in mammals (38,39). Because we did not assess life

spans in this study, the effect of increased levels of IGF-1 by RJ/pRJ treatment on life span was not clear. However, previous studies reported that RJ/pRJ extended the life span in mice and *Caenorhabditis elegans* (10,11). Further studies are required to evaluate the effects and mechanisms of RJ/pRJ on life span.

Dietary supplementation with 1%–5% RJ/pRJ would be too great in an amount and would not be feasible for humans. Generally, dietary supplementation intake in animals cannot be directly converted into human dietary intake. Thus, we did a pilot study to examine the effect of RJ on muscle strength and physical performance in free-living elderly patients (Identifier: UMIN000004057, Trial Registration: http://www.umin.ac.jp/ctr/index.htm). We found that the intake of RJ (low dose: 1.2 g/day; high dose: 4.8 g/day) for 3 months improved muscle strength and physical performance in the elderly patients. Based on this pilot study, we are performing a randomized, double-blinded, placebo-controlled trial to confirm the effects of RJ on muscle strength and physical performance of the elderly patients (Identifier: UMIN000009648, Trial Registration: http://www.umin.ac.jp/ctr/index.htm).

In conclusion, in vivo, RJ/pRJ treatment increased the muscle weight, grip strength, regenerating capacity of injured muscles, and serum IGF-1 levels compared with controls in aged mice. In vitro, pRJ increased the cell proliferation rate, promoted the cell differentiation, and activated Akt-signaling pathway compared with controls in isolated satellite cells from aged mice. These findings suggest that RJ/pRJ treatment may have a beneficial effect on the prevention of age-related sarcopenia through increasing the systemic IGF-1 levels and activating Akt-signaling pathways in satellite cells.

CONFLICT OF INTERESTS

All the authors have no conflicts of interest to disclose.

REFERENCES
1. Nations United. *Department of Economic and Social Affairs Population Division: World Population Ageing 2009*. New York: United Nations; 2009:11.
2. Altun M, Grönholdt-Klein M, Wang L, Ulfhake B. Cellular degradation machineries in age-related loss of muscle mass (Sarcopenia). In: Nagata T, ed. *Senescence*. San Francisco, CA: Academia.edu; 2012:269–286.
3. Hawke TJ, Garry DJ. Myogenic satellite cells: physiology to molecular biology. *J Appl Physiol*. 2001;91:534–551.
4. Clemmons DR. Role of IGF-I in skeletal muscle mass maintenance. *Trends Endocrinol Metab*. 2009;20:349–356.
5. Welle S. Cellular and molecular basis of age-related sarcopenia. *Can J Appl Physiol*. 2002;27:19–41.
6. Sacco A, Doyonnas R, Kraft P, Vitorovic S, Blau HM. Self-renewal and expansion of single transplanted muscle stem cells. *Nature*. 2008;456:502–506.
7. Giovannini S, Marzetti E, Borst SE, Leeuwenburgh C. Modulation of GH/IGF-1 axis: potential strategies to counteract sarcopenia in older adult. *Mech Ageing Dev*. 2008;129:593–601.

8. Viuda-Martos M, Ruiz-Navajas Y, Fernández-López J, Pérez-Alvarez JA. Functional properties of honey, propolis, and royal jelly. *J Food Sci*. 2008;73:R117–R124.
9. Sabatini AG, Marcazzan GL, Caboni MF, Bogdanov S, Almeida-Muradian LBd. Quality and standardisation of Royal Jelly. *J ApiProduct ApiMedical Sci*. 2009;1:1–6.
10. Inoue S, Koya-Miyata S, Ushio S, Iwaki K, Ikeda M, Kurimoto M. Royal Jelly prolongs the life span of C3H/HeJ mice: correlation with reduced DNA damage. *Exp Gerontol*. 2003;38:965–969.
11. Honda Y, Fujita Y, Maruyama H, et al. Lifespan-extending effects of royal jelly and its related substances on the nematode Caenorhabditis elegans. *PLoS One*. 2011;6:e23527.
12. Kamakura M, Mitani N, Fukuda T, Fukushima M. Antifatigue effect of fresh royal jelly in mice. *J Nutr Sci Vitaminol (Tokyo)*. 2001;47:394–401.
13. Matsui T, Yukiyoshi A, Doi S, Sugimoto H, Yamada H, Matsumoto K. Gastrointestinal enzyme production of bioactive peptides from royal jelly protein and their antihypertensive ability in SHR. *J Nutr Biochem*. 2002;13:80–86.
14. Vittek J. Effect of royal jelly on serum lipids in experimental animals and humans with atherosclerosis. *Experientia*. 1995;51:927–935.
15. Liu JR, Yang YC, Shi LS, Peng CC. Antioxidant properties of royal jelly associated with larval age and time of harvest. *J Agric Food Chem*. 2008;56:11447–11452.
16. Kohno K, Okamoto I, Sano O, et al. Royal jelly inhibits the production of proinflammatory cytokines by activated macrophages. *Biosci Biotechnol Biochem*. 2004;68:138–145.
17. Macaluso A, De Vito G. Muscle strength, power and adaptations to resistance training in older people. *Eur J Appl Physiol*. 2004;91:450–472.
18. Strasser EM, Wessner B, Roth E. Cellular regulation of anabolism and catabolism in skeletal muscle during immobilisation, aging and critical illness. *Wien Klin Wochenschr*. 2007;119:337–348.
19. Schaap LA, Pluijm SM, Deeg DJ, Visser M. Inflammatory markers and loss of muscle mass (sarcopenia) and strength. *Am J Med*. 2006;119:526.e9–526.17.
20. Meng SJ, Yu LJ. Oxidative stress, molecular inflammation and sarcopenia. *Int J Mol Sci*. 2010;11:1509–1526.
21. Sriram S, Subramanian S, Sathiakumar D, et al. Modulation of reactive oxygen species in skeletal muscle by myostatin is mediated through NF-κB. *Aging Cell*. 2011;10:931–948.
22. Okazaki T, Ebihara S, Asada M, et al. Macrophage colony-stimulating factor improves cardiac function after ischemic injury by inducing vascular endothelial growth factor production and survival of cardiomyocytes. *Am J Pathol*. 2007;171:1093–1103.
23. Yahiaoui L, Gvozdic D, Danialou G, Mack M, Petrof BJ. CC family chemokines directly regulate myoblast responses to skeletal muscle injury. *J Physiol*. 2008;586:3991–4004.
24. Okazaki T, Ebihara S, Asada M, Yamanda S, Niu K, Arai H. Erythropoietin promotes the growth of tumors lacking its receptor and decreases survival of tumor-bearing mice by enhancing angiogenesis. *Neoplasia*. 2008;10:932–939.
25. Nakajima R, Takao K, Huang SM, et al. Comprehensive behavioral phenotyping of calpastatin-knockout mice. *Mol Brain*. 2008;7:1–15.
26. Uezumi A, Fukada S, Yamamoto N, Takeda S, Tsuchida K. Mesenchymal progenitors distinct from satellite cells contribute to ectopic fat cell formation in skeletal muscle. *Nat Cell Biol*. 2010;12:143–152.
27. Niu K, Asada M, Okazaki T, et al. Adiponectin pathway attenuates malignant mesothelioma cell growth. *Am J Respir Cell Mol Biol*. 2012;46:515–523.
28. Fukada S, Yamaguchi M, Kokubo H, et al. Hesr1 and Hesr3 are essential to generate undifferentiated quiescent satellite cells and to maintain satellite cell numbers. *Development*. 2011;138:4609–4619.
29. Asada M, Ebihara S, Yamanda S, et al. Depletion of serotonin and selective inhibition of 2B receptor suppressed tumor angiogenesis by

inhibiting endothelial nitric oxide synthase and extracellular signal-regulated kinase ½ phosphorylation. *Neoplasia.* 2009;11:408–417.

30. Yamanda S, Ebihara S, Asada M, et al. Role of ephrinB2 in non-productive angiogenesis induced by Delta-like 4 blockade. *Blood.* 2009;113:3631–3639.

31. Okazaki T, Sakon S, Sasazuki T, et al. Phosphorylation of serine 276 is essential for p65 NF-kappaB subunit-dependent cellular responses. *Biochem Biophys Res Commun.* 2003;300:807–812.

32. Okazaki T, Ni A, Baluk P, et al. Capillary defects and exaggerated inflammatory response in the airways of EphA2-deficient mice. *Am J Pathol.* 2009;174:2388–2399.

33. Chevenne D, Porquet D. Growth hormone (GH) and insulin-like growth factor 1 (IGF-1) in nutritional status. *Ann Biol Clin (Paris).* 1995;53:527–538.

34. Campillo B, Paillaud E, Bories PN, Noel M, Porquet D, Le Parco JC. Serum levels of insulin-like growth factor-1 in the three months following surgery for a hip fracture in elderly: relationship with nutritional status and inflammatory reaction. *Clin Nutr.* 2000;19:349–354.

35. Ponzer S, Tidermark J, Brismar K, Söderqvist A, Cederholm T. Nutritional status, insulin-like growth factor-1 and quality of life in elderly women with hip fractures. *Clin Nutr.* 1999;18:241–246.

36. McWhirter JP, Ryan MF, Pennington CR. An evaluation of insulin-like growth factor-1 as an indicator of nutritional status. *Clin Nutr.* 1995;14:74–80.

37. Kandarian SC, Jackman RW. Intracellular signaling during skeletal muscle atrophy. *Muscle Nerve.* 2006;33:155–165.

38. Carter CS, Ramsey MM, Ingram RL, et al. Models of growth hormone and IGF-1 deficiency: applications to studies of aging processes and lifespan determination. *J Gerontol A Biol Sci Med Sci.* 2002;57:B177–B188.

39. Chiba T, Yamaza H, Shimokawa I. Role of insulin and growth hormone/insulin-like growth factor-I signaling in lifespan extension: rodent longevity models for studying aging and calorie restriction. *Curr Genomics.* 2007;8:423–428.

CrossMark
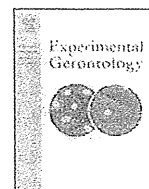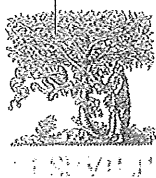
Coffee treatment prevents the progression of sarcopenia in aged mice in vivo and in vitro

Yinting Guo [a,b], Kaijun Niu [a,*], Tatsuma Okazaki [c], Hongmei Wu [a], Takeo Yoshikawa [d], Takashi Ohrui [e], Katsutoshi Furukawa [f], Masakazu Ichinose [c], Kazuhiko Yanai [d], Hiroyuki Arai [f], Guowei Huang [a], Ryoichi Nagatomi [g]

[a] Department of Nutrition and Food Science, School of Public Health, Tianjin Medical University, Tianjin, China
[b] Department of Behavioral Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan
[c] Department of Respiratory Medicine, Graduate School of Medicine, Tohoku University, Sendai, Japan
[d] Department of Pharmacology, Graduate School of Medicine, Tohoku University, Sendai, Japan
[e] Division of Geriatric Pharmacotherapy, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan
[f] Department of Geriatrics and Gerontology, Division of Brain Sciences, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan
[g] Division of Biomedical Engineering for Health & Welfare, Tohoku University Graduate School of Biomedical Engineering, Sendai, Japan

ARTICLE INFO

ABSTRACT

Sarcopenia is characterized by the age-related loss of muscle mass and strength, which results in higher mortality in aged people. One of the mechanisms of the sarcopenia is the loss in the function and number of muscle satellite cells. Chronic low-grade inflammation plays a central role in the pathogenesis of age-related sarcopenia. Accumulating evidence suggests that coffee, one of the most widely consumed beverages in the world, has potential pharmacological benefits such as anti-inflammatory and anti-oxidant effects. Since these effects may improve sarcopenia and the functions of satellite cells, we examined the effects of coffee on the skeletal muscles in an animal model using aged mice. In vivo, coffee treatment attenuated the decrease in the muscle weight and grip strength, increased the regenerating capacity of injured muscles, and decreased the serum pro-inflammatory mediator levels compared to controls. In vitro, using satellite cells isolated from aged mice, coffee treatment increased the cell proliferation rate, augmented the cell cycle, and increased the activation level of Akt intracellular signaling pathway compared to controls. These findings suggest that the coffee treatment had a beneficial effect on age-related sarcopenia.

