Worker honeybees produce royal jelly (RJ) in their hypopharyngeal and mandibular glands (8). RJ has been used worldwide for many years as commercially available medical products and health foods and has been considered beneficial to health. These days, a modified RJ product, protease-treated RJ (pRJ), has been developed to improve digestion and absorption compared with regular RJ. Accumulating evidence suggests that RJ is rich in a wide variety of nutrients, including vitamins, minerals, and more than 20 amino acids (9). RJ also has numerous potential pharmacological capacities, such as prolonging the life span (in mice and nematodes) (10,11) and reducing fatigue (12), hypertension (13), and hypercholesterolemia, as well as antioxidant and anti-inflammatory effects (8,14–16).

Because these effects of RJ might have a potential to improve sarcopenia and the functions of satellite cells (17–21), we hypothesized that RJ might have a beneficial effect on the prevention of sarcopenia. Furthermore, we hypothesized that this effect might involve IGF-1. To the best of our knowledge, few studies have examined the effects of RJ on muscles in elderly patients or aged animals or the relationship between RJ and IGF-1. Thus, in this study, we examined the effects of the RJ/pRJ on muscle weight, muscle strength, satellite cell functions, the regenerating capacity of the skeletal muscles in vivo and in vitro, and the involvement of IGF-1 in an animal model using aged mice.

Methods

Culture Conditions of Satellite Cells and Cell Proliferation Assay

Sorted satellite cells from untreated, aged mice were cultured in growth medium containing high-glucose Dulbecco's modified Eagle's medium with 20% fetal bovine serum (MP Biomedicals, Morgan Irvine, CA), 2.5 ng/mL basic fibroblast growth factor (Invitrogen, Eugene, OR), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma, St. Louis, MO). Satellite cells under eight passages were used in this study. Differentiation was induced as previously shown with some modifications in differentiation medium containing high-glucose Dulbecco's modified Eagle's medium, 5% horse serum (Sigma), penicillin, and streptomycin for several days (22). RJ and pRJ were dissolved in water, sterilized by a filter, and then added to the culture medium at the following concentrations: 100, 200, 500, or 1000 μg/mL. Some cells were serum starved for overnight and then stimulated with 10 nM insulin (Sigma), which is a potent activator of Akt, for 5 minutes. The cells were cultured for 24, 48, or 72 hours, and the number of cells was determined by water-soluble tetrazolium-8 (WST-8, DOJINDO, Tokyo, Japan) assay using a cell-counting kit (23,24).

Mice and Dietary 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 guidelines established by the Tohoku University Committee on Animal Research. At the age of 21 months, mice were divided into five groups according to the diets provided for each group and maintained for the next 3 months, with 10 mice in each group. The five groups of diets were normal diet (controls), diet mixed with 1% weight RJ (1% RJ), diet mixed with 5% weight RJ (5% RJ), diet mixed with 1% weight pRJ (1% pRJ), and diet mixed with 5% pRJ (5% pRJ). All diets were manufactured by Oriental Yeast Co., Ltd. (Chiba, Japan), stored at 4°C, and sealed in plastic bags in vacuo until use to avoid oxidation. The base diet was composed of 20% milk casein, 0.3% cystine, 39.7% starch, 13.2% a-starch, 10% sucrose, 0.0014% cellulose, 1% vitamins, 3.5% mineral mixture, 0.25% choline bitartrate, and 0.5% *tert*-butylhydroquinone. The amounts of milk casein and starch were adjusted to equalize total proteins and calories between the groups in accordance with the amounts of added RJ/pRJ. Therefore, total energy and protein levels per weight were the same in all the diet groups. However, the amino acid contents were different among the groups. Dried RJ and pRJ powder was supplied by Institute for Bee Products & Health Science (Okayama, Japan). The vitamin and mineral components of RJ and pRJ were analyzed by Japan Food Research Laboratories (Tokyo, Japan) and are shown in Table 1. The mice had unrestricted access to food and water. After 3 months of the diet treatment, the grip strength was measured. Then, 25 mice (five mice from each group) were anesthetized and sacrificed, their sera were collected, and skeletal muscle samples were isolated. The other 19 (≥ 3 mice from each group) mice were sacrificed for evaluation of the regenerating capacity of injured skeletal muscles at 5 days after the injury.

Wire Hang Test

A wire mesh grid (10 × 10 cm) was used to assess the muscle strength. The mouse was placed on the wire mesh, then the mesh was inverted, and the mouse was forced to hang on the wire using its four limbs. The longest hanging time was recorded as the duration. The previously mentioned measuring process was repeated until the mouse could not hang on the wire mesh after the inversion. The number of repeated times is shown as the number of times (25).

Muscle Injury Model

After 3 months of the diet treatment, mice were anesthetized, and cardiotoxin from *Naja mossambica mossambica* (Sigma) dissolved in 100 μL phosphate-buffered saline (PBS) (10 μM) was injected into the tibialis anterior (TA) muscle. Five days later, the mice were sacrificed; and the

Table 1. Vitamin and Mineral Composition of Royal Jelly Products (mg/100 g)

Components	Royal Jelly	Protease-Treated Royal Jelly
Minerals		
Sodium	40.2	2050
Phosphorus	662	580
Iron	3.21	2.8
Calcium	40.6	44.8
Potassium	814	766
Magnesium	90.9	74.3
Copper	1.34	0.91
Zinc	6.61	5.62
Manganese	0.22	0.17
Selenium	>0.005	0.006
Vitamins		
Thiamine	0.96	0.84
Riboflavin	2.03	1.92
Vitamin B6	1.12	0.63
α-Tocopherol	0.1	>0.1
Folic acid	0.12	0.06
Pantothenic acid	12.8	14.5
Biotin	0.0467	0.0722
Inositol	>2	41
Niacin	14.8	15.4
Choline	620	480

TA muscles were isolated, frozen in 2-methylbutane precooled in liquid nitrogen, and stored at −80°C for following histological analysis (26).

Measurements of Muscle Weight and Isolation of Satellite Cells

The satellite cells were isolated according to a previous study (6) with some modifications. The large hindlimb muscles of mice including the TA muscle, triceps surae muscle, quadriceps muscle, biceps femoris muscle, gluteus maximus muscle, and iliopsoas muscle were isolated, and the weights of the muscles were measured. Next, nonmuscle tissues were removed under a dissection microscope; the muscles were subjected to enzymatic dissociation with 0.2% collagenase Type II (Worthington Biochemical Corporation, Lakewood, NJ) for 60 minutes and then with 0.04 U/mL dispase (Gibco BRL, Grand Island, NY) for 45 minutes. The cell suspension was filtered through a cell strainer (BD Bioscience, Franklin Lakes, NJ), incubated with antimouse CD16/CD32 monoclonal antibody (mAb, 2.4G2, BD Bioscience) to block Fc receptors and then with the following antibodies: fluorescein isothiocyanate–labeled anti-CD31, anti-CD45 (BD Bioscience), anti-CD11b, and anti-Sca-1 antibodies (eBioscience, San Diego, CA); PE-labeled anti-integrin-α7 (MBL, Nagoya, Japan); and Alexa 647-labeled anti-CD34 (BD Bioscience). The cells were counted and sorted by FACSAria II flow cytometer (BD Bioscience) as previously shown (27).

