第54回日本老年医学会学術集会記録
〈ワークショップ：老年医学教育のあり方を考える～学部教育から専門医教育まで～〉

5. 高齢者救急

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要　約　高齢者人口の増加から，高齢者救急患者は今後急増することが予想される．看取りも含めた在宅医療や，医療と介護の連携の習得とともに，急性期病院での救急医療，これに続く地域への退院支援をも含めて一体の高齢者医療システムとしての医学教育プログラムの展開が必要である．高齢者では救急対応が必要な急性疾患であっても，症状や経過が非定型的であることが多く，個人差も大きいことから，高齢者救急の現場で必要とされる検査の習得が必要である．また，高齢者救急例では，原因となる急性疾患以外にも，多臓器の合併症発症の予防，治療が必要とされ，恒常性維持機構易破綻，薬物副作用にも注意する．さらに治療に際しては輸液や循環器薬使用の習熟が必須である．病態や患者が置かれている社会的状況も考慮して個々に治療ゴールの設定が必要となる．

Key words：高齢者，救急医療

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高齢者で救急対応が必要な急性期疾患

高齢者の救急対象となる疾患では，脳卒中，急性冠症候群，肺炎，急性腹症などに対する診断，救急搬送基準，治療に習熟する必要がある．以下に要点を示す．

1）脳卒中

①一過性脳虚血発作も含め，疑いがあれば，速やかに脳卒中急性期医療機関へ搬送する．

②診断には画像検査(特に拡散強調画像)が望ましい．

③発症直後の降圧療法は高血圧性脳症やくも膜下出血以外は病型診断後でよい．

④一過性脳虚血発作も脳梗塞に準じて重要に扱う．ABCD^{2}スコアを参照[1]．

⑤75歳以上での血栓溶解療法は適応を慎重に判断する．

⑥急性期から合併症予防に取り組む．

2）急性冠症候群（ACS）

①ST上昇型急性ACSだけでなく，非ST上昇型ACSでも中等度リスク以上であれば，速やかに循環器急性期医療機関へ搬送する．

②高齢者では，無痛性ACS，せん妄や失神で初発するACSにも注意する．

③トロポニンTを測定する．

3）急性腹症

①腹痛が制御できない場合は，速やかに消化器対応急性期医療機関へ搬送する．

②高齢者においても，腹部大動脈瘤破裂，腸間膜動脈血栓症，絞扼性イレウス，消化管穿孔，胆嚢穿孔，ヘルニア嵌頓，結腸軸捻転など緊急手術を要する疾患に注意する．

4）肺炎

成人市中肺炎診療ガイドライン（2007年日本呼吸器学会）では下記の身体所見，年齢による重症度分類（A-DROPシステム）を採用している[2]．本邦では意識レベルはJapan Coma Scale（3-3-9度方式）が用いられる．ただし，高齢者ではⅠ・1～3程度の意識レベルは認知症などで日頃から存在する場合があり，肺炎に由来する意識障害であることを検討する必要がある．

高齢者救急の現場で必要とされる検査

高齢者救急例では，まずは気道確保，ショック，嘔吐への対応が必要である．また意識レベル，バイタルサイン（呼吸，脈拍，血圧，体温），SpO_2測定を繰り返し観察する．高齢者救急例では，症状が非定型的であり，また合併症も多発することから，全ての救急例に対して，血算（含，血小板，Neutro%），CRP，Na，K，BUN，Cr，CK，CK-MB，LDH，T.Bil，アルブミン，血糖，HbA1c，

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表1　高齢者救急に必要な情報

◆まずは気道確保，ショック，嘔吐への対応
◆意識レベル，バイタルサイン（呼吸，脈拍，血圧，体温），SpO_2測定を繰り返し観察．
◆全ての救急例に対して
血算（含，血小板，Neutro%），CRP，Na，K，BUN，Cr，CK，CK-MB，LDH，T.Bil，アルブミン，血糖，HbA1c，BNP，心電図
◆疑う疾患には集中して