© 2013 Elsevier Inc. All rights reserved.

1. Introduction

The population of aged people over 60 years old is currently growing at the rate of 2.6% per year, which is more than twice the rate of the total population in the world (United Nations, 2009). In general, aging is accompanied by frailty, functional limitations, and disabilities that interfere with the activities of daily life. These factors reduce the quality of life of aged people and eventually cause their loss of autonomy in daily life. Sarcopenia is the age-related loss of the muscle mass and strength, which causes frailty, functional limitations in daily living, disabilities, and finally, a higher mortality rate in aged people (Altun et al., 2012).

Satellite cells are resident myogenic progenitors in the skeletal muscles. They play a central role in the growth and regeneration of the skeletal muscles (Hawke and Garry, 2001). In response to stimulation,

satellite cells form myoblasts, fuse together and generate new fibers (Clemmons, 2009). The age-related functional disability and decrease in the number of satellite cells contribute to the development of sarcopenia (Welle, 2002). Thus, maintaining the functions of satellite cells and their numbers might reduce sarcopenia and, furthermore, might improve the regenerating capacity of the skeletal muscles in aged people.

Chronic low-grade inflammation plays a central role in the pathogenesis of age-related sarcopenia (Beyer et al., 2012). With aging, the levels of serum pro-inflammatory mediators such as tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) increase, and are inversely related to muscle mass, muscle strength, and disability in aged people (Cohen et al., 1997; Ferrucci et al., 1999; Greiwe et al., 2001; Visser et al., 2002). TNF-α decreases the expression levels of MyoD messenger RNA, a well-established skeletal muscle-specific transcription factor that directly regulates the expression of myogenic proteins and resulting in muscle wasting (Cai et al., 2004; Guttridge et al., 2000). IL-6 induces skeletal muscle atrophy in mice (Haddad et al., 2005; Tsujinaka et al., 1996).

Coffee is one of the most widely consumed beverages in the world, and it has been considered to have beneficial capacities to one's health. Accumulating evidence suggests that coffee intake has numerous potential pharmacological benefits, such as antioxidant (Sato et al., 2011), antitumor (Franco, 2008), anti-diabetic (Ong et al., 2012), and anti-inflammatory effects (Chang et al., 2010; Shen et al., 2010).

Since the above effects of coffee might have the potential to improve sarcopenia and the functions of satellite cells (Macaluso and De Vito, 2004; Meng and Yu, 2010; Schaap et al., 2006; Sriram et al., 2011; Strasser et al., 2007), we hypothesized that coffee intake might have a beneficial effect on the prevention of sarcopenia. Furthermore, we hypothesized that this effect might involve inflammation. To the best of our knowledge, few studies have examined the effect of coffee intake on muscles in aged people or aged animals, or the relationship between coffee intake and pro-inflammatory mediator levels. Thus, in the present study, we examined the effects of coffee treatment on muscle weight, muscle strength, satellite cell functions, regenerating capacity of the skeletal muscles in vivo and in vitro, and the involvement of pro-inflammatory mediators in an animal model using aged mice.

2. Material and methods

2.1. Mice and drink treatment

Male C57BL/6 mice were obtained from Clea Japan (Tokyo, Japan) and maintained under specific pathogen-free conditions with unrestricted access to food and water. Experiments were carried out in accordance with the guidelines established by the Tohoku University Committee on Animal Research. At the age of 27-months, mice were divided into 2 groups according to the beverages provided for each group and maintained for the next 4 weeks, with 12 mice in each group. The 2 groups were: normal water (controls) and coffee. Ready-to-drink coffee (Clear®) was purchased from Nescafe (Sizuoka, Japan) and diluted with distilled water at 1:2 dilutions. In this study, all of the coffee products were from the same lot number. Japan Food Research Laboratories (Tokyo, Japan) analyzed the main components of the coffee. The coffee used in this study contained: coffee bean, ≈2.5 g/100 ml; chlorogenic acid, 0.07 mg/ml; anhydrous caffeine, 0.45 mg/ml; polyphenols, 1.4 mg/ml; specific gravity, 1.005 (at 20 °C). The water and coffee were changed every day. The mice had unrestricted access to food and water. Two mice in the control and one mouse in the treatment group died of natural causes during the treatment period. After 4 weeks of treatment, 4 mice from the control group and 5 mice from the coffee group were anesthetized, sacrificed, their sera were collected, and the weights of the hind limb muscles were measured. The hind limb muscles included the tibialis anterior muscle (TA), triceps surae muscle, quadriceps muscle, biceps femoris muscle, gluteus maximus muscle, and iliopsoas muscle. For evaluation of the myogenic progenitor proliferation and differentiation and fusion capacity of injured skeletal muscles, mice were sacrificed 3 or 5 days after the injury, respectively, 3 mice in each group.

2.2. Isolation and culture conditions of satellite cells and cell proliferation assay

Satellite cells were isolated from untreated, 28-month-old mice according to our previous study (Niu et al., 2013). Briefly, the hind limb muscles including the TA, triceps surae muscle, quadriceps muscle, biceps femoris muscle, gluteus maximus muscle, and iliopsoas muscle were isolated, non-muscle tissues were removed, and then the muscles were subjected to enzymatic dissociation with 0.2% collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ, USA) for 60 min, then with 0.04 U/ml dispase (Gibco BRL, Grand Island, NY, USA) for 45 min. The cell suspension was filtered through a cell strainer (BD Bioscience, Franklin Lakes, NJ, USA), incubated with anti-mouse CD16/CD32 monoclonal antibody (mAb, 2.4G2, BD Bioscience) to

block Fc receptors, then with the following antibodies: FITC-labeled anti-CD31, anti-CD45 (BD Bioscience), anti-CD11b, and anti-Sca-1 antibodies (eBioscience, San Diego, CA, USA); PE-labeled anti-Integrin α7 (MBL, Nagoya, Japan); Alexa 647-labeled anti-CD34 (BD Bioscience). The cells were sorted by a FACSAria™ II flow cytometer (BD Bioscience) as previously described (Niu et al., 2013).

Sorted satellite cells were cultured in growth medium containing high-glucose Dulbecco's modified Eagle's medium (HG-DMEM) with 20% fetal bovine serum (FBS) (MP Biomedicals, Morgan Irvine, CA, USA), 2.5 ng/ml basic fibroblast growth factor (bFGF, Invitrogen, Eugene, OR, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA). Satellite cells under 8 passages were used in this study. Diluted coffee solution was sterilized by a filter then added to the culture medium at the following concentrations: 10, 30, 50, or 100 μg/ml. The cells were cultured for 72 h, and the number of cells was determined by a water-soluble tetrazolium-8 (WST-8, DOJINDO, Tokyo, Japan) assay using a cell counting kit (Okazaki et al., 2008).

2.3. Locomotor activity recordings

Freely moving locomotor activity was recorded by an infrared ray sensor system (SUPERMEX®; Muromachi-Kikai, Tokyo, Japan) that consisted of 12 small compartments divided by walls on a large shelf. Each compartment (width: 40 cm × depth: 50 cm × height: 35 cm) was equipped with a ceiling sensor that can detect heat energy radiated from a mouse. The system detected mouse movement by recording changes in heat energy in the covered field. Mice were individually placed in a plastic cage (width: 19 cm × depth: 27.5 cm × height: 17 cm) and then put into the system shelf. Counts were measured every 10 min (Inoue et al., 1996). Locomotor activity was consecutively measured on days 0–1, 5–6, 10–11, and 19–20.

2.4. Grip strength test

Grip strength was measured by an electronic grip strength meter (MK-380; Muromachi Kikai). Mice were put on the fence and pulled back slowly. The point at which mice released the fence was determined as the grip strength. The measurements were repeated 3 times and maximal readings were taken (Arai et al., 2001). The grip strength was measured twice a week.

2.5. Cell-cycle analysis by flow cytometry

DNA synthesis in cells was evaluated by measuring BrdU incorporation (BrdU Flow Kits; BD Biosciences, San Jose, CA, USA) by flow cytometry. Briefly, 5×10^5 cells were cultured overnight. Then, the cells were stimulated for the next 72 h with 10, 30, 50, or 100 μg/ml coffee bean extract sterilized with a 0.22 μm filter. The cells were labeled with BrdU during the final 2 h of stimulation. The cells were then permeabilized, fixed, and stained with an anti-BrdU antibody coupled with FITC according to the manufacturer's protocol. Flow cytometry data were collected using a logarithmic scale, and the percentage of BrdU-positive cells was determined (Niu et al., 2012).

2.6. Muscle injury model

After 4 weeks of the coffee treatment, the mice were anesthetized and cardiotoxin from Naja mossambica mossambica (Sigma) dissolved in 100 μl phosphate-buffered saline (PBS) (10 μM) was injected into the TA. Three or five days later, the mice were sacrificed, the TA muscles were isolated, frozen in 2-methylbutane precooled in liquid nitrogen, and stored at −80 °C for the following histological analysis (Uezumi et al., 2010).

2.7. Immunohistochemistry and immunocytochemistry

Frozen muscle tissues were sectioned from a region approximately 3 mm from the top of the TA (8 μm in thickness) using a cryostat. For embryonic myosin heavy chain (eMyHC) staining, frozen sections were fixed with acetone/methanol (50%/50%) for 30 s at −20 °C. Specimens were blocked with 1% BSA, 0.1% Triton X-100 in PBS at room temperature for 45 min, then incubated with anti-eMyHC antibody (F1.652, DSHB, Iowa City, IA, USA) at a 1:2 dilution at 4 °C overnight, followed by Rodamine conjugated-secondary antibody staining (Chemicon International, Temecula, CA, USA) at room temperature in the dark for 1 h. Finally, the sections were mounted in Vectashield Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector labs, Burlingame, CA, USA). In vivo, the regenerating capacity of the injured skeletal muscles was evaluated by quantifying the percentage of eMyHc-immunoreactive area per field (Fukada et al., 2012). Ten randomly selected fields at ×200 magnification were measured in each sample. ImageJ software was used to quantify the eMyHC-immunoreactive areas per field. For ki-67 staining, after quenching endogenous peroxidase with 3% H_2O_2 in PBS for 15 min, the sections were incubated with primary antibodies at 4 °C overnight (anti-ki67 antibody, 1:40 dilution; DAKO, Tokyo, Japan), followed by incubation with biotinylated anti-rabbit immunoglobulin G antibody using Histofine (Max-PO (Multi), Nichirei Bioscience, Osaka, Japan) according to the manufacturer's instructions. Then, the antibody complex was visualized with 3,30-diaminobenzidine tetrahydrochloride (MERCK, Darmstadt, Germany) (Niu et al., 2012). Images were taken using a phase-contrast and fluorescence microscope BZ9000 (Keyence, Osaka, Japan) (Asada et al., 2009).

2.8. Western blot analysis

Akt and phosphorylated-Akt (phospho-Akt) proteins of the satellite cells were detected by western blot analysis. Some cells were serum-starved overnight, then stimulated with 10 nM insulin (Sigma), which is a potent activator of Akt, for 5 min as a positive control. Western blot analysis was performed with a SDS-PAGE Electrophoresis System as describe previously (Yamanda et al., 2009). In brief, the cells were rinsed twice with ice-cold PBS and lysed using RIPA Lysis Buffer (Upstate, Temecula, CA, USA). The extracted protein fraction was electrophoresed in a sodium dodecyl sulphate-10% polyacrylamide gel and then transferred onto an Immobilon transfer membrane (Millipore, Bedford, MA, USA). The amount of protein loaded onto the gels was 36 μg per well. The membranes were immunoblotted with the primary antibodies to Akt and phospho-Akt (Cell Signaling, Boston, MA, USA) at 1:1000 dilutions. Then the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Cell Signaling) at 1:25,000 dilution and the protein bands were detected with an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK) (Yamanda et al., 2009).