Immunohistochemistry and Immunocytochemistry

Frozen muscle tissues were sectioned from a region approximately 3 mm from the top of the TA muscle (8 μm in thickness) using a cryostat. For embryonic myosin heavy chain (eMyHC) staining, frozen sections or cultured cells were fixed with acetone/methanol (50%/50%) for 30 seconds at −20°C. Specimens were blocked with 1% bovine serum albumin and 0.1% Triton X-100 in PBS at room temperature for 45 minutes and then incubated with anti-eMyHC antibody (F1.652, DSHB, Iowa City, IA) at 1:2 dilution at 4°C overnight, followed by Rodamine-conjugated secondary antibody staining (Chemicon International, Temecula, CA) at room temperature in the dark for 1 hour. For PAX7 staining, cultured cells were fixed with PBS containing 4% paraformaldehyde at room temperature for 20 minutes and then blocked with 1% bovine serum albumin and 0.1% Triton X-100 in PBS at room temperature for 45 minutes. After blocking, the cells were incubated with anti-Pax-7 antibody (R&D Systems, Minneapolis, NE) at 1:50 dilution at 4°C overnight followed by Alexa 488–coupled antimouse IgG antibody (Invitrogen) at 1:200 dilution at room temperature for 1 hour. Finally, the sections or cells were mounted in Vectashield Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector labs, Burlingame, CA). In vivo, the regenerating capacity of the injured skeletal muscles was evaluated by quantifying the percentage of eMyHc-immunoreactive area per field (28). Ten randomly selected fields at ×200 magnification were measured in each sample. ImageJ software was used to quantify the eMyHC-immunoreactive areas per field. In vitro, the degree of differentiation of satellite cells of the aged mice was evaluated by the maximum diameter of the cells by Adobe Photoshop CS2 software (San Jose, CA). The muscle sections were stained for hematoxylin and eosin also. Images were taken using a phase-contrast and fluorescence microscope BZ9000 (Keyence, Osaka, Japan) (29).

Western Blot Analysis

PAX7, Type I IGF receptor (IGF-IR), Akt, and phosphorylated Akt (phospho-Akt) proteins were detected by Western blot analysis. In brief, the cells were rinsed twice with ice-cold PBS and lysed using RIPA Lysis Buffer (Upstate, Temecula, CA). The extracted protein fraction was electrophoresed in a sodium dodecyl sulfate and 10% polyacrylamide gel and then transferred onto an Immobilon transfer membrane (Millipore, Bedford, MA). The amount of protein loaded onto the gels was 36 μg per well. The membranes were immunoblotted with the primary antibodies to PAX7 (DSHB) at 1:100 dilution, GAPDH, IGF-IR, Akt, and phospho-Akt (Cell Signaling, Boston, MA) at 1:1000 dilution. Then, the membranes were incubated with horseradish peroxidase–conjugated antirabbit immunoglobulin G (Cell Signaling) at 1:25,000 dilution, and the protein bands were detected with an

enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK) (30).

Enzyme-Linked Immunosorbent Assay

After RJ/pRJ treatment, the mice were anesthetized with diethyl ether, and blood samples were isolated from the inferior vena cava. The serum levels of interleukin-1α (IL-1α), IL-1β, IL-6, tumor necrosis factor-α, and IGF-1 were measured using a specific ELISA kit (R&D Systems) according to the manufacturer's instructions, respectively (22).

Statistical Analysis

Data are presented as mean ± standard deviation. Differences were analyzed by one-way analysis of variance test (Post hoc, Tukey). A level of $p < 0.05$ was accepted as statistically significant. All in vitro experiments were repeated at least three times.

Results

Isolation and Characterization of Satellite Cells

As an initial step, we tried to identify the effect of RJ/pRJ on satellite cells. The characterization of satellite cells by cell surface markers has been established only very recently (6). Therefore, according to that study, we first tried to isolate satellite cells with some modifications. We enzymatically dissociated mononuclear cells from the mouse hind-limb muscles (Figure 1A, circle: upper muscles were isolated from a right leg, and lower muscles were isolated from a left leg, from left to right, TA, triceps surae, quadriceps, divided biceps femoris into two, gluteus maximus, and