疑い疾患名	診断に必要な検査
急性冠症候群	トロポニンT，心エコー
重症不整脈	心エコー，心電図モニター
心不全急性増悪	心エコー，中心静脈圧，胸部CT，SpO_2，$PaCO_2$
脳卒中，頭蓋内疾患	頭部CT，MRI：特にDWI
肺炎	$PaCO_2$，Neutro%，胸部Xp，胸部CT，痰培，結核を除外
播種性血管内凝固症候群（DIC）	血小板，FDP，フィブリノゲン，PT，AT-Ⅲ
敗血症	血液培養2連検，エンドトキシン，β-Dグリカン
急性腎不全（脱水・DIC・薬剤）	eGFR（血清クレアチニン，年齢，性），尿酸
消化管出血	潜血（便：吐物），血算，BUN/Cr，腹部Xp：CT：エコー
急性腹症	腹部Xp：CT：エコー：MRI
大動脈解離	血圧左右差，胸部Xp（縦隔拡大），単純CT，造影CT
高血圧緊急症	レニン活性，アルドステロン，カテコラミン
肺塞栓症	Dダイマー，心エコー，造影CT，肺シンチグラム，肺動脈造影

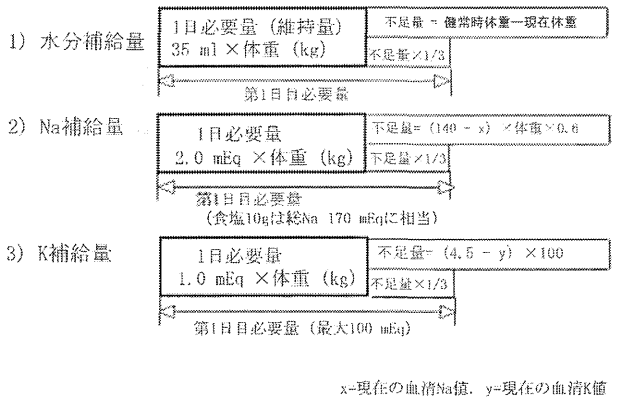

図1　維持量および欠乏量の計算

BNP，心電図検査を行う．また，疑われる疾患には集中して特殊検査を行う（表1）．救急の現場では必要最小限の検査に留める．不要な検査を多数行うことは結果的には診断を遅らせる．

輸液に対する基本知識の習得

高齢者救急例においては，経口摂取が不能または禁忌となり輸液が必要となる例が多く，輸液に関する基本的知識の習得が必要である．まずは，輸液の種類と適応，特に細胞外液補充液，1～4号輸液の特性と特徴に関する知識は是非とも必要である．また，水，Na，Kにつき，維持量と欠乏量が計算できることが必要である．

1）輸液の種類と適応

①細胞外液補充液：血漿と電解質濃度がほぼ同じ組成を持つ．Na欠乏型脱水（出血，嘔吐，下痢による脱水），ショックに適用．生理食塩水，リンゲル液，乳酸加リンゲル液など．

②1号液（開始液）：Kイオンを含んでいないのが特徴．緊急時などの病態不明時の水分・電解質補給の第一選択薬．通常1本のみ使用し，病態が判明すれば他の輸液に移行する．

③2号液（脱水補給液）：細胞内に多い電解質（K^+，Mg^{2+}，HPO_4^{2-}）を含むのが特徴．利尿がついた後の低カリウム血症や細胞内電解質が不足する脱水に用いられる．

④3号液（維持液）：水分・電解質の1日必要量が組成の基準．経口摂取不能または不充分な患者の水分・電解質の補給と維持に用いられる．

⑤4号液（術後回復液）：電解質濃度が最も低く，水分の補給を目的とした輸液．腎機能の未熟な新生児や小児，腎機能が低下している高齢者や術後早期の患者に用いられる．

2）維持量および欠乏量の計算（図1）

輸液の水，電解質（特にNa，K）の維持量（1日単位で計算），欠乏量（総量で計算，1日補給量はその1/3，3日間かけて補正）の計算は日常的に行えることが必要である．維持量に関しては，水分補給量は35 m*l*×体重（kg），Na補給量は2.0 mEq×体重（kg），K補給量