2.9. Enzyme-Linked Immunosorbent Assay (ELISA)

Sera were isolated from the inferior vena cava of the mice (Okazaki et al., 2007). The serum levels of IL-1α, IL-1β, IL-6, TNF-α, and IGF-1 were measured using a specific ELISA kit (R&D Systems) according to the manufacturer's instructions, respectively (Okazaki et al., 2007). The minimum detectable levels were 2.5, 3.0, 1.6, 5.1, and 3.5 pg/ml for IL-1α, IL-1β, IL-6, TNF-α, and IGF-1, respectively.

2.10. Statistical analysis

To determine the sample size, a power analysis was performed based on the results of previously performed research (Niu et al., 2013) and a preliminary experiment. The mean hind limb muscle-weight divided by the body weight of 28-month-old mice was 0.009.

We estimated ≥20% (standard deviation: 0.0005) difference between the control and coffee groups in the hind limb muscle-weight divided by the body weight. Assuming an alpha error of 0.05 with a power of 0.90, we calculated a necessary sample size of 3 to show a significant effect. Based on this calculation and to ensure reasonable data, we increased the sample size to 4 or 5 in this study. The same calculation was applied to determine the sample size of myogenic progenitor proliferation and regenerating capacity test in vivo. Based on previously performed research (Niu et al., 2013) and a preliminary experiment, we estimated ≥30% (mean Ki67-positive cell number per field in control group ≈ 40 cells, SD ≈ 3.5) and ≥70% (mean relative eMyHc positive area in control group ≈ 9%, SD ≈ 1.8) difference in the myogenic progenitor proliferation and regenerating capacity test, respectively, between the control and coffee treatment groups. Assuming an alpha error of 0.05 with a power of 0.90 we calculated a necessary sample size of 3 to show a significant effect both in the myogenic progenitor proliferation and regenerating capacity test.

Data were presented as mean ± standard error (SE). Differences were analyzed by one-way analysis of variance (ANOVA) test (Post hoc, Tukey). The Spearman correlation coefficient (r) was calculated to evaluate the relationship between two continuous variables. All the tests for statistical significance were 2 sided, and $p < 0.05$ was considered statistically significant. All in vitro experiments were repeated at least 3 times. In this study, the main experiments such as grip strength measurement, cardiotoxin injection, and histological quantifications were blindly carried out.

3. Results

3.1. Coffee-treated mice had greater muscle weight and grip strength than controls

To examine the effects of coffee treatment on aged mice in vivo, we divided 27-month-old mice into 2 groups and treated them with either normal water (controls) or coffee for 4 weeks. Two mice in the control and one mouse in the treatment group died of natural causes during the treatment period. These mice were excluded from the analysis. During the intervention period, the body weight changed similarly in the coffee-treated and control groups (Fig. 1A) (p value > 0.89). The amounts of daily diet intake and drink were not different between the groups (Fig. 1B and C). We also examined the effect of coffee on locomotor activity in the aged mice. Experiments were performed under light: dark cycles of 12 h:12 h. Locomotor activity was not different between the groups (Fig. 1D). A previous study compared hind limb muscles-weight divided by body weight between 2, 8, and 24 month old mice and showed progressive loss of hind limb muscles-weight / body weight with aging, suggesting the progression of sarcopenia with aging (Niu et al., 2013). The hind limb muscles included the TA, triceps surae, quadriceps, biceps femoris, gluteus maximus, and iliopsoas muscles. Coffee treatment significantly increased the hind limb muscle-weight compared to controls (Fig. 1E). Similarly, we measured the weight of the bilateral hind limb muscles and divided by the body weight. Coffee treatment increased the weight of the hind limb muscles per body weight compared to control by 13.1% (0.011 ± 0.0005 for the coffee treatment group vs 0.0098 ± 0.0007 for the control group, mean ± SE, Fig. 1F). To examine the effect of coffee on the muscle strength, we performed the grip strength test. Consistent with the effect of coffee on the muscle mass, the coffee group had greater grip strength than the controls, suggesting that coffee improved the grip strength (Fig. 1G). Furthermore, a comparison between before and after the treatment period within the controls showed that grip strength decreased after the treatment period compared to before, suggesting the progression of age-related atrophy in muscle function during this period. In contrast, no significant changes were observed within the coffee group during the treatment period. These data suggested that coffee
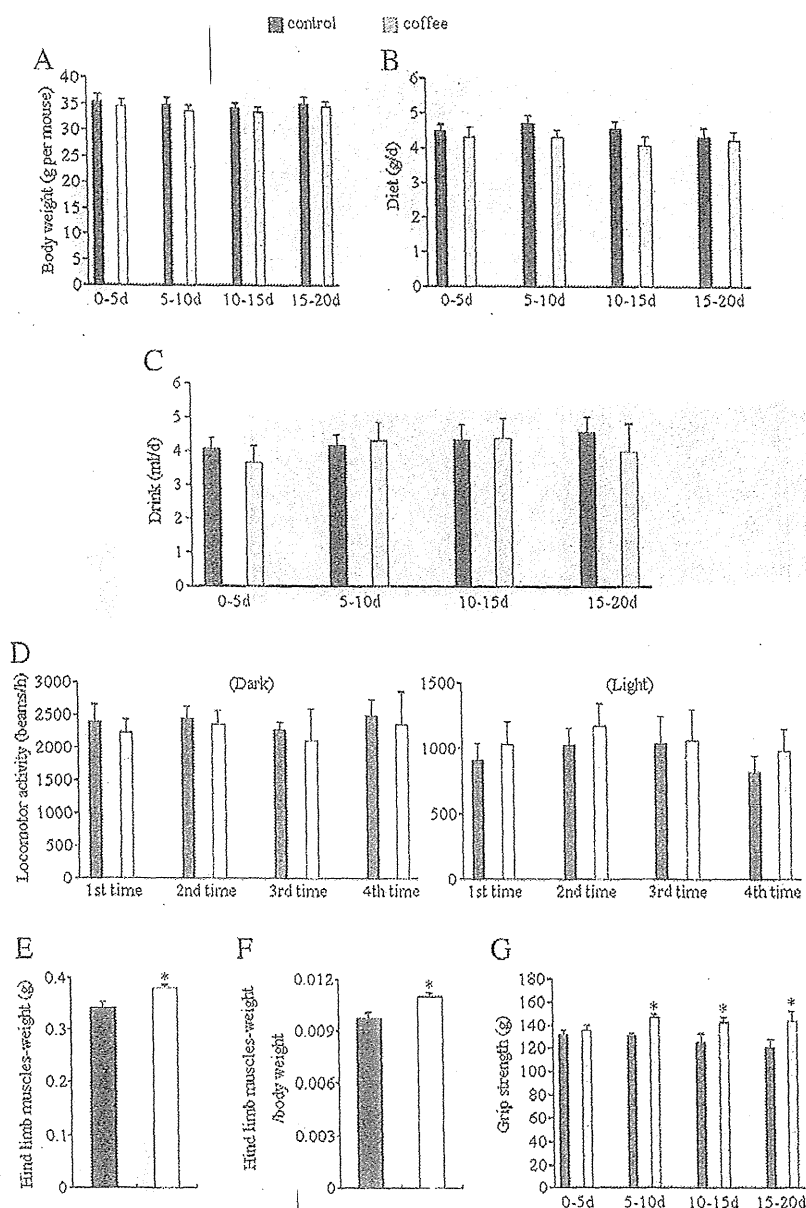
Fig. 1. Effects of coffee treatment on aged mice in vivo. Twenty-seven month-old mice were treated with coffee for 4 weeks. (A–C) Coffee treatment did not change body weight (A), the amount of the daily diet intake (B), or the volume of water intake (C). (D) Coffee did not change the daily locomotor activity levels. (E) The coffee-treated group had greater hind limb muscle-weight than the controls. (F) The coffee-treated group had greater hind limb muscles-weight per body weight than the controls. (G) The coffee-treated group had greater grip strength than controls. Columns are mean ± SE, $n \geq 4$ in each group. *$p < 0.05$ compared with control.

treatment prevented the progression of atrophy in muscle weight and function in the aged mice.

3.2. Coffee treatment accelerated the regeneration of injured skeletal muscles

We next examined the effect of coffee treatment on the regenerating capacity of the skeletal muscles in aged mice in vivo by injuring the TA muscles with cardiotoxin injection and observed their regeneration. We isolated the muscles 3 or 5 days after the cardiotoxin injection. To determine the effect of coffee on the cell proliferation rate, we immunohistochemically stained muscle tissues for the cell proliferation marker Ki67 three days after the injury (Fig. 2A, left panels). The number of Ki67 immunoreactive cells was greater in the coffee group than that in controls, suggesting a greater cell proliferation rate in the coffee

group (Fig. 2A, right panel). To confirm the regenerating capacity of the skeletal muscles, we immunohistochemically stained the muscle tissues for eMyHc, which is a marker of immature myotubes including regenerating muscles, 5 days after the injury (Fig. 2B, left panels). Quantification of the eMyHc immunoreactive area showed greater immunoreactive areas in the coffee group than in the controls (Fig. 2B, right panel). These results suggested that coffee treatment accelerated the regeneration of the injured skeletal muscles.

3.3. Coffee treatment decreased serum pro-inflammatory mediator levels

Since coffee has been suggested to have an anti-inflammatory effect, we examined the effect of coffee treatment on serum pro-inflammatory mediator levels in the aged mice. We chose IL-1α, IL-1β, IL-6, and TNF-α as pro-inflammatory mediators (Okazaki et al., 2003, 2009), and
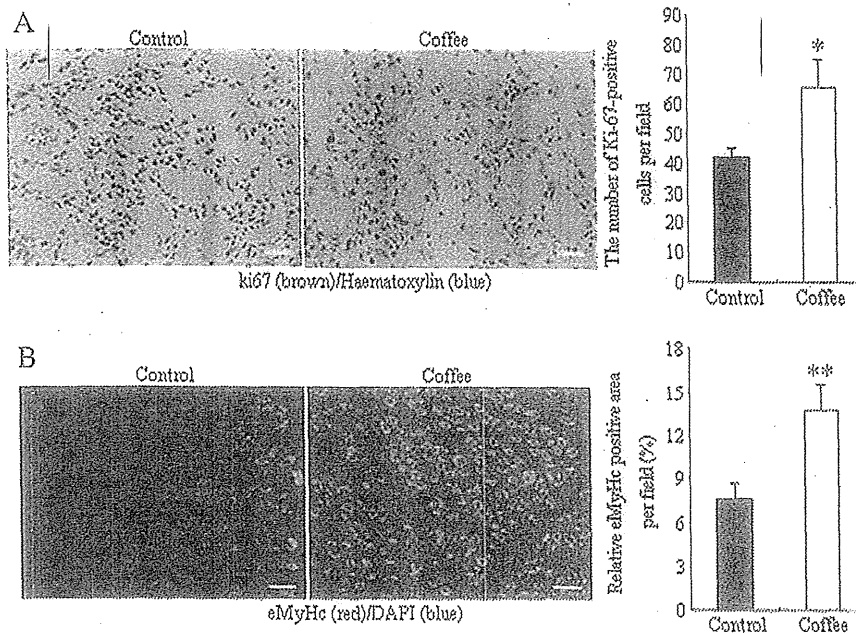
Fig. 2. Coffee treatment accelerated the regeneration of the injured skeletal muscles in aged mice. After 4 weeks of coffee treatment, we injected cardiotoxin into the tibialis anterior muscles of the mice to injure the muscles and isolated them 3 days later for (A) or 5 days later for (B). (A) The left panels are immunohistochemical staining for ki67 of the injured tibialis anterior muscles. The right graph is quantification of the number of ki-67 positive cells per visual microscopic field for each group (10 randomly selected fields at ×200 magnification per sample were quantified). (B) The left panels are immunohistochemical staining for eMyHC and DAPI of the injured tibialis anterior muscles. The right graph is quantification of the percentage of eMyHc-immunoreactive area per field for each group (10 randomly selected fields at ×200 magnification per sample). Scale bars, 100 μm. Columns are mean ± SE, n = 3 in each group. $*p < 0.05$ and $**p < 0.01$ compared with controls.

measured their levels in the serum. The levels of IL-1α, IL-6, and TNF-α were decreased in coffee treated group compared to controls (Fig. 3A). The correlation coefficients suggested a relationship between the serum pro-inflammatory mediator levels and the grip strength (r = −0.38, r = −0.34, r = −0.42, and r = −0.36 for IL-1α, IL-1β, IL-6, and TNF-α, respectively [$p < 0.05$ for all]). The correlation coefficients also suggested a significant relationship between several serum pro-inflammatory mediator levels and the muscle weight (r = −0.52, r = −0.39, r = −0.69, and r = −0.63 for IL-1α, IL-1β, IL-6, and TNF-α, respectively [$p < 0.05$ for IL-6 and TNF-α]). Since IGF-1 plays a central role in stimulating satellite cells, we also measured the serum levels of IGF-1. The serum levels of IGF-1 were not different (Fig. 3B).