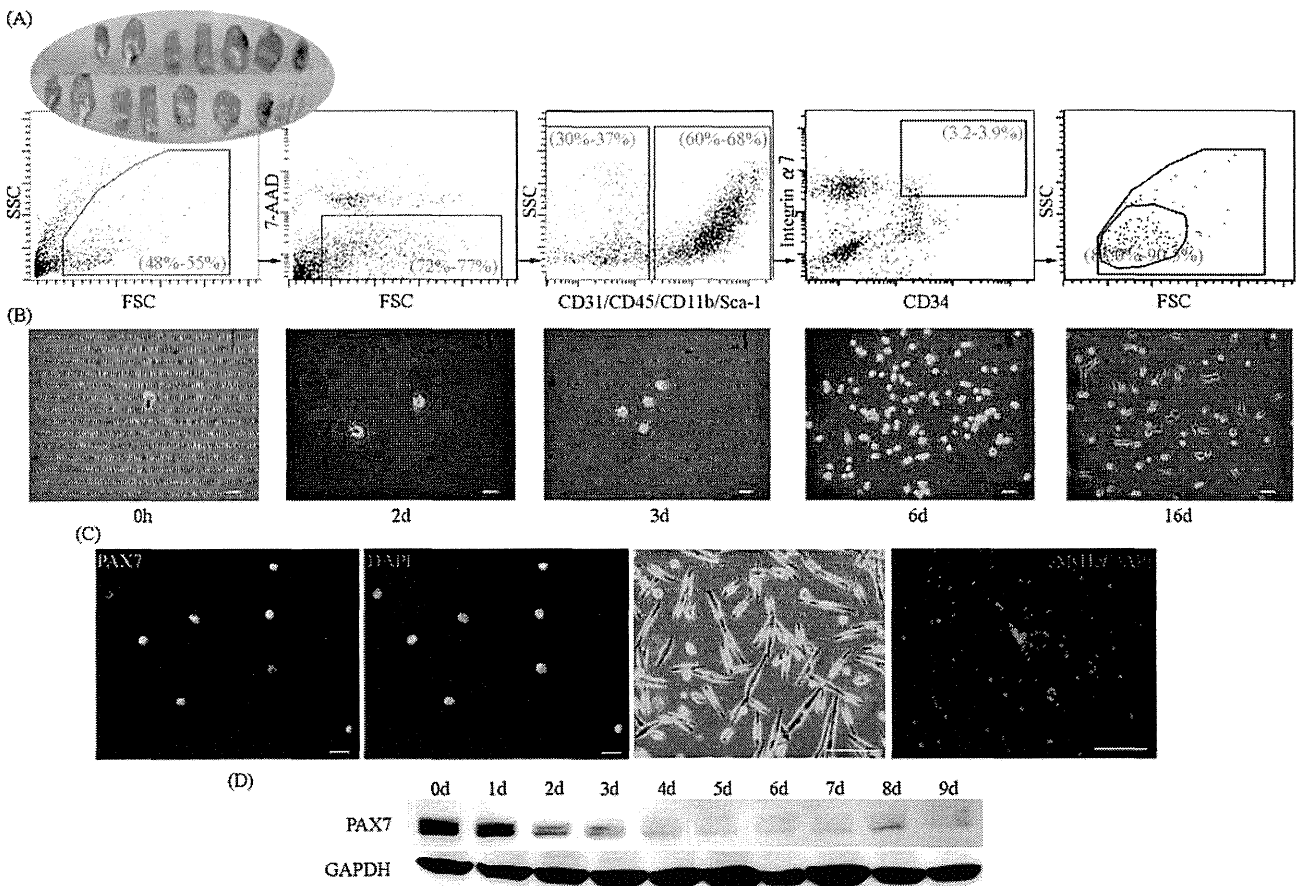

Figure 1. The isolation and characterization of the satellite cells. (**A**) A flow cytometer sorted the satellite cells from the hind-limb muscles after enzymatic dissociation (shown in circle on the left: upper muscles were isolated from a right leg and lower muscles were isolated from a left leg; from left to right: tibialis anterior, triceps surae, quadriceps, divided biceps femoris into two, gluteus maximus, and iliopsoas muscles) by gating for 7-AAD negative, then for CD31, CD45, CD11b, and Sca1 negative, and finally for integrin-α7 and CD34 positive. Numbers in the gates show percentage of the cells in each gate among total cells. (**B**) The light phase-contrast microscopy shows the morphology of the isolated satellite cells cultured in proliferation medium for the indicated time periods. Scale bars: 50 μm (left three panels) and 25 μm (right two panels). (**C**) The sorted cells were immunoreactive with satellite cell marker Pax7 and nucleus marker DAPI after 2 days in the proliferation medium (left two panels: Pax7 in green and DAPI in blue). After 3 days in the differentiation medium, some cells formed tube-like shapes (the third panel from the left) and some cells were immunoreactive with an immature myotube marker eMyHc (right panel: eMyHc in red and DAPI in blue). Scale bars: 20 μm (left two panels) and 50 μm (right two panels). (**D**) Western blot analysis shows the levels of PAX7 protein in the satellite cells after the induction of differentiation for the indicated time periods. The GAPDH protein is a loading control.

iliopsoas muscle) and sorted them according to the cell surface markers (Figure 1A). We characterized satellite cells as 7-AAD (a dead cell marker) negative, CD31 (an endothelial cell marker) negative, CD45 (a pan-hematopoietic cell marker) negative, CD11b (a myeloid cell marker) negative, Sca1 (a mesenchymal cell marker) negative, and integrin-α7 and CD34 positive (Figure 1A). We cultured the sorted cells in growth medium for several days and noted the proliferation of these cells, which suggested that these cells had the potential to re-enter the cell cycle (Figure 1B). After 2 days in the growth medium, the sorted cells were immunoreactive with satellite cell–specific transcriptional factor Pax7 and nucleus marker DAPI (Figure 1C, two panels in the left). To examine the potential of these cells to differentiate into myotubes, we cultured the cells in differentiation medium for 3 days (Figure 1C, two panels in the right). The cells fused and were immunoreactive with an immature myotube marker embryonic myosin heavy chain (eMyHc, Figure 1C, the right panel), suggesting that the cells differentiated into myotubes. The sorted cells were cultured in the differentiation medium, and the expression levels of Pax7 gradually decreased in a time-dependent manner after the induction of differentiation (Figure 1D). These data suggested that the sorted cells had the characteristics of satellite cells and the potential to differentiate into myotubes.

Effects of RJ/pRJ on the Satellite Cells of the Aged Mice In Vitro

To examine the effect of RJ/pRJ 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 RJ/pRJ for 24 hours (Figure 2A, left panel), 48 hours (Figure 2A, center panel), or 72 hours (Figure 2A, right