表2　高齢者救急で使用頻度の高い循環器用薬

対象疾患	薬剤名	商品名	用量	摘要
心不全	フロセミド	ラシックス	初回 10-20 mg 静注，持続静注は 2.5 mg/時	反応尿量確認（300-1000 ml/2- 数時間），不応時（重症，腎機能低下，低 Alb 血症）は他剤．血圧低下，低 K に注意．
	ドパミン	イノバン等	2-10γ持続静注	腎血流量増加作用．（≧5γで昇圧作用）
	ミルリノン	ミルリーラ	初回 50 μg/kg 静注，0.25-0.75γ持続静注	心筋細胞内の cAMP 濃度上昇
	カルペリチド	ハンプ	0.0125-0.2γ持続静注	利尿作用（hANP）
高血圧緊急症	ニカルジピン	ペルジピン	0.5-6γ持続静注	脳卒中急性期は過降圧に注意
	ジルチアゼム	ヘルベッサー	5-15γ持続静注	脳卒中急性期は過降圧に注意
	ニトログリセリン	ミリスロール	5-100 μg/分持続静注	脳卒中急性期は過降圧に注意
心室頻拍・心室細動	リドカイン	リドカイン オリベス	初回 1-1.5 mg/kg，追加 0.5-0.75 mg/kg，最大 3 mg/kg，静注	電気的除細動で復帰しても頻回に再発するとき
	アミオダロン	アンカロン	初回 150-300 mg，3-5 分後に追加 150 mg，静注	電気的除細動で復帰しても頻回に再発するとき
頻脈性心房細動	ベラパミル	ワソラン	5～10 mg，静注．	2 分以上で（WPW 症候群には禁忌）
	ジルチアゼム	ヘルベッサー	0.25 mg/kg 静注．	2 分以上で（WPW 症候群には禁忌）
	ジゴキシン	ジゴシン	0.125-0.25 mg，2 時間毎総量 1 mg まで，静注．	上記 2 剤に不応時，または心不全時（LVEF＜40%）．（WPW 症候群には禁忌）
心房細動（48 時間以内）	基礎疾患のある心房細動では電気的徐細動を優先．心機能に問題のない発症48時間以内の除細動には下記の Na チャンネル遮断薬による除細動を試みる．			
	ピルジカイニド	サンリズム	1 mg/kg まで静注	血圧・心電図監視下で 10 分かけ．
	シベンゾリン	シベノール	1.4 mg（0.1 ml）/kg 静注	血圧・心電図監視下で5分以上かけ．
	ジソピラミド	リスモダン P	50～100 mg 静注	5 分以上かけゆっくり
	フレカイニド	タンボコール	1～2 mg/kg，静注 総投与量は 150 mg まで	血圧・心電図監視下で 10 分かけ．三環系抗鬱薬の併用で QRS 幅延長，心室頻拍
	プロカインアミド	アミサリン	総量は 17 mg/kg	20 mg/分で静注
心房細動	48 時間以上持続している場合には原則として 3 週間以上の抗凝血薬療法が必須であり，さもなければ経食道心エコーによって左房内血栓が否定されてから除細動を行うことが推奨される．			
	ヘパリン	各種	5,000 U ボーラス後 14,000 U/日持続静注	塞栓予防
	ワーファリン	ワーファリン	0.5 mg-5.5 mg/日　経口	塞栓予防，PT-INR 1.6-2.5 に調節
房室ブロック	めまいや失神，呼吸困難等の症状血行動態の悪化を伴う場合は不安定と判断し，根治的には経皮ペーシング～経静脈ペーシングを準備する．ペーシングの準備中に，アトロピン，アドレナリン，ドパミン，イソプロテレノールを使用する．			
	アトロピン	アトロピン	0.5 mg を 3～5 分ごと，総投与量 0.04 mg/kg まで	狭隅角緑内障/尿閉に注意．Mobitz2 型Ⅱ度房室ブロックでは使用せず，以下の投薬，不応時は経皮ペーシング施行．
	アドレナリン	ボスミン	2～10 μ/分，持続静注	1 型 QT 延長症候群で torsade des pointes を誘発
	ドパミン	イノバン等	2～10γ，持続静注	≧5γで昇圧作用．1 型 QT 延長症候群で torsade des pointes を誘発
	イソプロテレノール	プロタノール	0.01～0.03γ持続静注	心拍数が 50 以上になるように量を増減．薬物中毒による徐脈には禁忌

文献 3，4 より改変

は1.0 mEq×体重（kg）が基本となる．また欠乏量に関しては，水分不足量＝健常時体重－現在体重，Na不足量＝(140－x)×体重×0.6，K不足量＝(4.5－y)×100で計算され，1日補給量は，維持量＋欠乏量×1/3が基本となる．

カリウム投与には様々な投与制限があり，①シングルボーラスショットは禁忌，②濃度：40 mEq/L以下（末梢），③速度：20 mEq/hr以下，④投与量：100 mEq/日以下，⑤尿量：0.5 mL/kg/hr以上が安全，⑥K排泄障害時は高K血症に注意，⑦心電図でのモニターが必要，などの基本事項の遵守が必要である．

循環器用薬の使い方

高齢者救急例では，虚血性心疾患例以外でも，急性期疾患に伴いショックや心不全に陥る例は多く，循環器用薬の使い方に習熟しておく必要がある（表2）．循環器用薬では持続点滴投与を行う薬剤も多く，この場合用いられる投与単位であるγ＝μg/kg体重/分にも習熟し，実際の用量，投与速度が瞬時に計算できるように習熟が求められる．