3.4. Effects of coffee on the satellite cells of the aged mice in vitro

To examine the effect of coffee on the proliferation rate of the satellite cells of the aged mice in vitro, we isolated satellite cells from aged mice and stimulated them with coffee in growth medium for 72 h (Fig. 4A). Under the growing condition, coffee treatment increased the number of proliferating satellite cells compared to controls in a dose-dependent manner in vitro. Next, to examine the effect of coffee on the cell cycles of the satellite cells, we cultured the satellite cells for 72 h with coffee and measured DNA synthesis by BrdU incorporation using flow cytometry (Fig. 4B). The coffee-treated group had a greater BrdU incorporation rate than the controls (Fig. 4C). These results suggested that coffee enhanced the DNA synthesis of the proliferating satellite cells of the aged mice. The Akt signaling pathway plays a key role in the proliferation and cell cycle progression of the satellite cells (Giovannini et al., 2008; Kandarian and Jackman, 2006). Therefore, we next examined the activation level of Akt by western blot for Akt and the activated form of Akt, phosphorylated Akt. Coffee treatment increased the intensity of the bands of phosphorylated Akt compared to controls, which suggests that coffee treatment increased the activation
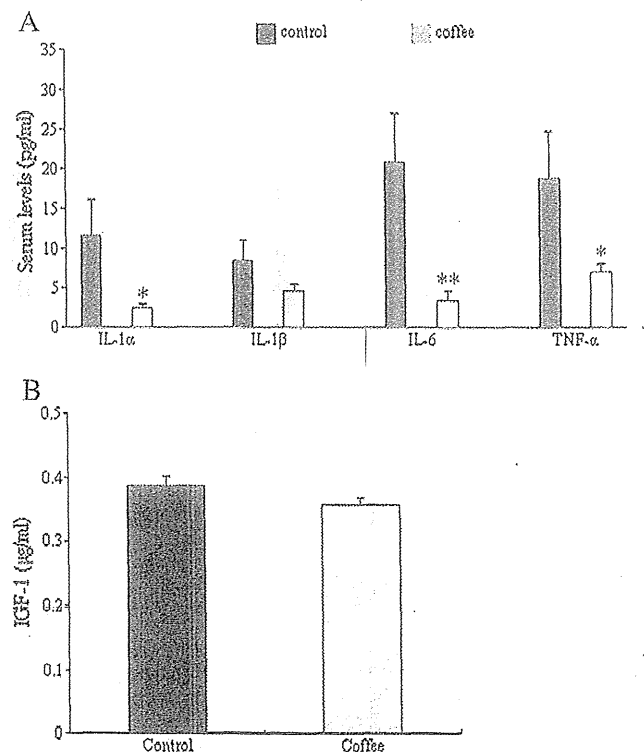

Fig. 3. Coffee treatment decreased the serum pro-inflammatory mediator levels, but did not affect the serum IGF-1 levels. ELISA determined serum levels of pro-inflammatory mediators and IGF-1 in aged mice treated with coffee for 4 weeks. (A) Coffee treatment significantly decreased the serum levels of IL-1α, IL-6, and TNF-α compared to controls. (B) Coffee treatment did not change the serum levels of the IGF-1 levels. Columns are mean ± SE, ≥4 in each group. $*p < 0.05$, and $**p < 0.01$ compared with control.
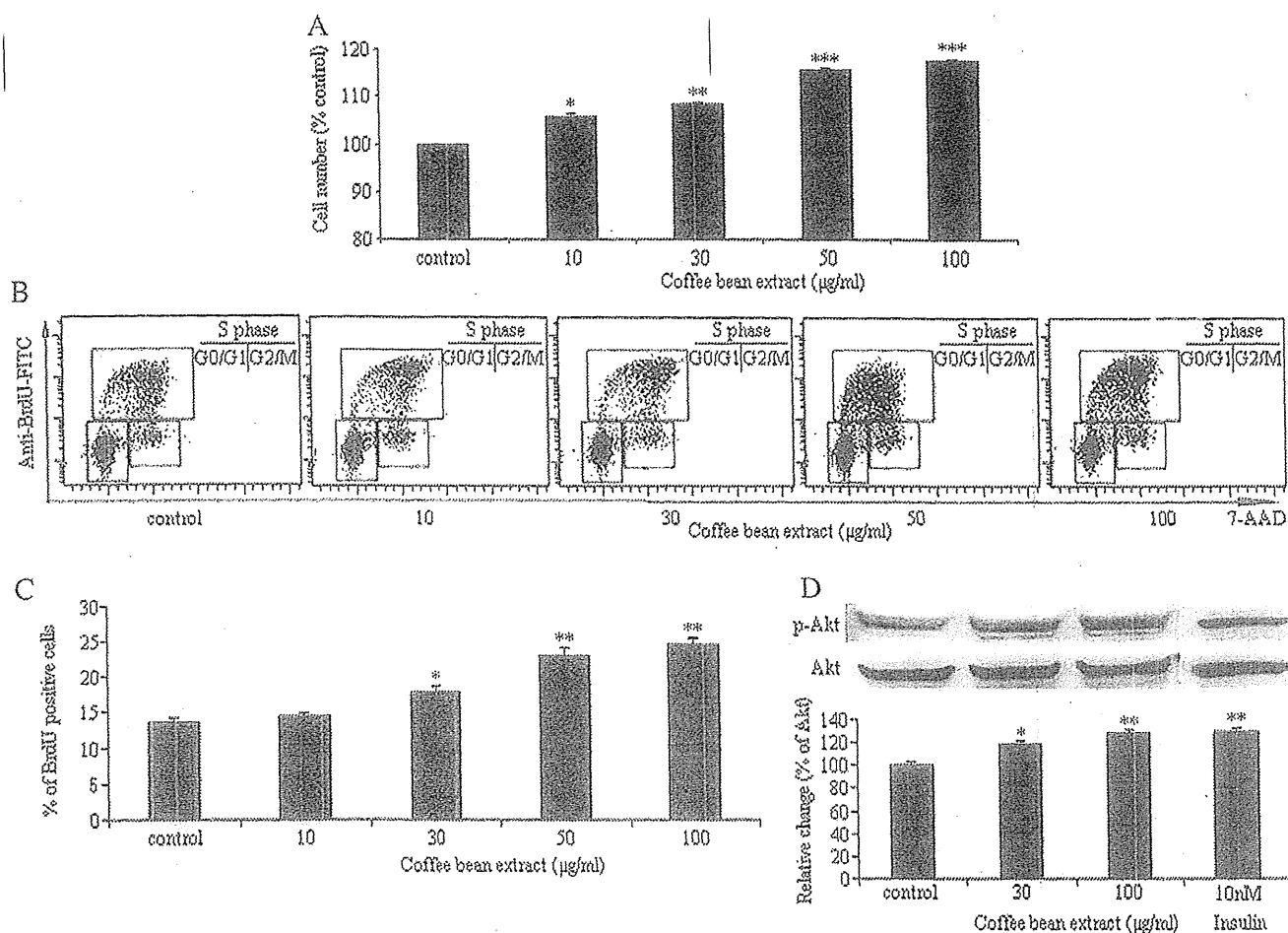
Fig. 4. Effects of coffee on the satellite cells of aged mice in vitro. (A) The satellite cells of aged mice were treated with the indicated concentrations of coffee bean extract for 72 h and the cell proliferation rate was measured. (B) Bromodeoxyuridine (BrdU)/7-AAD incorporation was evaluated by flow cytometry after stimulating the cells with water or indicated concentrations of coffee for 72 h. The regions were set on the G0/G1, S phase and G2/M populations. Representative data are shown. (C) The satellite cells of aged mice were cultured for 72 h with or without the indicated concentrations of coffee, and BrdU-positive percentages were calculated. (D) The satellite cells of aged mice were pretreated with coffee for 72 h, then the western blot analysis detected activated form of Akt (phospho-Akt) and total Akt. The densitometry quantified the band intensities. The graph shows the phospho-Akt band intensities normalized to the Akt band intensities. Representative of 3 independent experiments. Columns are mean ± SE. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.0001$, compared with control.

level of Akt (Fig. 4D). As a positive control, we activated Akt in satellite cells with insulin (Fig. 4D). These results suggested that coffee treatment increased the proliferation rate and augmented DNA synthesis through the Akt signaling pathway in the satellite cells isolated from aged mice in vitro.

4. Discussion

In this study, using aged mice, we showed that coffee treatment increased the skeletal muscle weight, grip strength, regenerating capacity of injured skeletal muscles, and decreased the serum pro-inflammatory mediator levels compared to controls in vivo. In vitro, coffee treatment increased the cell proliferation rate, augmented DNA synthesis, and activated the Akt signaling pathway compared to controls in the satellite cells of aged mice.

Coffee treatment increased the number of proliferating satellite cells isolated from aged mice and augmented their cell cycle compared to controls, which could be the mechanism responsible for the increase in the skeletal muscle weight and grip strength, and accelerated the regeneration of injured skeletal muscles compared to controls. Since these effects possibly antagonized the loss of muscle mass and strength, the results suggested that coffee treatment might improve sarcopenia in aged mice. The coffee-treated group had greater grip strength than the

controls. However, comparison between before and after the treatment period within the same groups showed that the grip strength did not change in the coffee-treated groups, whereas the grip strength decreased after the same period in the controls. This result suggested that coffee treatment did not improve but rather attenuated the progression of the decrease in muscle strength. Therefore, the effects of coffee on skeletal muscles might be attenuating the atrophy rather than improving the muscle mass and strength in aged mice. Satellite cells play an essential role in the regeneration of skeletal muscles (Lepper et al., 2011). Although skeletal muscle has the capacity to regenerate itself, this process is not activated in the gradual age-related loss of muscle fibers. The endocrine, autocrine, and paracrine environment in old muscle is less supportive of the activation, proliferation, and differentiation of satellite cells than in young muscle (Welle, 2002). The current results showed that coffee treatment increased the number of proliferating satellite cells, augmented their cell cycle in vivo and in vitro, and accelerated the differentiation and regeneration in vivo. These results suggested that coffee augmented the satellite cell activation. The decreased inflammatory levels by coffee treatment may contribute to the prevention of sarcopenia, as well. The combination of augmented satellite cell activation and decreased inflammatory levels by coffee treatment might antagonize the degenerative environment in old muscles and might prevent the sarcopenia.

Inflammation plays an important role in age-related sarcopenia (Beyer et al., 2012; Jensen, 2008). Therefore, the decreased serum levels of pro-inflammatory mediators after coffee treatment might be one of the mechanisms of the effects of coffee. Results from several experimental studies showed that coffee extracts inhibited inflammation in animal models (J.Y. Kim et al., 2006; Paur et al., 2010). A human clinical trial also demonstrated that coffee intake had beneficial effects on subclinical inflammation (Kempf et al., 2010). Our findings are consistent with these observations. Coffee contains many components and some of them have immunomodulatory effects. For example, a component of coffee, kahweol, inhibited the effect of TNF-α-induced protein and mRNA expression of the adhesion molecules, vascular cell adhesion molecule 1 and intercellular adhesion molecule 1, in human endothelial cells in vitro (H.G. Kim et al., 2006). Kahweol also inhibited the inflammatory response induced by carrageenan in a rat using an acute air pouch inflammation model (J.Y. Kim et al., 2006). These results strongly suggested that some components in coffee have significant anti-inflammatory effects in vitro and in vivo. Moreover, accumulating evidence suggests that coffee is a good source of antioxidant (Svilaas et al., 2004). Because oxidative stress causes inflammation (Butt and Sultan, 2011), the decrease in inflammation by coffee may be partly mediated through the antioxidant mechanism. Furthermore, some studies also reported that caffeine can increase skeletal muscle contractions by increasing calcium ion release (Olorunshola and Achie, 2011). Since this is a study of a complex product, we could not evaluate the effect of each component in the prevention of sarcopenia. However, the results suggested that whole coffee improved sarcopenia in aged mice. Further studies are required to evaluate the effects and mechanisms of the components of coffee on sarcopenia.