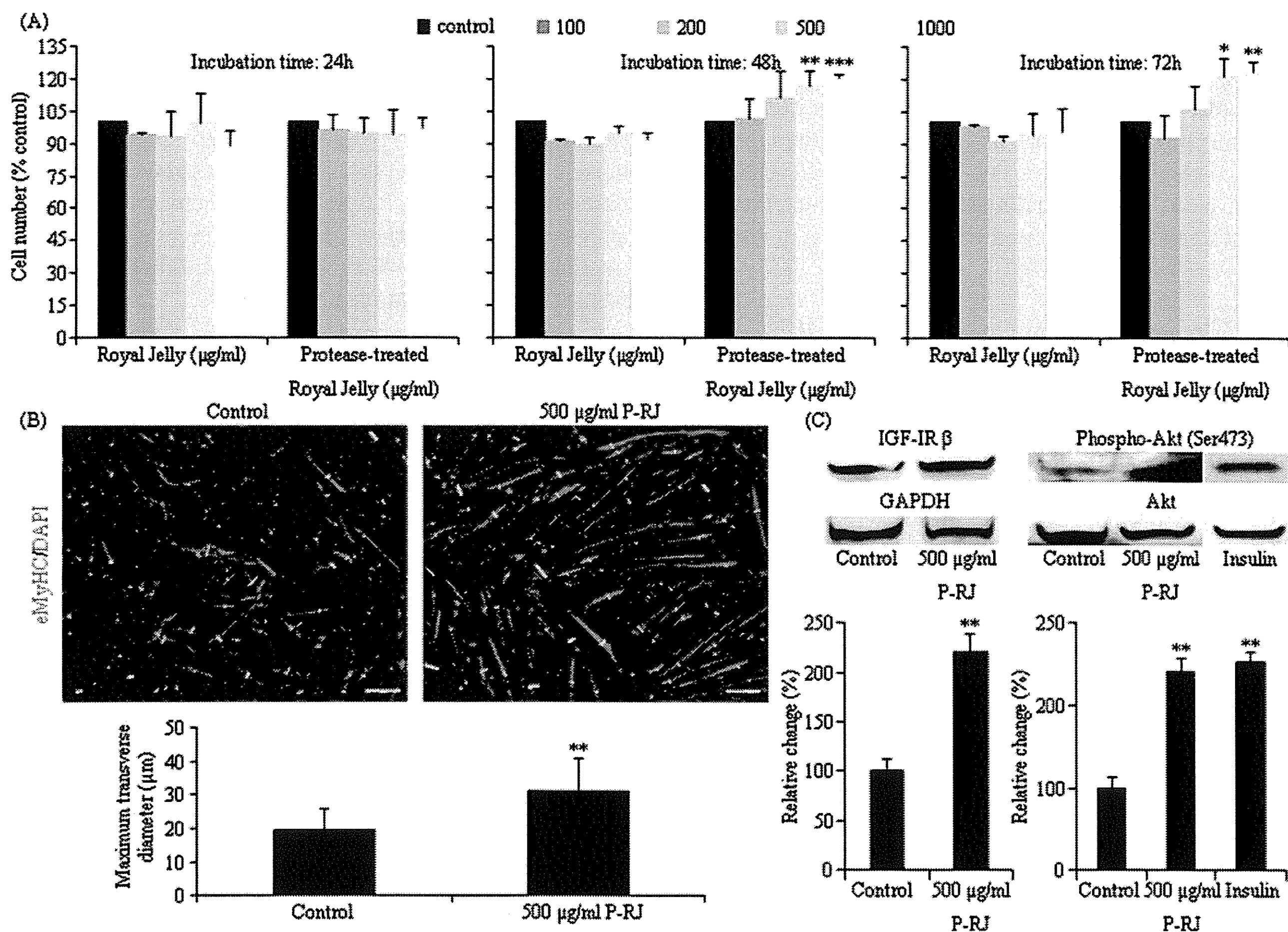

Figure 2. Effects of RJ/pRJ on the satellite cells of aged mice in vitro. (**A**) The satellite cells of the aged mice were treated with the indicated concentrations of RJ or pRJ for 24, 48, or 72 h, and the cell proliferation rate was measured at each time point. (**B**) The satellite cells of aged mice were cultured in differentiation medium with pRJ or without pRJ (control) for 5 days, then immunohistochemically stained for eMyHC in red and for DAPI in blue to evaluate their differentiation into myotubes. The maximum diameter of each myotube was marked with a green line (upper panels). We randomly selected 50 myotubes per field at ×400 magnification, measured the maximum diameter of each myotube for 10 randomly selected fields per sample, measured the diameter, and calculated for each group (lower panel). Scale bars: 100 μm. (**C**) The satellite cells of aged mice were pretreated with pRJ (500 μg/mL) for 48 h, then the Western blot analysis detected IGF-1 receptor (IGF-IR), GAPDH, activated form of Akt (phospho-Akt), and total Akt. Insulin (10 nM) was used as a positive control. The densitometry quantified the band intensities. The graphs show the IGF-IR band intensities normalized by the GAPDH band intensities, and phospho-Akt band intensities normalized by the Akt band intensities. This figure is the representative of three independent experiments. Columns are mean ± *SD*. *$p < .05$, **$p < .01$, and ***$p < .0001$, compared with control.

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.

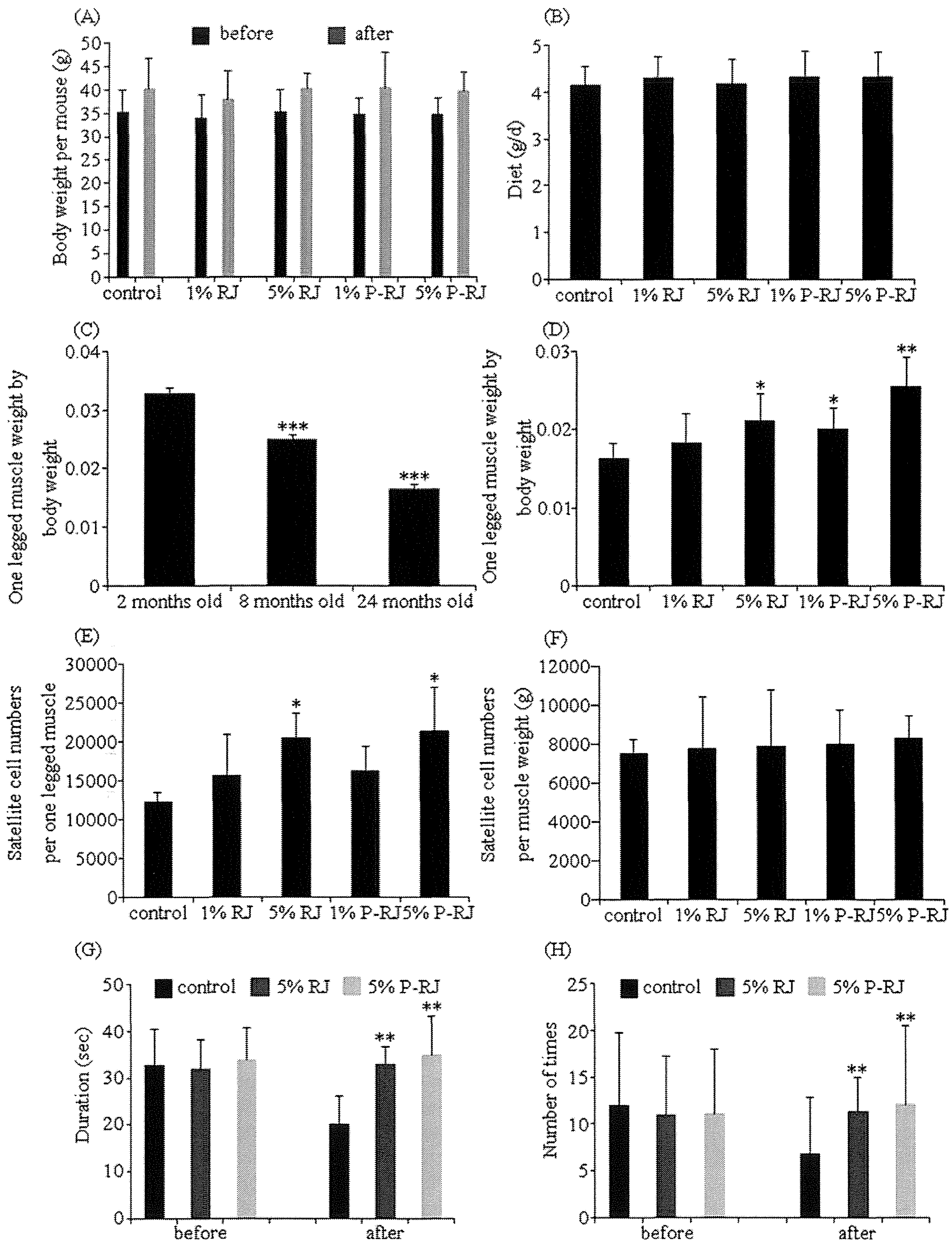

Figure 3. Effects of RJ/pRJ treatment on aged mice in vivo. Twenty-one-month-old mice were treated with a diet mixed with 1% weight RJ (1% RJ), diet with 5% weight RJ (5% RJ), diet with 1% weight pRJ (1% pRJ), or diet with 5% pRJ (5% pRJ) for following 3 months. (**A**) Control, RJ, or pRJ treatment 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.