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Royal Jelly Prevents the Progression of Sarcopenia in Aged Mice In Vivo and In Vitro

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Sarcopenia is characterized by the age-related loss of muscle mass and strength. One of the mechanisms of sarcopenia is the loss in the function and number of muscle satellite cells. Royal jelly (RJ) is a health food used worldwide. To obtain better digestion and absorption than RJ, protease-treated RJ (pRJ) has been developed. RJ and pRJ have been suggested to have potential pharmacological benefits such as prolonging the life span and reducing fatigue. Because these effects may improve sarcopenia and the functions of satellite cells, we examined the effects of RJ or pRJ treatment on the skeletal muscles in an animal model using aged mice. In vivo, RJ/pRJ treatment attenuated the decrease in the muscle weight and grip strength and increased the regenerating capacity of injured muscles and the serum insulin-like growth factor-1 levels compared with controls. In vitro, using isolated satellite cells from aged mice, pRJ treatment increased the cell proliferation rate, promoted cell differentiation, and activated Akt intracellular signaling pathway compared with controls. These findings suggest that RJ/pRJ treatment had a beneficial effect on age-related sarcopenia.

Key Words: Aged mice—Sarcopenia—Satellite cells—Royal jelly—Insulin-like growth factor-1—Akt signaling.

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THE population of people aged 60 and older is currently growing at the rate of 2.6% per year, which is more than twice the rate of growth of the total population in the world (1). 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 the elderly patients 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 the elderly patients (2).

Satellite cells are resident myogenic progenitors in the skeletal muscles. They play a central role in the growth and regeneration of the skeletal muscles (3). In response to stimulation, satellite cells form myoblasts, fuse together, and generate new fibers (4). The age-related functional disability and decrease in the number of satellite cells contribute to the development of sarcopenia (5). Thus, maintaining the functions of satellite cells and their numbers may reduce sarcopenia and, furthermore, may improve the regenerating capacity of the skeletal muscles in the elderly patients. However, to isolate satellite cells, specific cell surface markers were not available until recently (6).

Among the factors that stimulate satellite cells, insulin-like growth factor-1 (IGF-1) plays a central role. IGF-1 stimulates satellite cell proliferation, their differentiation into myoblasts, and, finally, their differentiation into myotubes (4). IGF-1 is the most important mediator of muscle growth and repair (7). Furthermore, a recent study suggested the potential of IGF-1 to improve sarcopenia in the elderly patients (7).