In vitro, coffee activated the Akt signaling pathway in satellite cells isolated from aged mice. Since coffee contains a wide variety of components, it is not clear which component(s) activated Akt. However, the effect of coffee on Akt activation is controversial. In several types of cancer cells, coffee decreased the Akt activation level (Choi et al., 2011; Oh et al., 2009). In contrast, in the cells of a Parkinson's disease model, coffee activated Akt and prevented apoptotic cell death (Nakaso et al., 2008). Combined with previous studies, our results suggested that the effect of coffee on Akt activation level might depend on the cell type. Since most satellite cells in aged animals are in a quiescent state, coffee might not activate Akt in vivo. However, satellite cells are activated by increased muscle loading and some of these cells fuse with apparently undamaged myofibers as part of the hypertrophy process (Adams, 2006). Furthermore, a recent study identified apoptotic cells as a new promoter of myoblast fusion (Hochreiter-Hufford et al., 2013), Therefore, some satellite cells might be in an active state in aged animals and coffee treatment might augment their Akt activation in vivo.

Several studies showed that exercise alone did not affect muscle function in aged animal models (Derbre et al., 2012; Leiter et al., 2011, 2012). A previous study indicated that the systemic environment of old animals is a crucial factor for maintaining and improving the function of satellite cells (Brack et al., 2007). In fact, another study suggested that nitric oxide and exercise together promoted muscle function in aged mice (Leiter et al., 2012). Therefore, the effect of exercise on muscle function in aged mice may depend on systemic and/or muscle environments. The decreased systemic inflammatory levels of the aged mice by coffee treatment may be one reason for the discrepancy in the effects on muscle function between exercise and coffee treatment.

Previous studies suggested that caffeine intake increased physical activity and energy metabolism, and decreased body weight (Magkos and Kavouras, 2004). However, in the present study, coffee intake did not change these parameters. The difference in age might partly explain this discrepancy.

A limitation of this study was that we could not clearly rule out whether caffeine, a major biological active component of coffee, or whole coffee itself produced stimulatory effects on proliferation in satellite cells. Several studies have shown that caffeine affected the proliferation rate and activation levels of Akt and inhibited reactive oxygen species in other cell types including epithelial, neuronal, cancer, and vascular smooth muscle cells (Mercer et al., 2012; Miwa et al., 2011, 2013; Nakaso et al., 2008; Sahu et al., 2013; Sarobo et al., 2012). Further study is required to clarify the exact effects and mechanisms of caffeine or other coffee components on the functions of satellite cells isolated from aged mice.

In conclusion, in vivo, coffee treatment increased the muscle weight, grip strength, regenerating capacity of injured muscles, and decreased serum pro-inflammatory mediator levels compared to controls in aged mice. In vitro, coffee increased the cell proliferation rate, augmented the cell cycle, and increased the activation level of the Akt signaling pathway compared to controls in satellite cells isolated from aged mice. These findings suggested that coffee treatment might have a beneficial effect on the prevention of age-related sarcopenia through decreasing the systemic inflammation and activating the Akt signaling pathway in satellite cells.

Conflict of interests

All the authors declare no conflicts of interest to disclose.

References

Adams, G.R., 2006. Satellite cell proliferation and skeletal muscle hypertrophy. Appl. Physiol. Nutr. Metab. 31, 782–790.

Altun, M., Grönholdt-Klein, M., Wang, L., Ulfhake, B., 2012. Senescence. In: Nagata, T. (Ed.), Cellular Degradation Machineries in Age-Related Loss of Muscle Mass (Sarcopenia). Academia.edu, San Francisco, pp. 269–286 (Chapter 13).

Arai, K., Matsuki, N., Ikegaya, Y., Nishiyama, N., 2001. Deterioration of spatial learning performances in lipopolysaccharide-treated mice. Jpn. J. Pharmacol. 87, 195–201.

Asada, M., Ebihara, S., Yamanda, S., Niu, K., Okazaki, T., Sora, I., Arai, H., 2009. Depletion of serotonin and selective inhibition of 2B receptor suppressed tumor angiogenesis by inhibiting endothelial nitric oxide synthase and extracellular signal-regulated kinase 1/2 phosphorylation. Neoplasia 11, 408–417.

Beyer, I., Mets, T., Bautmans, I., 2012. Chronic low-grade inflammation and age-related sarcopenia. Curr. Opin. Clin. Nutr. Metab. Care 15, 12–22.

Brack, A.S., Conboy, M.J., Roy, S., Lee, M., Kuo, C.J., Keller, C., Rando, T.A., 2007. Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. Science 317, 807–810.

Butt, M.S., Sultan, M.T., 2011. Coffee and its consumption: benefits and risks. Crit. Rev. Food Sci. Nutr. 51, 363–373.

Cai, D., Frantz, J.D., Tawa Jr., N.E., Melendez, P.A., Oh, B.C., Lidov, H.G., Hasselgren, P.O., Frontera, W.R., Lee, J., Glass, D.J., Shoelson, S.E., 2004. IKKbeta/NF-kappaB activation causes severe muscle wasting in mice. Cell 119, 285–298.

Chang, W.C., Chen, C.H., Lee, M.F., Chang, T., Yu, Y.M., 2010. Chlorogenic acid attenuates adhesion molecules upregulation in IL-1beta-treated endothelial cells. Eur. J. Nutr. 49, 267–275.

Choi, M.J., Park, E.J., Oh, J.H., Min, K.J., Yang, E.S., Kim, Y.H., Lee, T.J., Kim, S.H., Choi, Y.H., Park, J.W., Kwon, T.K., 2011. Cafestol, a coffee-specific diterpene, induces apoptosis in renal carcinoma Caki cells through down-regulation of anti-apoptotic proteins and Akt phosphorylation. Chem. Biol. Interact. 190, 102–108.

Clemmons, D.R., 2009. Role of IGF-I in skeletal muscle mass maintenance. Trends Endocrinol. Metab. 20, 349–356.

Cohen, H.J., Pieper, C.F., Harris, T., Rao, K.M., Currie, M.S., 1997. The association of plasma IL-6 levels with functional disability in community-dwelling elderly. J. Gerontol. A Biol. Sci. Med. Sci. 52, M201–M208.

Derbre, F., Gomez-Cabrera, M.C., Nascimento, A.L., Sanchis-Gomar, F., Martinez-Bello, V.E., Tresguerres, J.A., Fuentes, T., Gratas-Delamarche, A., Monsalve, M., Vina, J., 2012. Age-associated low mitochondrial biogenesis may be explained by lack of response of PGC-1alpha to exercise training. Age (Dordr.) 34, 669–679.

Ferrucci, L., Harris, T.B., Guralnik, J.M., Tracy, R.P., Corti, M.C., Cohen, H.J., Penninx, B., Pahor, M., Wallace, R., Havlik, R.J., 1999. Serum IL-6 level and the development of disability in older persons. J. Am. Geriatr. Soc. 47, 639–646.

Franco, R., 2008. Coffee and cancer. Med. Clin. (Barc.) 131, 633–635.

Fukada, S., Yamaguchi, M., Kokubo, H., Ogawa, R., Uezumi, A., Yoneda, T., Matev, M.M., Motohashi, N., Ito, T., Zolkiewska, A., Johnson, R.L., Saga, Y., Miyagoe-Suzuki, Y., Tsujikawa, K., Takeda, S., Yamamoto, H., 2012. Hesr1 and Hesr3 are essential to generate undifferentiated quiescent satellite cells and to maintain satellite cell numbers. Development 138, 4609–4619.

Giovannini, S., Marzetti, E., Borst, S.E., Leeuwenburgh, C., 2008. Modulation of GH/IGF-1 axis: potential strategies to counteract sarcopenia in older adults. Mech. Ageing Dev. 129, 593–601.

Greiwe, J.S., Cheng, B., Rubin, D.C., Yarasheski, K.E., Semenkovich, C.F., 2001. Resistance exercise decreases skeletal muscle tumor necrosis factor alpha in frail elderly humans. FASEB J. 15, 475–482.

Guttridge, D.C., Mayo, M.W., Madrid, L.V., Wang, C.Y., Baldwin Jr., A.S., 2000. NF-kappaB-induced loss of MyoD messenger RNA: possible role in muscle decay and cachexia. Science 289, 2363–2366.

Haddad, F., Zaldivar, F., Cooper, D.M., Adams, G.R., 2005. IL-6-induced skeletal muscle atrophy. J. Appl. Physiol. 98, 911–917.

Hawke, T.J., Garry, D.J., 2001. Myogenic satellite cells: physiology to molecular biology. J. Appl. Physiol. 91, 534–551.

Hochreiter-Hufford, A.E., Lee, C.S., Kinchen, J.M., Sokolowski, J.D., Arandjelovic, S., Call, J.A., Klibanov, A.L., Yan, Z., Mandell, J.W., Ravichandran, K.S., 2013. Phosphatidylserine receptor BAI1 and apoptotic cells as new promoters of myoblast fusion. Nature 497, 263–267.

Inoue, I., Yanai, K., Kitamura, D., Taniuchi, I., Kobayashi, T., Niimura, K., Watanabe, T., 1996. Impaired locomotor activity and exploratory behavior in mice lacking histamine H1 receptors. Proc. Natl. Acad. Sci. U. S. A. 93, 13316–13320.

Jensen, G.L., 2008. Inflammation: roles in aging and sarcopenia. JPEN J. Parenter. Enteral Nutr. 32, 656–659.

Kandarian, S.C., Jackman, R.W., 2006. Intracellular signaling during skeletal muscle atrophy. Muscle Nerve 33, 155–165.

Kempf, K., Herder, C., Erlund, I., Kolb, H., Martin, S., Carstensen, M., Koenig, W., Sundvall, J., Bidel, S., Kuha, S., Tuomilehto, J., 2010. Effects of coffee consumption on subclinical inflammation and other risk factors for type 2 diabetes: a clinical trial. Am. J. Clin. Nutr. 91, 950–957.

Kim, J.Y., Kim, D.H., Jeong, H.G., 2006. Inhibitory effect of the coffee diterpene kahweol on carrageenan-induced inflammation in rats. Biofactors 26, 17–28.

Kim, H.G., Kim, J.Y., Hwang, Y.P., Lee, K.J., Lee, K.Y., Kim, D.H., Jeong, H.G., 2006. The coffee diterpene kahweol inhibits tumor necrosis factor-alpha-induced expression of cell adhesion molecules in human endothelial cells. Toxicol. Appl. Pharmacol. 217, 332–341.

Leiter, J.R., Peeler, J., Anderson, J.E., 2011. Exercise-induced muscle growth is muscle-specific and age-dependent. Muscle Nerve 43, 828–838.

Leiter, J.R., Upadhaya, R., Anderson, J.E., 2012. Nitric oxide and voluntary exercise together promote quadriceps hypertrophy and increase vascular density in female 18-mo-old mice. Am. J. Physiol. Cell Physiol. 302, C1306–C1315.

Lepper, C., Partridge, T.A., Fan, C.M., 2011. An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. Development 138, 3639–3646.

Macaluso, A., De Vito, G., 2004. Muscle strength, power and adaptations to resistance training in older people. Eur. J. Appl. Physiol. 91, 450–472.

Magkos, F., Kavouras, S.A., 2004. Caffeine and ephedrine: physiological, metabolic and performance-enhancing effects. Sports Med. 34, 871–889.

Meng, S.J., Yu, L.J., 2010. Oxidative stress, molecular inflammation and sarcopenia. Int. J. Mol. Sci. 11, 1509–1526.