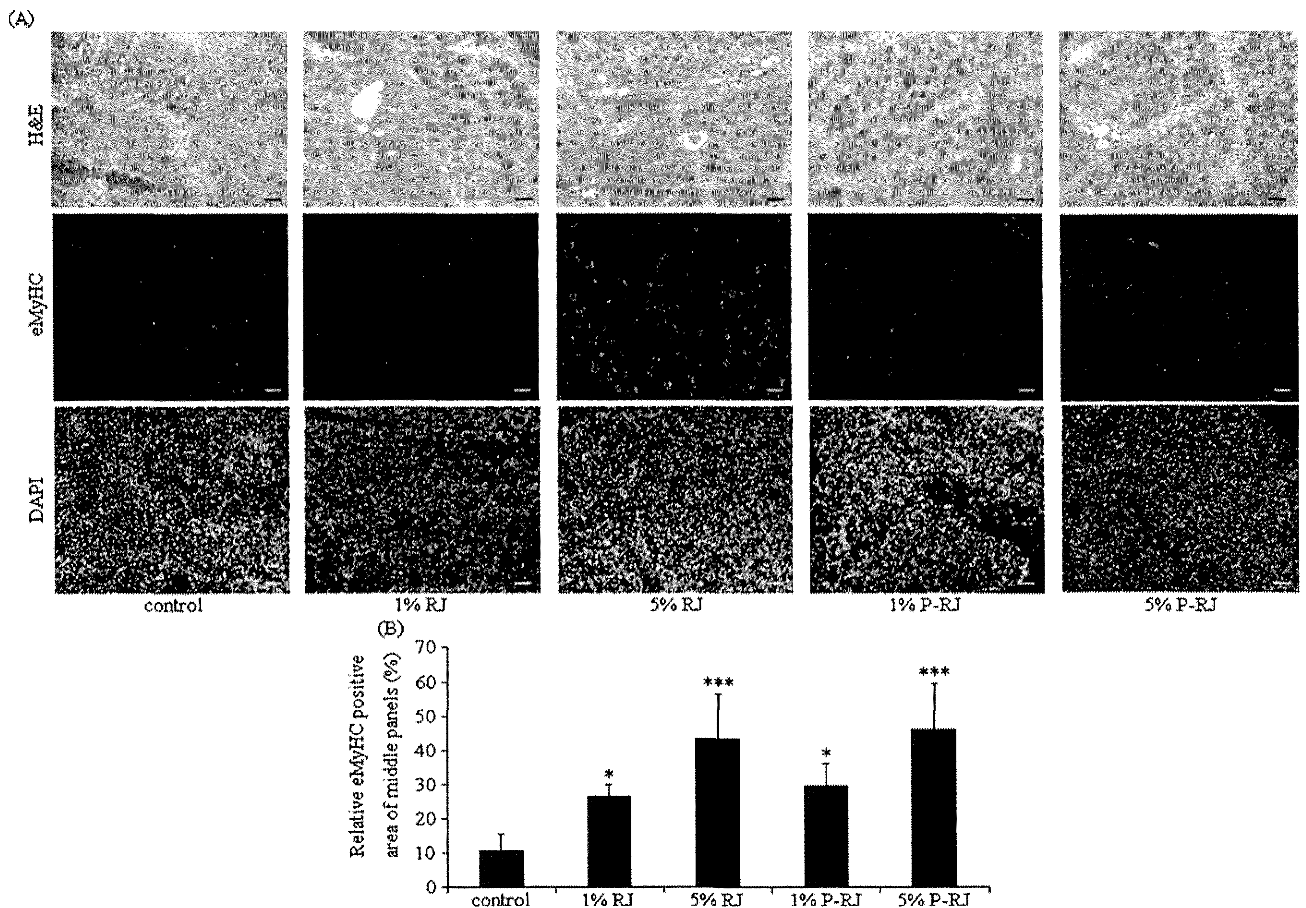

Figure 4. RJ/pRJ treatment accelerated the regeneration of the injured skeletal muscles in aged mice. After 3 months of RJ/pRJ treatment, we injected cardiotoxin into the tibialis anterior muscles of the aged mice to injure the muscles and isolated them 5 days later. (**A**) Hematoxylin and eosin staining (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*, $n \geq 3$ 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

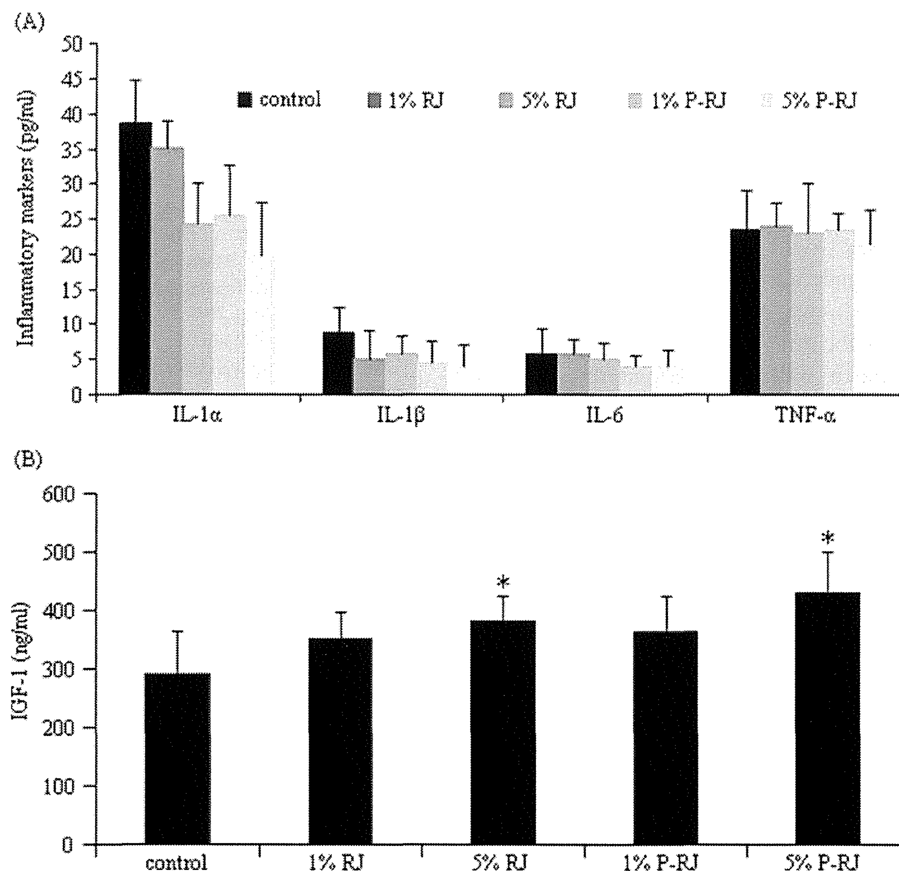

Figure 5. RJ/pRJ treatment increased the serum IGF-1 levels but did not affect the serum proinflammatory mediator levels. ELISA determined serum levels of proinflammatory mediators and IGF-1 in aged mice treated with RJ/pRJ for 3 months. (**A**) RJ/pRJ treatment did not change the serum levels of the 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.