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

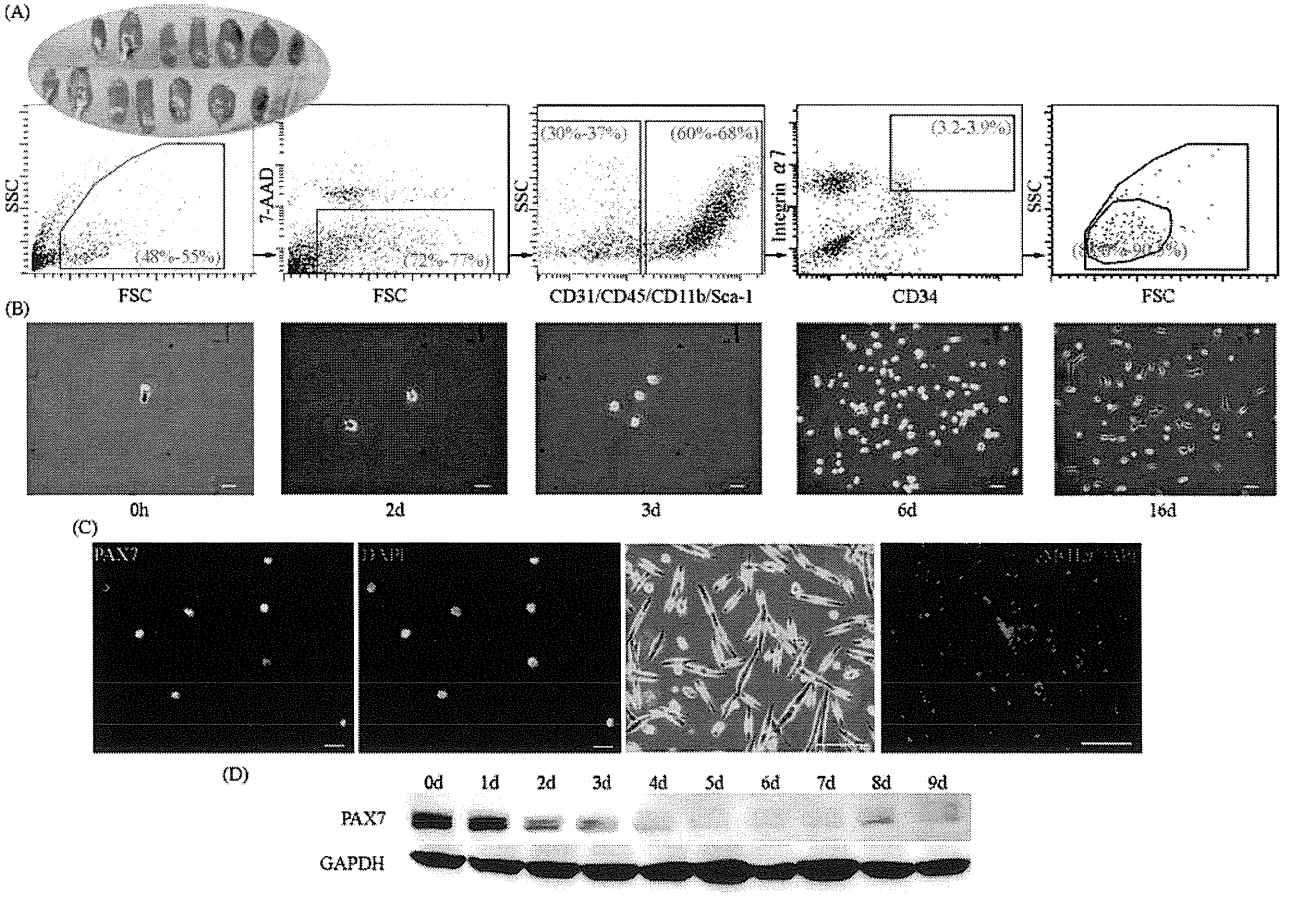

Figure 1. The isolation and characterization of the satellite cells. (**A**) A flow cytometer sorted the satellite cells from the hind-limb muscles after enzymatic dissociation (shown in circle on the left: upper muscles were isolated from a right leg and lower muscles were isolated from a left leg; 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