Mercer, J.R., Gray, K., Figg, N., Kumar, S., Bennett, M.R., 2012. The methyl xanthine caffeine inhibits DNA damage signaling and reactive species and reduces atherosclerosis in ApoE(−/−) mice. Arterioscler. Thromb. Vasc. Biol. 32, 2461–2467.

Miwa, S., Sugimoto, N., Shirai, T., Hayashi, K., Nishida, H., Ohnari, I., Takeuchi, A., Yachie, A., Tsuchiya, H., 2011. Caffeine activates tumor suppressor PTEN in sarcoma cells. Int. J. Oncol. 39, 465–472.

Miwa, S., Yano, S., Tome, Y., Sugimoto, N., Hiroshima, Y., Uehara, F., Mii, S., Kimura, H., Hayashi, K., Efimova, E.V., Fujiwara, T., Tsuchiya, H., Hoffman, R.M., 2013. Dynamic color-coded fluorescence imaging of the cell-cycle phase, mitosis, and apoptosis demonstrates how caffeine modulates cisplatinum efficacy. J. Cell. Biochem. 114, 2454–2460.

Nakaso, K., Ito, S., Nakashima, K., 2008. Caffeine activates the PI3K/Akt pathway and prevents apoptotic cell death in a Parkinson's disease model of SH-SY5Y cells. Neurosci. Lett. 432, 146–150.

Niu, K., Asada, M., Okazaki, T., Yamanda, S., Ebihara, T., Guo, H., Zhang, D., Nagatomi, R., Arai, H., Kohzuki, M., Ebihara, S., 2012. Adiponectin pathway attenuates malignant mesothelioma cell growth. Am. J. Respir. Cell Mol. Biol. 46, 515–523.

Niu, K., Guo, H., Guo, Y., Ebihara, S., Asada, M., Ohrui, T., Furukawa, K., Ichinose, M., Yanai, K., Kudo, Y., Arai, H., Okazaki, T., Nagatomi, R., 2013. Royal jelly prevents the progression of sarcopenia in aged mice in vivo and in vitro. J. Gerontol. A Biol. Sci. Med. Sci. http://dx.doi.org/10.1093/gerona/glt041.

Oh, J.H., Lee, J.T., Yang, E.S., Chang, J.S., Lee, D.S., Kim, S.H., Choi, Y.H., Park, J.W., Kwon, T.K., 2009. The coffee diterpene kahweol induces apoptosis in human leukemia U937 cells through down-regulation of Akt phosphorylation and activation of JNK. Apoptosis 14, 1378–1386.

Okazaki, T., Sakon, S., Sasazuki, T., Sakurai, H., Doi, T., Yagita, H., Okumura, K., Nakano, H., 2003. Phosphorylation of serine 276 is essential for p65 NF-kappaB subunit-dependent cellular responses. Biochem. Biophys. Res. Commun. 300, 807–812.

Okazaki, T., Ebihara, S., Asada, M., Yamanda, S., Saijo, Y., Shiraishi, Y., Ebihara, T., Niu, K., Mei, H., Arai, H., Yambe, T., 2007. Macrophage colony-stimulating factor improves cardiac function after ischemic injury by inducing vascular endothelial growth factor production and survival of cardiomyocytes. Am. J. Pathol. 171, 1093–1103.

Okazaki, T., Ebihara, S., Asada, M., Yamanda, S., Niu, K., Arai, H., 2008. Erythropoietin promotes the growth of tumors lacking its receptor and decreases survival of tumor-bearing mice by enhancing angiogenesis. Neoplasia 10, 932–939.

Okazaki, T., Ni, A., Baluk, P., Ayeni, O.A., Kearley, J., Coyle, A.J., Humbles, A., McDonald, D.M., 2009. Capillary defects and exaggerated inflammatory response in the airways of EphA2-deficient mice. Am. J. Pathol. 174, 2388–2399.

Olorunshola, K.V., Achie, L.N., 2011. Caffeine alters skeletal muscle contraction by opening of calcium ion channels. Curr. Res. J. Biol. Sci. 3, 521–525.

Ong, K.W., Hsu, A., Tan, B.K., 2012. Chlorogenic acid stimulates glucose transport in skeletal muscle via AMPK activation: a contributor to the beneficial effects of coffee on diabetes. PLoS One 7, e32718.

Paur, I., Balstad, T.R., Kolberg, M., Pedersen, M.K., Austenaa, L.M., Jacobs Jr., D.R., Blomhoff, R., 2010. Extract of oregano, coffee, thyme, clove, and walnuts inhibits NF-kappaB in monocytes and in transgenic reporter mice. Cancer Prev. Res. (Phila.) 3, 653–663.

Sahu, S., Kauser, H., Ray, K., Kishore, K., Kumar, S., Panjwani, U., 2013. Caffeine and modafinil promote adult neuronal cell proliferation during 48 h of total sleep deprivation in rat dentate gyrus. Exp. Neurol. 248, 470–481.

Sarobo, C., Lacorte, L.M., Martins, M., Rinaldi, J.C., Moroz, A., Scarano, W.R., Delella, F.K., Felisbino, S.L., 2012. Chronic caffeine intake increases androgenic stimuli, epithelial cell proliferation and hyperplasia in rat ventral prostate. Int. J. Exp. Pathol. 93, 429–437.

Sato, Y., Itagaki, S., Kurokawa, T., Ogura, J., Kobayashi, M., Hirano, T., Sugawara, M., Iseki, K., 2011. In vitro and in vivo antioxidant properties of chlorogenic acid and caffeic acid. Int. J. Pharm. 403, 136–138.

Schaap, L.A., Pluijm, S.M., Deeg, D.J., Visser, M., 2006. Inflammatory markers and loss of muscle mass (sarcopenia) and strength. Am. J. Med. 119 (526), e9–e17.

Shen, T., Park, Y.C., Kim, S.H., Lee, J., Cho, J.Y., 2010. Nuclear factor-kappaB/signal transducers and activators of transcription-1-mediated inflammatory responses in lipopolysaccharide-activated macrophages are a major inhibitory target of kahweol, a coffee diterpene. Biol. Pharm. Bull. 33, 1159–1164.

Sriram, S., Subramanian, S., Sathiakumar, D., Venkatesh, R., Salerno, M.S., McFarlane, C.D., Kambadur, R., Sharma, M., 2011. Modulation of reactive oxygen species in skeletal muscle by myostatin is mediated through NF-κB. Aging Cell 10, 931–948.

Strasser, E.M., Wessner, B., Roth, E., 2007. Cellular regulation of anabolism and catabolism in skeletal muscle during immobilisation, aging and critical illness. Wien. Klin. Wochenschr. 119, 337–348.

Svilaas, A., Sakhi, A.K., Andersen, L.F., Svilaas, T., Strom, E.C., Jacobs Jr., D.R., Ose, L., Blomhoff, R., 2004. Intakes of antioxidants in coffee, wine, and vegetables are correlated with plasma carotenoids in humans. J. Nutr. 134, 562–567.

Tsujinaka, T., Fujita, J., Ebisui, C., Yano, M., Kominami, E., Suzuki, K., Tanaka, K., Katsume, A., Ohsugi, Y., Shiozaki, H., Monden, M., 1996. Interleukin 6 receptor antibody inhibits muscle atrophy and modulates proteolytic systems in interleukin 6 transgenic mice. J. Clin. Invest. 97, 244–249.

Uezumi, A., Fukada, S.I., Yamamoto, N., Takeda, S., Tsuchida, K., 2010. Mesenchymal progenitors distinct from satellite cells contribute to ectopic fat cell formation in skeletal muscle. Nat. Cell Biol. 12, 143–153.

United Nations, 2009. Department of Economic and Social Affairs Population Division: World Population Ageing 2009. United Nations, New York 11.

Visser, M., Pahor, M., Taaffe, D.R., Goodpaster, B.H., Simonsick, E.M., Newman, A.B., Nevitt, M., Harris, T.B., 2002. Relationship of interleukin-6 and tumor necrosis factor-alpha with muscle mass and muscle strength in elderly men and women: the Health ABC Study. J. Gerontol. A Biol. Sci. Med. Sci. 57, M326–M332.

Welle, S., 2002. Cellular and molecular basis of age-related sarcopenia. Can. J. Appl. Physiol. 27, 19–41.

Yamanda, S., Ebihara, S., Asada, M., Okazaki, T., Niu, K., Ebihara, T., Koyanagi, A., Yamaguchi, N., Yagita, H., Arai, H., 2009. Role of ephrinB2 in nonproductive angiogenesis induced by Delta-like 4 blockade. Blood 113, 3631–3639.

Hindawi

Research Article

Treatment of Posttraumatic Stress Disorder Using the Traditional Japanese Herbal Medicine Saikokeishikankyoto: A Randomized, Observer-Blinded, Controlled Trial in Survivors of the Great East Japan Earthquake and Tsunami

Takehiro Numata,[1] Shen GunFan,[2] Shin Takayama,[3] Satomi Takahashi,[4] Yasutake Monma,[5] Soichiro Kaneko,[1] Hitoshi Kuroda,[6] Junichi Tanaka,[7] Seiki Kanemura,[7] Masayuki Nara,[7] Yutaka Kagaya,[3] Tadashi Ishii,[7] Nobuo Yaegashi,[1] Masahiro Kohzuki,[2] and Koh Iwasaki[4]

[1] Department of Obstetrics and Gynecology, Tohoku University Graduate School of Medicine, No. 1-1, Seiryo-Machi, Aoba Ward, Sendai City 980-8574, Japan
[2] Department of Internal Medicine and Rehabilitation Science, Tohoku University Graduate School of Medicine, No. 1-1, Seiryo-Machi, Aoba Ward, Sendai City 980-8574, Japan
[3] Comprehensive Education Center for Community Medicine, Tohoku University Graduate School of Medicine, No. 2-1, Seiryo-Machi, Aoba Ward, Sendai City 980-8575, Japan
[4] Center for Traditional Asian Medicine, National Sendai-Nishitaga Hospital, No. 2-11-11, Kagitorihoncho, Taihaku Ward, Sendai City 982-8555, Japan
[5] Graduate Medical Education Center, Tohoku University Hospital, No. 1-1, Seiryo-Machi, Aoba Ward, Sendai City 980-8574, Japan
[6] Division of General Medicine, Saitama Medical Center, Jichi Medical University, No. 1-847, Amanumacho, Oomiya Ward, Saitama City 330-8503, Japan
[7] Department of Education and Support for Community Medicine, Tohoku University Hospital, No. 1-1, Seiryo-Machi, Aoba Ward, Sendai City 980-8574, Japan

Correspondence should be addressed to Shin Takayama; takayama@med.tohoku.ac.jp

The Great East Japan earthquake and tsunami caused immense damage over a wide area of eastern Japan. Hence, many survivors are at high risk for posttraumatic stress disorder (PTSD). This randomized, observer-blinded, controlled trial examined the efficacy and safety of the traditional Japanese herbal formula saikokeishikankyoto (SKK) in the treatment of PTSD among survivors of this disaster. Forty-three participants with an Impact of Event Scale-Revised (IES-R) score ≥ 25 were randomized into SKK ($n = 21$) and control ($n = 22$) groups. The primary endpoint was the change in IES-R scores from baseline till after 2 weeks of treatment. Intergroup statistical comparisons were performed. The magnitude of changes in total IES-R scores differed significantly between the two groups ($P < 0.001$). Post hoc analysis showed that the total IES-R score improved significantly in the SKK group from 49.6 $\pm$ 11.9 to 25.5 $\pm$ 17.0 ($P < 0.001$). Subscale scores improved significantly in the SKK group (avoidance, $P = 0.003$; hyperarousal, $P < 0.001$; intrusion, $P < 0.001$). Two-week treatment with SKK significantly improved IES-R scores among PTSD patients. This traditional medicine may be a valid choice for the treatment of psychological and physical symptoms in PTSD patients.