Funding

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Conflict of Interests

All the authors have no conflicts of interest to disclose.

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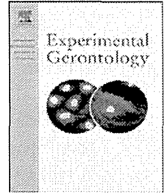

Coffee treatment prevents the progression of sarcopenia in aged mice in vivo and in vitro

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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.

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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).

Abbreviations: TNF-α, tumor necrosis factor-alpha; IL-6, interleukin 6; TA, tibialis anterior; eMyHC, embryonic myosin heavy chain.

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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 $\alpha7$ (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

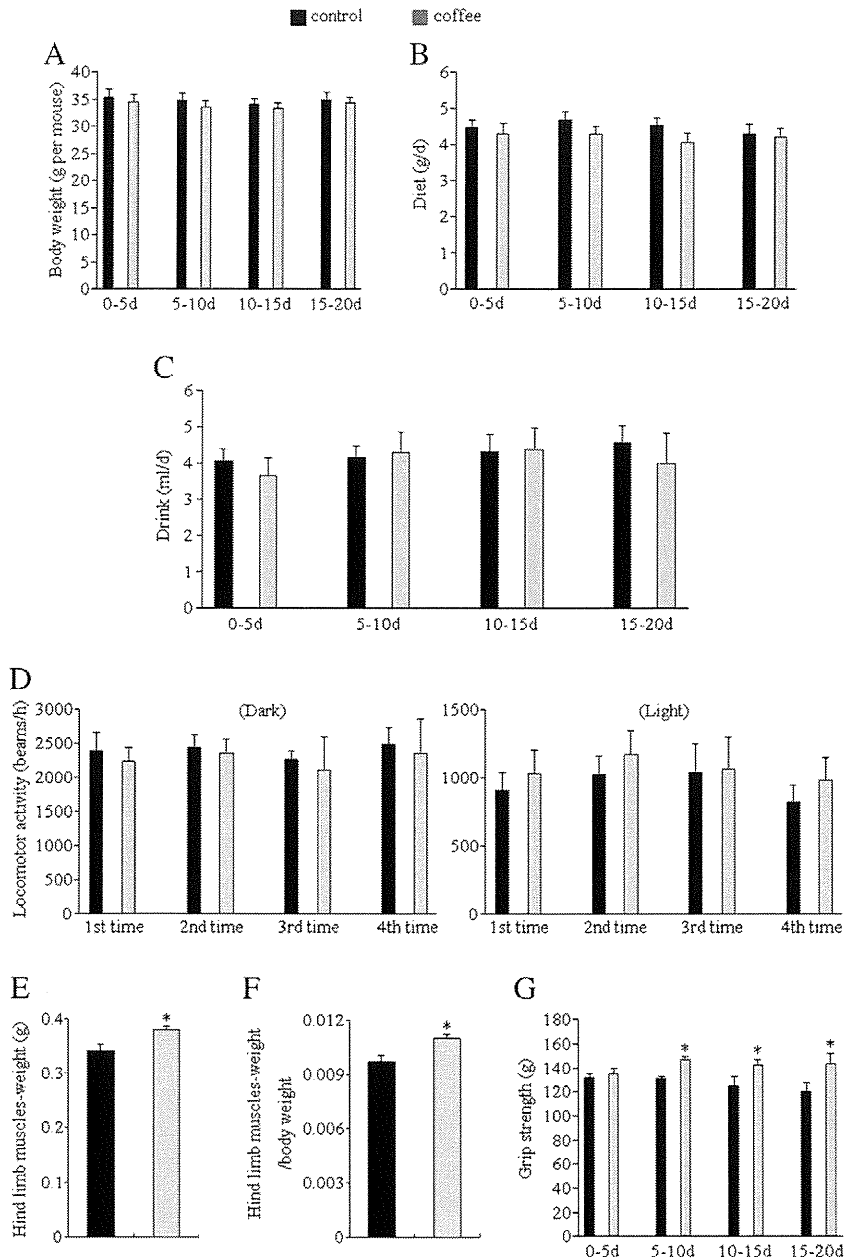

Fig. 1. Effects of coffee treatment on aged mice in vivo. Twenty-seven month-old mice were treated with coffee for 4 weeks. (A–C) Coffee treatment did not change body weight (A), the amount of the daily diet intake (B), or the volume of water intake (C). (D) Coffee did not change the daily locomotor 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 ≥ 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

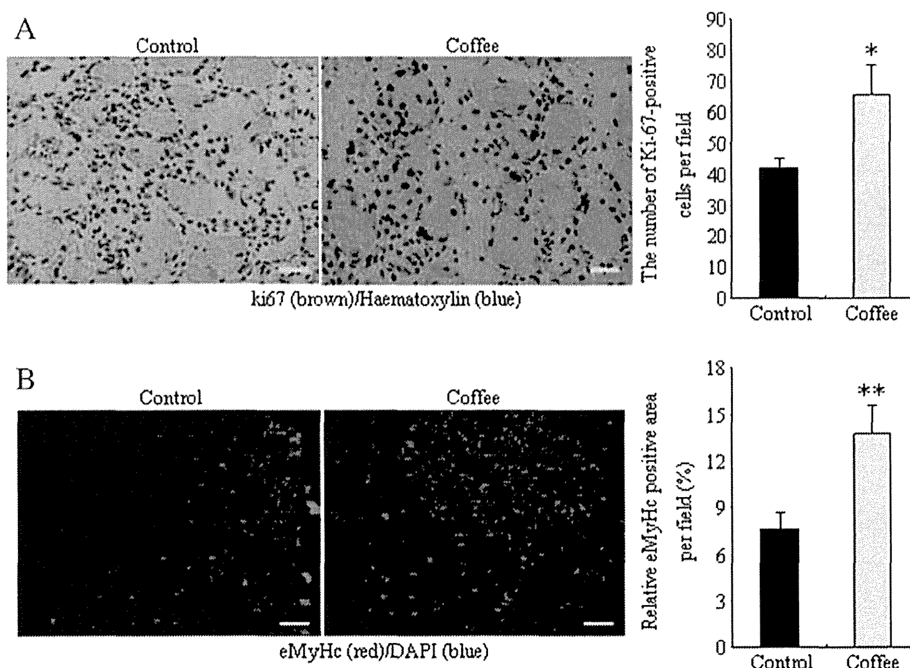

Fig. 2. Coffee treatment accelerated the regeneration of the injured skeletal muscles in aged mice. After 4 weeks of coffee treatment, we injected cardiotoxin into the tibialis anterior muscles of the mice to injure the muscles and isolated them 3 days later for (A) or 5 days later for (B). (A) The left 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