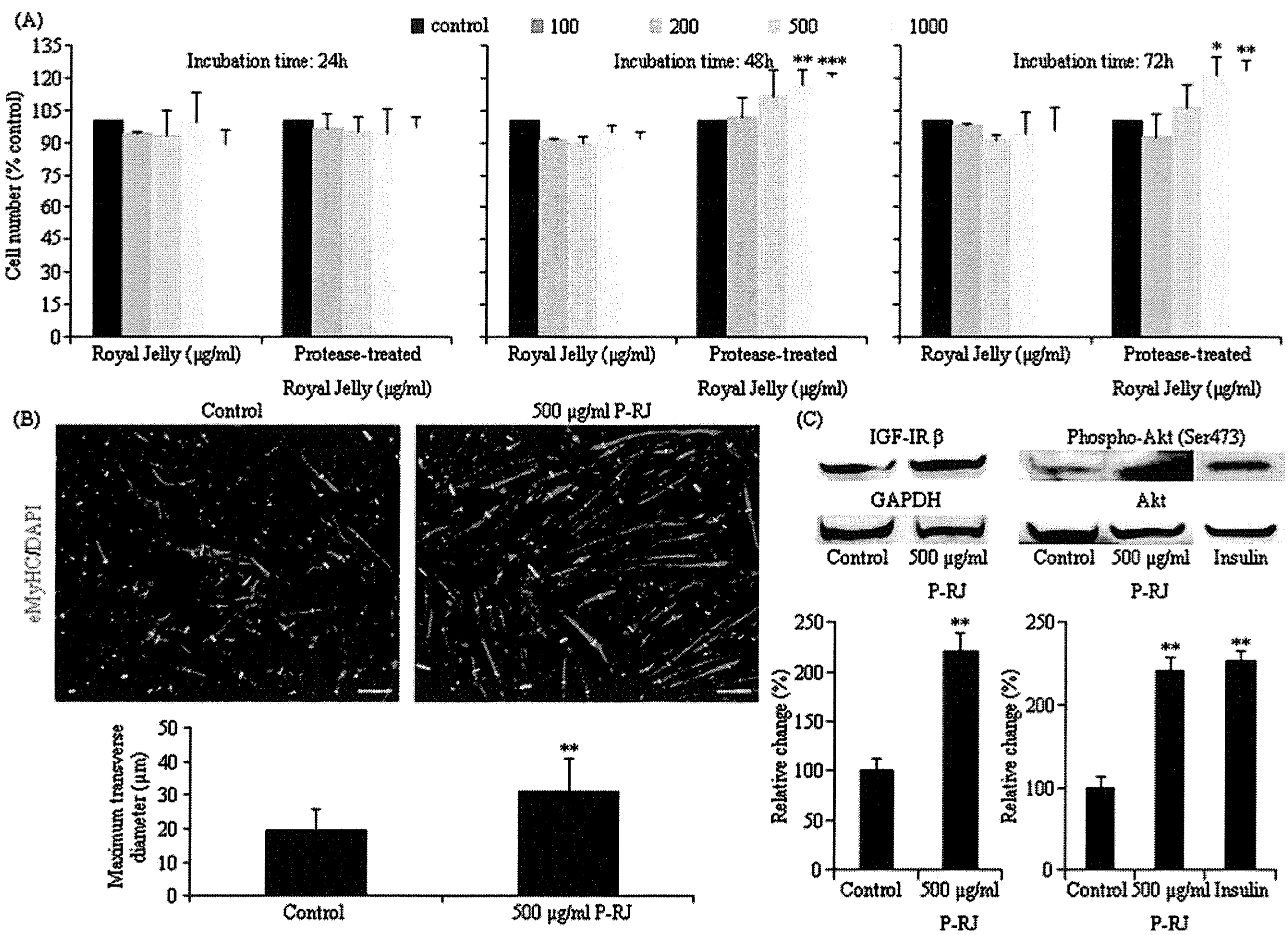

Figure 2. Effects of RJ/pRJ on the satellite cells of aged mice in vitro. (**A**) The satellite cells of the aged mice were treated with the indicated concentrations of RJ or pRJ for 24, 48, or 72 h, and the cell proliferation rate was measured at each time point. (**B**) The satellite cells of aged 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.