1. Introduction

Two years have passed since March 11, 2011, the day the Great East Japan earthquake and tsunami claimed 20,000 lives in a flash. Although people have been able to move from shelters to temporary housing, heaps of rubble and radioactive pollution prevent the rehabilitation of many areas. While some commercial fishing has resumed, inshore fish are polluted with radiation and cannot be sold. The towns and cities inundated by the tsunami are, even now, a wasteland. The disaster caused posttraumatic stress disorder (PTSD) in many people [1]. Many patients have visited our clinic for PTSD treatment, but psychotherapists are in short supply. We attempted to treat them using mood stabilizers, antidepressants, and hypnotics, but these medications are not always effective; some of these patients then suffer from intractable drug dependency. A Chinese herbal formula was reported to be effective for patients with PTSD after the Sichuan earthquake in 2008 [2]. Saikokeishikankyoto (SKK, Chaihu-Guizhi-Ganjiang-Tang in Chinese) is a traditional Japanese-Chinese herbal formula that has a marked effect on some PTSD patients. We therefore conducted a randomized observer-blinded trial of the efficacy and safety of SKK for the treatment of PTSD.

2. Methods

2.1. Participants. Subjects were recruited from the outpatient clinic at the National Sendai-Nishitaga Hospital in Sendai, Japan. The required sample size was calculated as 20 subjects for each arm, with an alpha error of 0.05 and power of 0.8. All participants completed an intake assessment that included medical history, physical examination, and standard blood examination as well as an Impact of Event Scale-Revised score (IES-R) (see the following pattern) to assess the severity of PTSD at baseline.

Scoring the Impact of Event Scale-Revised Questionnaire

Impact of Event Scale-Revised Questionnaire

Instructions. Below is a list of difficulties people sometimes have after stressful life events. Please read each item, and then indicate how distressing each difficulty has been for you during the past seven days with respect to _____, which occurred on _____. How much were you distressed or bothered by these difficulties?

Item Response Anchors are as follows: 0 = not at all; 1 = a little bit; 2 = moderately; 3 = quite a bit; 4 = extremely.

The Intrusion subscale is the mean item response of items 1, 2, 3, 6, 9, 14, 16, and 20. Thus, scores can range from 0 to 4. The avoidance subscale is the mean item response of items 5, 7, 8, 11, 12, 13, 17, and 22. Thus, scores can range from 0 to 4. The hyperarousal subscale is the mean item response of items 4, 10, 15, 18, 19, and 21. Thus, scores can range from 0 to 4. Consider the following.

(1) Any reminder brought back feelings about it.
(2) I had trouble staying asleep.
(3) Other things kept making me think about it.
(4) I felt irritable and angry.
(5) I avoided letting myself get upset when I thought about it or was reminded of it.
(6) I thought about it when I did not mean to.
(7) I felt as if it had not happened or was not real.
(8) I stayed away from reminders of it.
(9) Pictures about it popped into my mind.
(10) I was jumpy and easily startled.
(11) I tried not to think about it.
(12) I was aware that I still had a lot of feelings about it, but I did not deal with them.
(13) My feelings about it were kind of numb.
(14) I found myself acting or feeling like I was back at that time.
(15) I had trouble falling asleep.
(16) I had waves of strong feelings about it.
(17) I tried to remove it from my memory.
(18) I had trouble concentrating.
(19) Reminders of it caused me to have physical reactions, such as sweating, trouble breathing, nausea, or a pounding heart.
(20) I had dreams about it.
(21) I felt watchful and on-guard.
(22) I tried not to talk about it.

Total IES-R score: _____.

The inclusion criteria were as follows: (1) survivors of the Great East Japan earthquake and tsunami who were older than 20 years and diagnosed with PTSD according to the Diagnostic and Statistical Manual (DSM)-IV TR; (2) IES-R score ≥ 25 (cutoff point).

Exclusion criteria were as follows: (1) major medical illness such as neoplastic disease, acute inflammation, or any other disease that would most likely prevent the completion of this study; (2) psychosis due to other disorders such as schizophrenia, depression, and/or dementia; (3) delirium due to drugs, alcohol, metabolic intoxication, or inflammation; and (4) use of neuroleptics, antianxiety drugs, antiepileptic drugs, antidepressants, or herbal remedies during the past 2 months.

Participants underwent a complete diagnostic assessment, including medical history, physical examination, laboratory tests, and the Structured Clinical Interview for DSM-IV TR and IES-R. Eligible subjects were randomized into either the SKK or control group. Random numbers were generated using computer software. Treatment codes were held by the corresponding investigator, who was isolated from the patients and outcome data.

The study protocol was performed with the intention to treat. SKK extract was prescribed to the patients in the SKK

group. The SKK extract (TJ-11) was processed by Tsumura (Tokyo, Japan), and it contained the following mixture of dried herbs: Bupleuri Radix (6 g), Trichosanthis Kirilowii (3 g), Cinnamomi Cassiae (3 g), Radix Scutellariae Baicalensis (3 g), Concha Ostreae (3 g), Glycyrrhizae Radix (2 g), and dried Zingiberis Rhizoma (2 g). These herbs are registered in the Pharmacopoeia of Japan version 15. Each participant in the SKK group received 2.5 g of SKK powder (1.17 g extract) 3 times a day for 2 weeks. The processes involved in the production and supply of SKK comply with Good Manufacturing Practices for Kampo products and are also approved by the Ministry of Health, Labour, and Welfare of Japan.

Participants understood that those randomized into the control group could receive any treatment after completion of the whole trial if they wanted. Patients were free to withdraw at any time. Clinical assessments were performed at the baseline and at the endpoint.

The primary clinical outcome measure was the severity of PTSD symptoms as measured by the total IES-R. The secondary outcome measures were 3 IES-R subscale scores as defined below. The intrusion subscale is the mean response to items 1, 2, 3, 6, 9, 14, 16, and 20. The avoidance subscale is the mean response to items 5, 7, 8, 11, 12, 13, 17, and 22. The hyperarousal subscale is the mean response to items 4, 10, 15, 18, 19, and 21.

2.2. Statistical Analysis. Analyses were performed as a modified intention to treat. If a patient dropped out, all outcome measures were assessed within a week. Statistical analysis was performed using the SPSS software (version 16, SPSS Japan Inc., Tokyo, Japan). Measurements of the mean and SD were calculated at the baseline and at the endpoint for all continuous primary and secondary measures. Baseline comparisons of group differences were conducted using independent samples t-tests for continuous variables and chi-square test for categorical variables. Comparisons between the SKK and the control group were performed by the two-way analysis of variance (ANOVA). The changes in each group from baseline to endpoint were compared using the paired t-test when the intergroup difference was significant ($P < 0.05$) according to the post hoc test.

This study was carried out in compliance with the ethical principles embodied in the Helsinki Declaration. Written informed consent was obtained from each participant prior to participation in this study. The study protocol was approved by the Institutional Review Board of Sendai-Nishitaga National Hospital in Sendai, Japan, and registered with the UMIN clinical trial registry (UMIN000010890, http://www.umin.ac.jp/ctr/index.htm).

3. Results

Of the 48 enrolled subjects, 5 were excluded according to the exclusion criteria. The remaining 43 participants were randomized into SKK ($n = 21$) and control ($n = 22$) groups. The background factors for each group are shown

TABLE 1: Background factors for SKK and control groups.

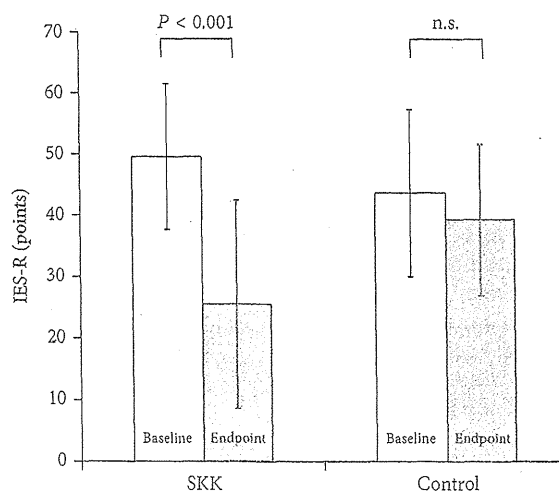| | Group | | P value |
	SKK	Control	
n	21	22	
Sex (m/f)	9/12	13/9	0.45
Age (year)	52.3 ± 13.0	48.0 ± 20.9	0.42
IES-R (baseline)	49.6 ± 11.6	43.7 ± 13.7	0.14

FIGURE 1: Changes in the total IES-R scores. The two-way analysis of variance (ANOVA) showed a significant difference between the groups ($P < 0.001$) and post hoc testing showed that total IES-R scores were significantly improved only in the SKK group.

in Table 1, with no significant difference except for the IES-R hyperarousal subscale score. One participant in the SKK group withdrew from the study on the third day because of coughing; all participants in the control group completed the study.

3.1. Changes in Total IES-R Scores. Two-way ANOVA analysis showed that the changes in total IES-R scores differed significantly between groups ($P < 0.001$). The post hoc testing showed that the total IES-R score was significantly improved from 49.6 ± 11.9 to 25.5 ± 17.0 in the SKK group ($P < 0.001$) but decreased from 43.7 ± 13.7 to 39.3 ± 12.4 in the control group. The latter trend was not significant (Figure 1).

3.2. Changes in IES-R Subscale Scores. The changes in IES-R subscale scores are shown in Figures 2(a), 2(b), and 2(c). Two-way ANOVA showed significant differences between groups ($P = 0.025$ for avoidance, $P = 0.005$ for hyperarousal, and $P = 0.001$ for intrusion), although there were baseline differences in scores on the hyperarousal subscale. The post hoc testing showed that all subscales improved significantly from baseline to the endpoint in the SKK group ((a) $P = 0.003$, (b) $P < 0.001$, and (c) $P < 0.001$), whereas only avoidance showed a significant change in the control group ((a) $P = 0.032$, (b) n.s., and (c) n.s.).
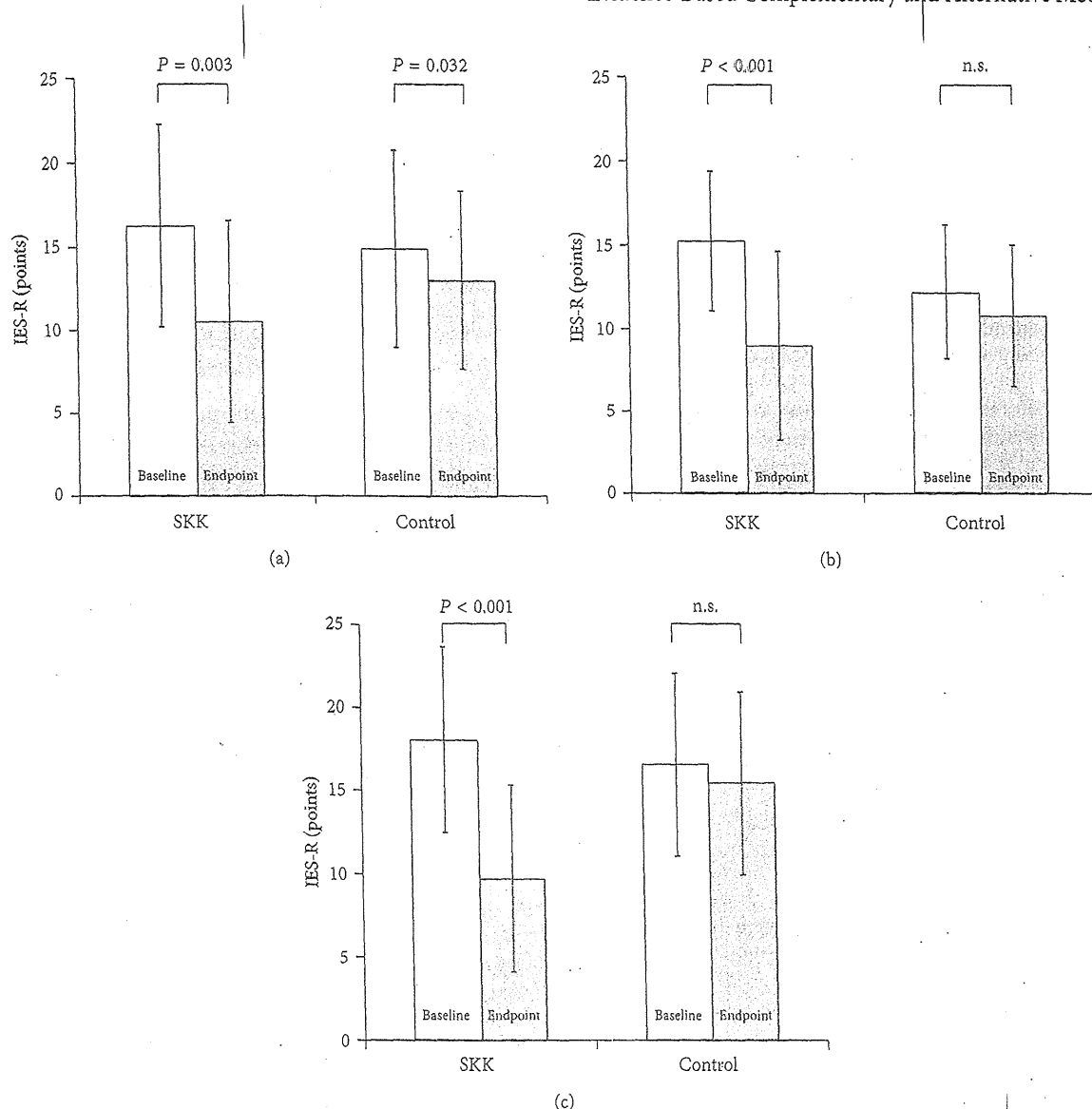
FIGURE 2: (a) Change in the IES-R avoidance subscale score. (b) Change in the IES-R hyperarousal subscale score. (c) Change in the IES-R intrusion subscale score. Two-way ANOVA showed that each subscale differed significantly between the groups ((a) $P = 0.025$, (b) $P = 0.005$, (c) $P = 0.001$). Post hoc testing showed that all subscale scores changed significantly from baseline to the endpoint in the SKK group ((a) $P = 0.003$, (b) $P < 0.001$, and (c) $P < 0.001$), whereas in the control group, only the avoidance subscale score showed a significant change ((a) $P = 0.032$, (b) n.s., and (c) n.s.).