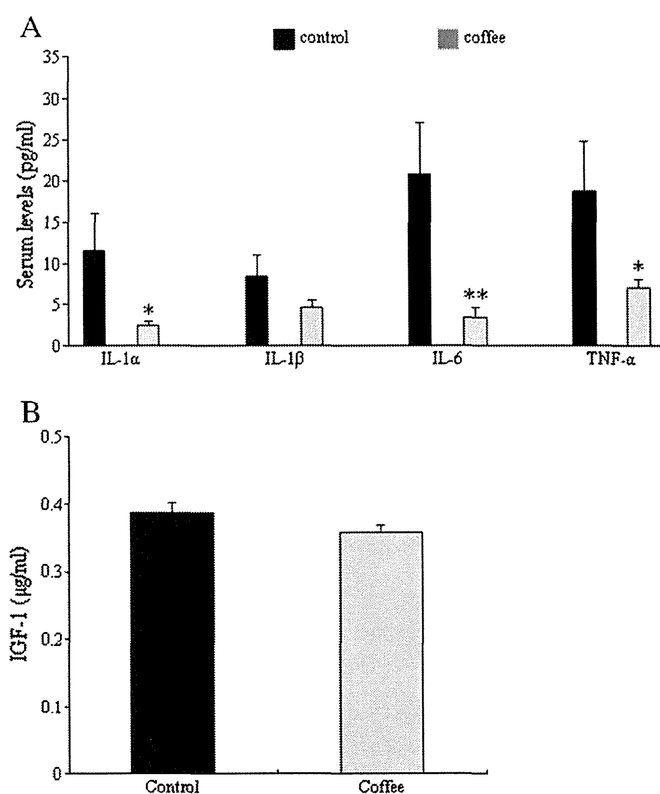

Fig. 3. Coffee treatment decreased the serum pro-inflammatory mediator levels, but did not affect the serum IGF-1 levels. ELISA determined serum levels of pro-inflammatory mediators and IGF-1 in aged mice treated with coffee for 4 weeks. (A) Coffee treatment significantly decreased the serum levels of 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.

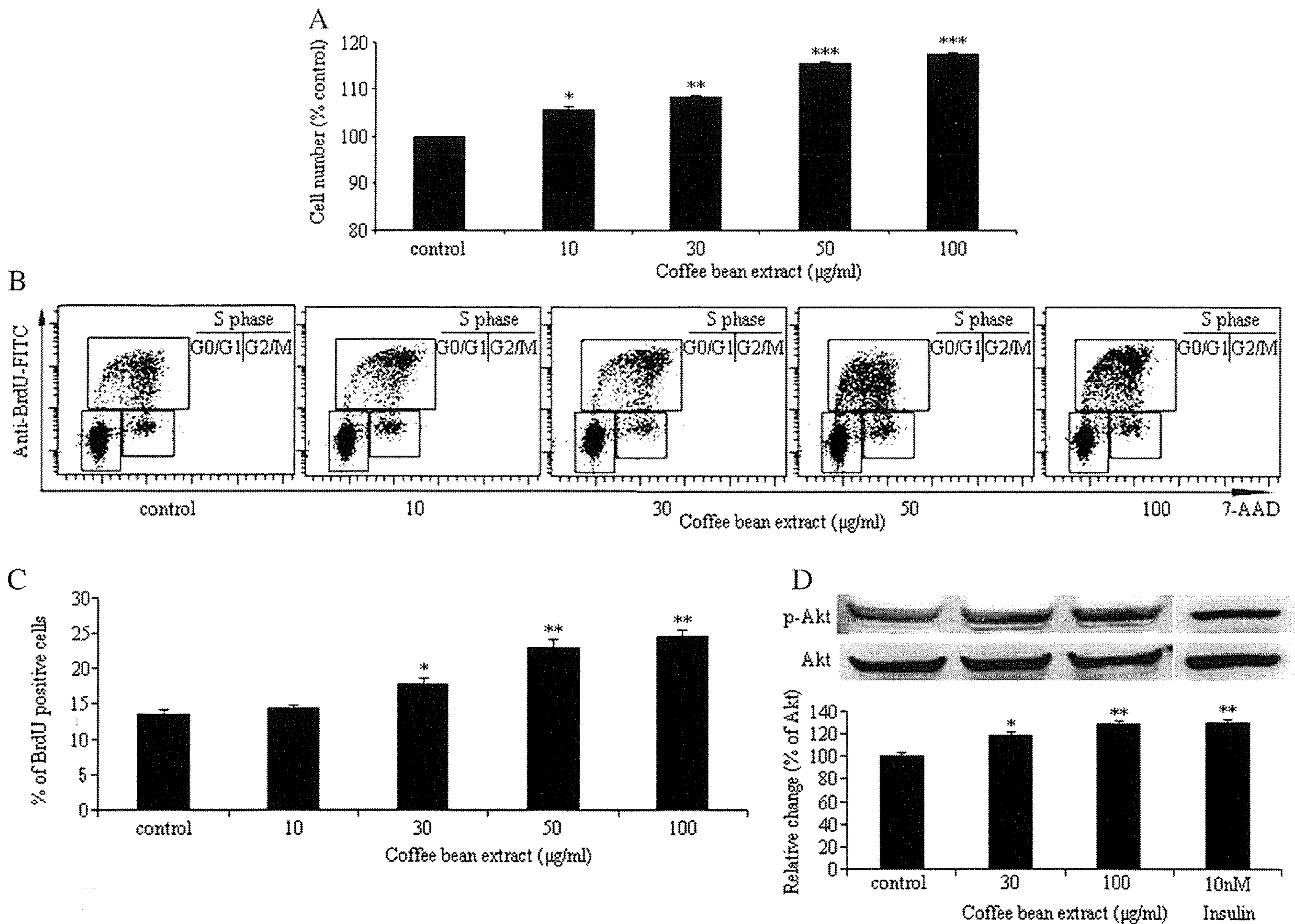

Fig. 4. Effects of coffee on the satellite cells of aged mice in vitro. (A) The satellite cells of aged mice were treated with the indicated concentrations of coffee bean extract for 72 h and the cell proliferation rate was measured. (B) Bromodeoxyuridine (BrdU)/7-AAD incorporation was evaluated by flow 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.