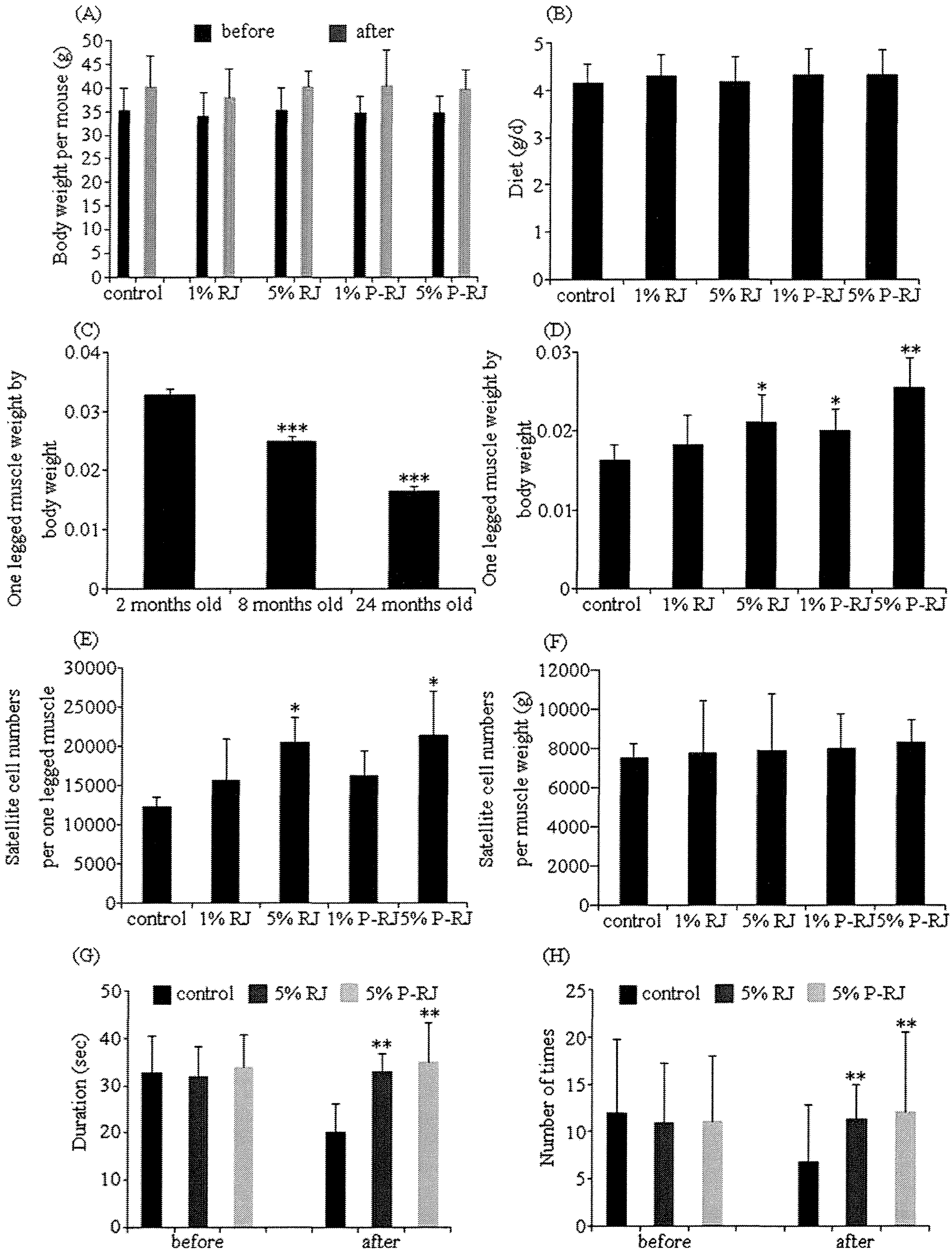

Figure 3. Effects of RJ/pRJ treatment on aged mice in vivo. Twenty-one-month-old mice were treated with a diet mixed with 1% weight RJ (1% RJ), diet with 5% weight RJ (5% RJ), diet with 1% weight pRJ (1% pRJ), or diet with 5% pRJ (5% pRJ) for following 3 months. (**A**) Control, RJ, or pRJ treatment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