3.3. Significant Improvements in Each Item in the IES-R. The IES-R items that differed significantly between groups were as follows: "any reminder brought back feelings about it" (Q1, $P < 0.001$); "other things kept making me think about it" (Q3, $P = 0.005$); "I thought about it when I did not mean to" (Q6, $P < 0.001$); "I found myself acting or feeling like I was back in that time" (Q14, $P = 0.003$); "reminders of it caused me to have physical reactions, such as sweating, trouble breathing, nausea, or a pounding heart" (Q19, $P = 0.001$); "I had dreams about it" (Q20, $P = 0.002$); and "I felt watchful and on-guard" (Q21, $P = 0.001$).

3.4. Adverse Events and Follow-Up. No participant showed any abnormal change in the blood examination results. One participant in the SKK group withdrew from the study on the third day because of light coughing. The cough was minor and continued for 3 days. When he came back to the clinic 2 weeks later, he showed no symptoms or signs. The relationship between the treatment and the cough is unknown. After the trial, some participants in the control group were treated using traditional medicines according to the rules of the traditional Chinese-Japanese medical decision. They were treated with different medicines, including SKK. No systematic data are available.

4. Discussion

The present study showed that SKK significantly improved PTSD caused by the disaster. A recent study showed that PTSD was strongly suspected in about 10% of all high school students in the city of Sendai at 9 months after the Great East Japan earthquake [3]. Both psychological and pharmacological treatments are effective for PTSD, but there is a severe shortage of psychologists and psychotherapists in the disaster-stricken area. General physicians and primary care doctors must routinely care for PTSD patients. Antidepressants, benzodiazepine, and antipsychotics are used to treat PTSD, but these medications have adverse effects. Drug dependency is common with the use of benzodiazepine. Selective serotonin reuptake inhibitors are also used to treat PTSD, but continuous treatment is often necessary to prevent relapse. Better pharmacological treatments that are both safe and effective are thus needed. Since the disaster, we have tried traditional herbal medicines, such as yokukansan (Yigan San), known to be effective for the treatment of mental disorders [4]. We observed that patients treated with SKK showed very clear improvements in PTSD symptoms. These clinical observations lead us to this trial. This study shows that SKK treatment resulted in the marked, rapid, and tolerable amelioration of PTSD symptoms in all participants, with no severe adverse events. In particular, SKK improved patient responses to the following items: "any reminder brought back feelings about it," "other things kept making me think about it," "I thought about it when I did not mean to," "I found myself acting or feeling like I was back in that time," "reminders of it caused me to have physical reactions, such as sweating, trouble breathing, nausea, or a pounding heart," and "I felt watchful and on-guard." These complaints may thus be indications for trying this medication. SKK also improved patient responses to "I had dreams about it." Sleep disturbance is commonly resistant to treatment [5] and can lead to drug abuse [6].

Several pharmacological mechanisms underlying the effects of SKK have been investigated [7–9]. Acute moderate to high stress activates serotonergic neurons in the hippocampus to release 5-hydroxytryptamine (5HT). 5HT then activates postsynaptic 5HT1A receptors that inhibit the process of hippocampal long-term potentiation [7]. The repeated administration of SKK significantly increases the 5HT level in the hippocampus and the corpus striatum and of NE and 5HT in the hippocampus [8]. SKK also regulates plasma interleukin-6 and soluble interleukin-6 receptor concentrations and improves depressed mood in climacteric women with insomnia [9]. These findings may partially explain the mechanisms of SKK action in the treatment of mental disorders.

4.1. Limitations. The present study has some limitations. First, we could not include a placebo for comparison. Japanese law requires that if a placebo is used, it must be produced using the same procedures as the actual drug. Placebo preparation is thus impractical in Japan for a small pilot study like ours. Placebo effects may have influenced our data. The number of participants in this study was rather small but nonetheless reached the statistically targeted sample size. The observation time was short (14 days), because this was the maximum waiting time allowed for participants in the control group. No serious adverse events were observed in the present study, which is noteworthy when considering that Radix Scutellariae Baicalensis is suspected of causing interstitial pneumonia [10]; this relationship is unclear because incidents are rare. Hypokalemia caused by *Glycyrrhiza* root is well known, and it is observed in 6% of elderly subjects [11]. Clinical signs should be carefully monitored because SKK contains these herbs. More elaborate studies are required in the future to elucidate the effect and mechanism of SKK on patients.

Despite these limitations, the present study reports that the familiar herbal medicine SKK is significantly efficacious for the treatment of PTSD, with no serious adverse events. This information may be valuable for general physicians and primary care doctors who care for PTSD patients in Japan because SKK is a well-known traditional herbal medicine. The history of its clinical use in eastern countries dates back to 2 AD. We previously reported that traditional medicine was beneficial in the disaster-stricken area, where the modern medical system and its infrastructures, such as electricity, hospitals, clinics, and logistics, were entirely destroyed [12–14]. In such a situation, traditional medicine could be used to treat daily symptoms that manifest as physical findings among evacuees. Traditional medicine should be considered as a powerful tool for the practice of disaster medicine.

5. Conclusions

SKK significantly improved IES-R scores after 2 weeks of treatment. This traditional medicine may be a treatment choice for psychological and physical symptoms in PTSD patients.

Abbreviations

PTSD: Posttraumatic stress disorder
IES-R: Impact of Event Scale-Revised score
DSM: Diagnostic and Statistical Manual
SKK: Saikokeishikankyoto
ANOVA: Analysis of variance.

Conflict of Interests

Shin Takayama has no personal competing financial or nonfinancial interests in this study; however, the Tohoku University Graduate School of Medicine did receive a grant from Tsumura, a Japanese manufacturer of Kampo medicine.

Authors' Contribution

Takehiro Numata took part in planning the study, performed the data analysis, and provided advice for writing the paper. Shen GunFan helped to plan the study and performed the data analysis. Shin Takayama took part in planning the study, provided advice for statistical analysis, and wrote the

paper. Satomi Takahashi and Yasutake Monma performed the data analysis. Soichiro Kaneko, Hitoshi Kuroda, Junichi Tanaka, Seiki Kanemura, and Masayuki Nara helped to plan the study and provided advice related to writing the paper. Yutaka Kagaya, Tadashi Ishii, Masahiro Kohzuki, and Nobuo Yaegashi were responsible for the study design and execution and assisted in writing the paper. Koh Iwasaki proposed the study. All authors read and approved the final paper. Takehiro Numata and Shen GunFan equally contributed to this work.

Acknowledgment

References

[1] D. Nishi, Y. Koido, N. Nakaya et al., "Peritraumatic Distress, watching television, and posttraumatic stress symptoms among rescue workers after the Great Japan earthquake," *PLOS ONE*, vol. 7, no. 4, 2012.

[2] X.-Z. Meng, F. Wu, P.-K. Wei et al., "A Chinese herbal formula to improve general psychological status in posttraumatic stress disorder: a randomized placebo-controlled trial on Sichuan earthquake survivors," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 691258, 11 pages, 2012.

[3] T. Takeda, M. Tadakawa, S. Koga et al., "Premenstrual symptoms and posttraumatic stress disorder in Japanese high school students 9 months after the Great East Japan Earthquake," *The Tohoku Journal of Experimental Medicine*, vol. 230, no. 3, pp. 151–154, 2013.

[4] K. Iwasaki, T. Satoh-Nakagawa, M. Maruyama et al., "A randomized, observer-blind, controlled trial of the traditional Chinese medicine Yi-Gan San for improvement of behavioral and psychological symptoms and activities of daily living in dementia patients," *Journal of Clinical Psychiatry*, vol. 66, no. 2, pp. 248–252, 2005.

[5] T. C. Neylan, C. R. Marmar, T. J. Metzler et al., "Sleep disturbances in the Vietnam generation: findings from a nationally representative sample of male Vietnam Veterans," *American Journal of Psychiatry*, vol. 155, no. 7, pp. 929–933, 1998.

[6] H. D. Chilcoat and N. Breslau, "Posttraumatic stress disorder and drug disorders: testing causal pathways," *Archives of General Psychiatry*, vol. 55, no. 10, pp. 913–917, 1998.

[7] F. Oosthuizen, G. Wegener, and B. H. Harvey, "Nitric oxide as inflammatory mediator in post-traumatic stress disorder (PTSD): evidence from an animal model," *Neuropsychiatric Disease and Treatment*, vol. 1, no. 2, pp. 109–123, 2005.

[8] T. Itoh, S. Michijiri, S. Murai et al., "Effects of Chaihu-Guizhi-Ganjiang-Tang on the levels of monoamines and their related substances, and acetylcholine in discrete brain regions of mice," *American Journal of Chinese Medicine*, vol. 24, no. 1, pp. 53–64, 1996.

[9] T. Ushiroyama, A. Ikeda, K. Sakuma, and M. Ueki, "Chai-hu-gui-zhi-gan-jiang-tang regulates plasma interleukin-6 and soluble interleukin-6 receptor concentrations and improves depressed mood in climacteric women with insomnia," *American Journal of Chinese Medicine*, vol. 33, no. 5, pp. 703–711, 2005.

[10] K. Takeshita, Y. Saisho, K. Kitamura et al., "Pneumonitis induced by ou-gon (scullcap)," *Internal Medicine*, vol. 40, no. 8, pp. 764–768, 2001.

[11] K. Iwasaki, K. Kosaka, H. Mori et al., "Open label trial to evaluate the efficacy and safety of yokukansan, A traditional Asian medicine, in dementia with Lewy bodies," *Journal of the American Geriatrics Society*, vol. 59, no. 5, pp. 936–938, 2011.

[12] S. Takayama, T. Kamiya, M. Watanabe et al., "Report on disaster medical operations with acupuncture/massage therapy after the great East Japan earthquake," *Integrative Medicine Insights*, vol. 7, pp. 1–5, 2012.

[13] S. Takayama, R. Okitsu, K. Iwasaki et al., "The role of oriental medicine in the great East Japan Earthquake disaster," *Kampo Medicine*, vol. 62, no. 5, pp. 621–626, 2011 (Japanese).

[14] S. Takayama, R. Okitsu, K. Iwasaki et al., "Role of oriental medicine in the great East Japan earthquake," *German Journal of Acupuncture and Related Tequniques*, vol. 55, no. 2, 2012 (German).