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Topics

高齢者誤嚥性肺炎予防の新戦略

大類 孝[1)]

Key Words 誤嚥性肺炎　不顕性誤嚥　大脳基底核病変　嚥下反射　咳反射

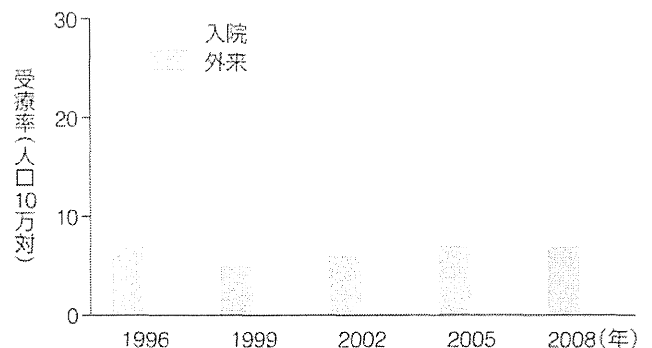
はじめに

抗菌薬の開発が目覚ましい現在でも肺炎による入院および死亡者数は増加傾向にあり，これまでわが国の疾患別死亡の第4位を占めてきたが，厚生労働省の2011年度の報告によれば，ついに脳血管障害を抜いて第3位になり，まさに現代病の様相を呈している．また，2010年度の人口動態統計によれば，肺炎による死亡者のなかで65歳以上の高齢者が占める割合は96.6%と極めて高い．本稿では，初めに高齢者肺炎の大部分を占める誤嚥性肺炎の発症の現状について明らかにし，次にその発症機序を解説し，最後にその予防策についてこれまでの筆者らのエビデンスを紹介しながら解説したい．

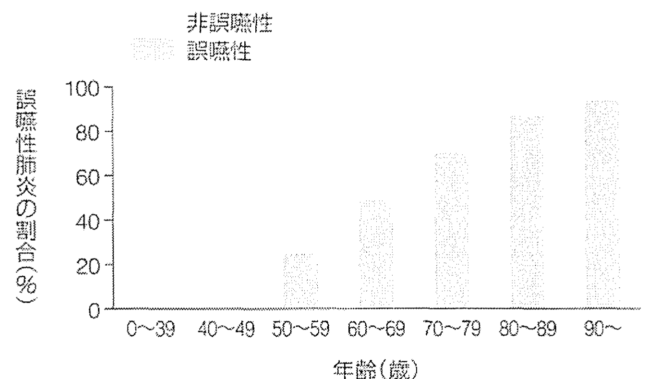
誤嚥性肺炎の概念

近年，外来で対処できる肺炎の患者数は横ばい状態にあるが，入院を要する肺炎の患者数は年々増加傾向にある(図1)．諸家の報告により異なるが，高齢者の肺炎のおよそ70%以上が誤嚥性肺炎であるといわれている(図2)[1)]．誤嚥(aspiration)とは，雑菌を含む唾液等の口腔・咽頭内容物，食物，稀に胃内容物を気道内に吸引することで，結果として生じる肺炎を広義の誤嚥性肺炎という．

誤嚥性肺炎(広義)は，臨床上おおまかにaspiration pneumonia(通常の誤嚥性肺炎)とaspiration pneumonitis(誤嚥性肺障害：メンデルソン症候群も含む)に分けられるが，両者はオーバーラップすることもある(表1)[2,3)]．Aspiration pneumoniaは，不顕性誤嚥(silent aspiration：無意識のうち



■ 図1　肺炎の受療率の年次推移(各年10月分)
肺炎による入院率は年々上昇している．
(国民衛生の動向，2011，2012を元に作成)



■ 図2　肺炎入院患者における誤嚥性および非誤嚥性肺炎の年齢別割合
(Teramoto et al, 2008)[1)]

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表1　誤嚥性肺炎(広義)の疾患概念

	Aspiration pneumonia（通常の誤嚥性肺炎）	Aspiration pneumonitis（誤嚥性肺障害：メンデルソン症候群等）
病因(引き金)	Silent aspiration(不顕性誤嚥)	Witnessed aspiration(顕性誤嚥)(嘔吐時等)
吸引物	雑菌を含む口腔・咽頭内容物	食物，胃液等の胃内容物(細菌は少なめ)
病態	細菌性肺炎	化学性肺炎(急性肺障害)
病原物質	細菌(黄色ブドウ球菌，腸内細菌，嫌気性菌等)	胃酸，ペプシン，食物等(稀に胃内の細菌)
頻度	高齢者に特に多い	少ない
危険因子	大脳基底核の脳血管障害，パーキンソン病，複数の抗精神病薬使用	麻酔，てんかん発作，鎮静剤の過量投与，広範な脳血管障害に伴う意識障害，認知症，球麻痺
治療	抗菌剤，補液，酸素投与	気道確保，補液，酸素投与，抗菌剤，グルココルチコイド

(Marik, 2001)[2] (Ohrui, 2005)[3]を参考に作成

に細菌を含む口腔・咽頭分泌物を微量に誤嚥する現象)を基にした細菌性肺炎であり，一方，aspiration pneumonitisは，意識障害時の嘔吐物(胃液を含む食物)の顕性誤嚥(周囲の者が明らかにそれと認識できる誤嚥)を基にした急性肺障害であり重症度が高い．他に，誤嚥性肺炎(広義)のなかにびまん性嚥下性細気管支炎および人工呼吸器関連肺炎が含まれる[4]．

誤嚥性肺炎(通常型)の危険因子

高齢者の肺炎の多くはaspiration pneumoniaであり，その危険因子として重要なものは不顕性誤嚥を併発しやすい大脳基底核の脳血管障害，脳変性疾患および認知症等の脳疾患である．その他の危険因子として，寝たきり状態(bed-ridden condition)，口腔内不衛生，胃食道逆流，抗精神病薬の多剤使用等が重要である．

誤嚥性肺炎(通常型)の発症機序

肺炎を繰り返す高齢者の多くは，不顕性誤嚥によって口腔内雑菌を気管や肺に吸引し，肺炎を発症するのではないかと考えられる．実際に当教室の研究によって，高齢の市中肺炎患者でも不顕性誤嚥が高率に認められることが明らかにされている[3]．さらに，通常，口腔・咽頭内容物が気道内に侵入すると，健常人では激しい咳によってこれを排除しようとする咳反射が働くが，肺炎を繰り返す高齢者ではこの咳反射の低下もしばしば認められる．

不顕性誤嚥は，脳血管障害のなかでも特に日本人に多い大脳基底核病変を有している人に多く認められる(図3)．大脳基底核は穿通枝領域にあり，もともと脳梗塞を起こしやすい部位であるが，その障害はこの部位にある黒質線条体から産生されるドーパミンを減少させる．ドーパミン産生の減少は，迷走神経知覚枝から咽頭や喉頭，気管の粘膜に放出されるサブスタンスP(SP)の量を減少させる[3]．SPは嚥下反射および咳反射の重要なトリガー(引き金)であるため，SPの減少は嚥下反射と咳反射を低下させる．実際に，繰り返し肺炎を起こす高齢者から得られた喀痰中のSPの量は，健常人に比べて減少していた[3]．高齢者肺炎患者では嚥下反射と咳反射の低下が認められ，不顕性誤嚥をベースに肺炎を発症するものと考えられる．