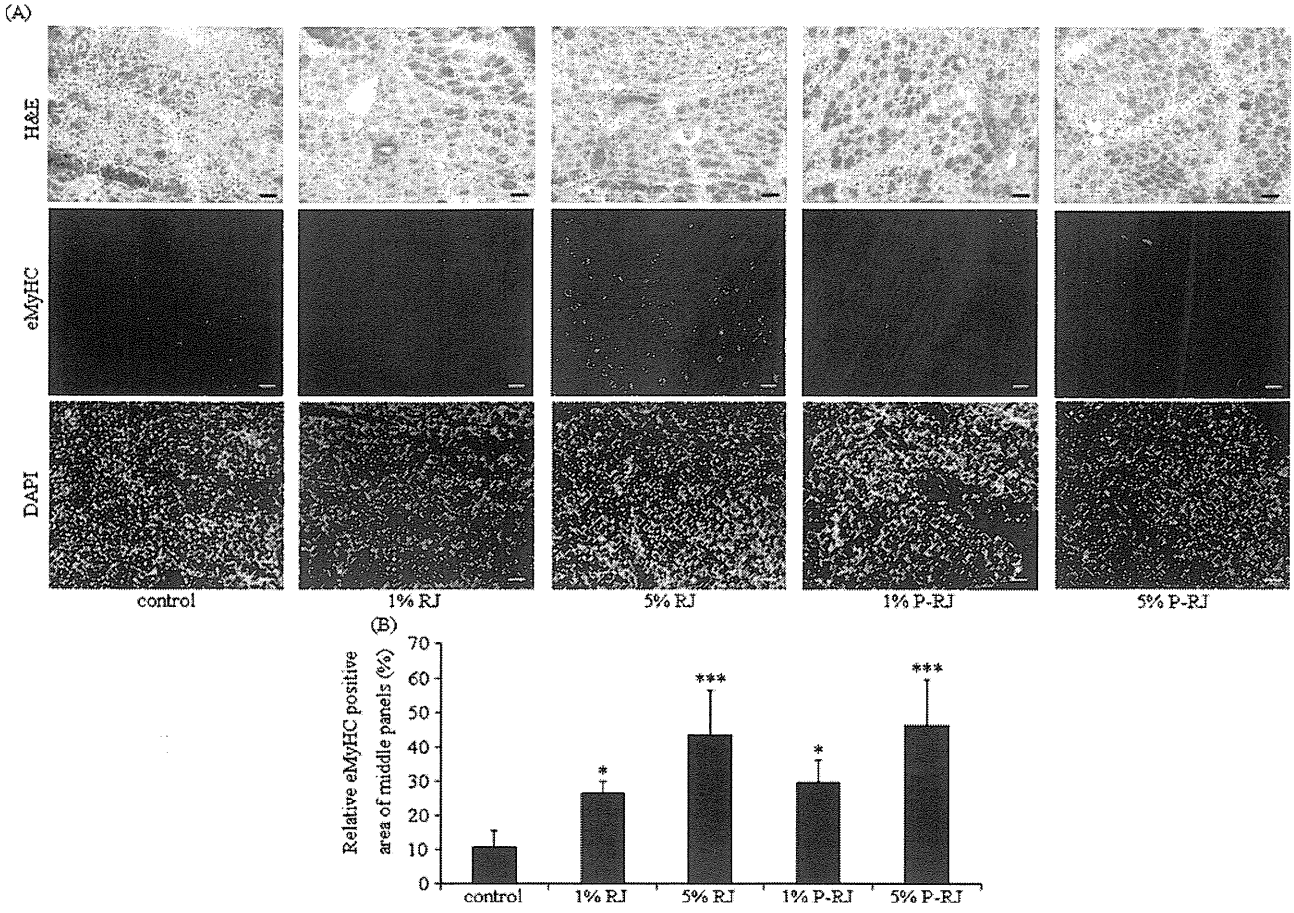

Figure 4. RJ/pRJ treatment accelerated the regeneration of the injured skeletal muscles in aged mice. After 3 months of RJ/pRJ treatment, we injected cardiotoxin into the tibialis anterior muscles of the aged mice to injure the muscles and isolated them 5 days later. (**A**) Hematoxylin and eosin staining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

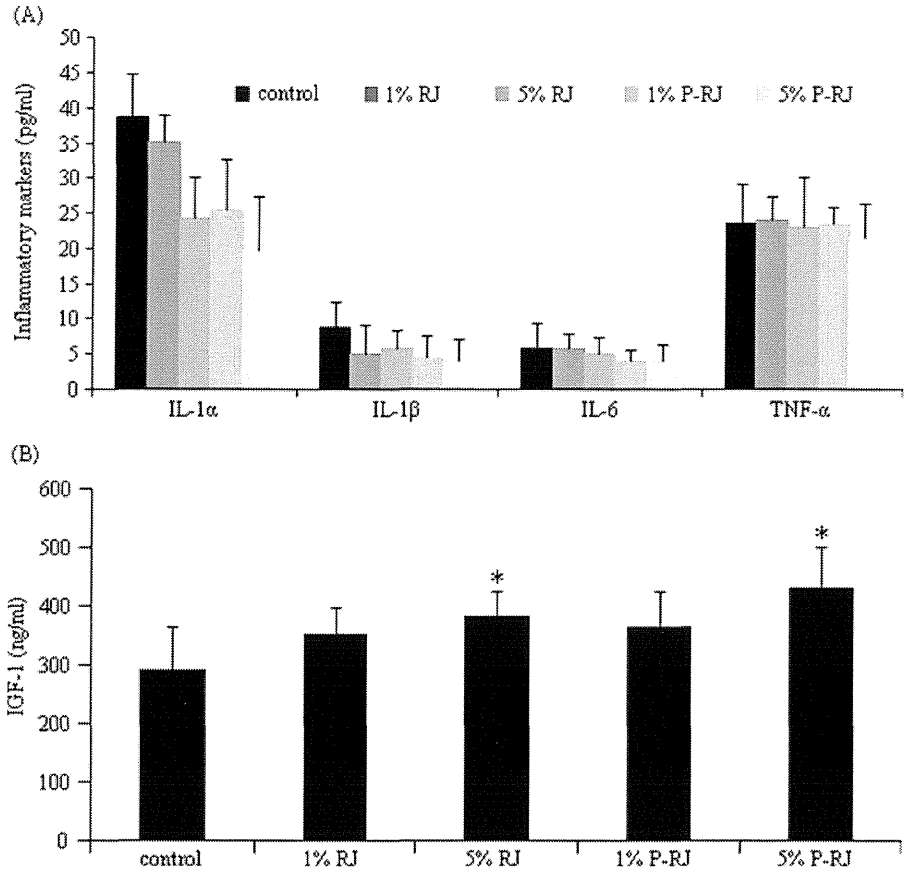

Figure 5. RJ/pRJ treatment increased the serum IGF-1 levels but did not affect the serum proinflammatory mediator levels. ELISA determined serum levels of proinflammatory mediators and IGF-1 in aged mice treated with RJ/pRJ for 3 months. (**A**) RJ/pRJ treatment did not change the serum levels of the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

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 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

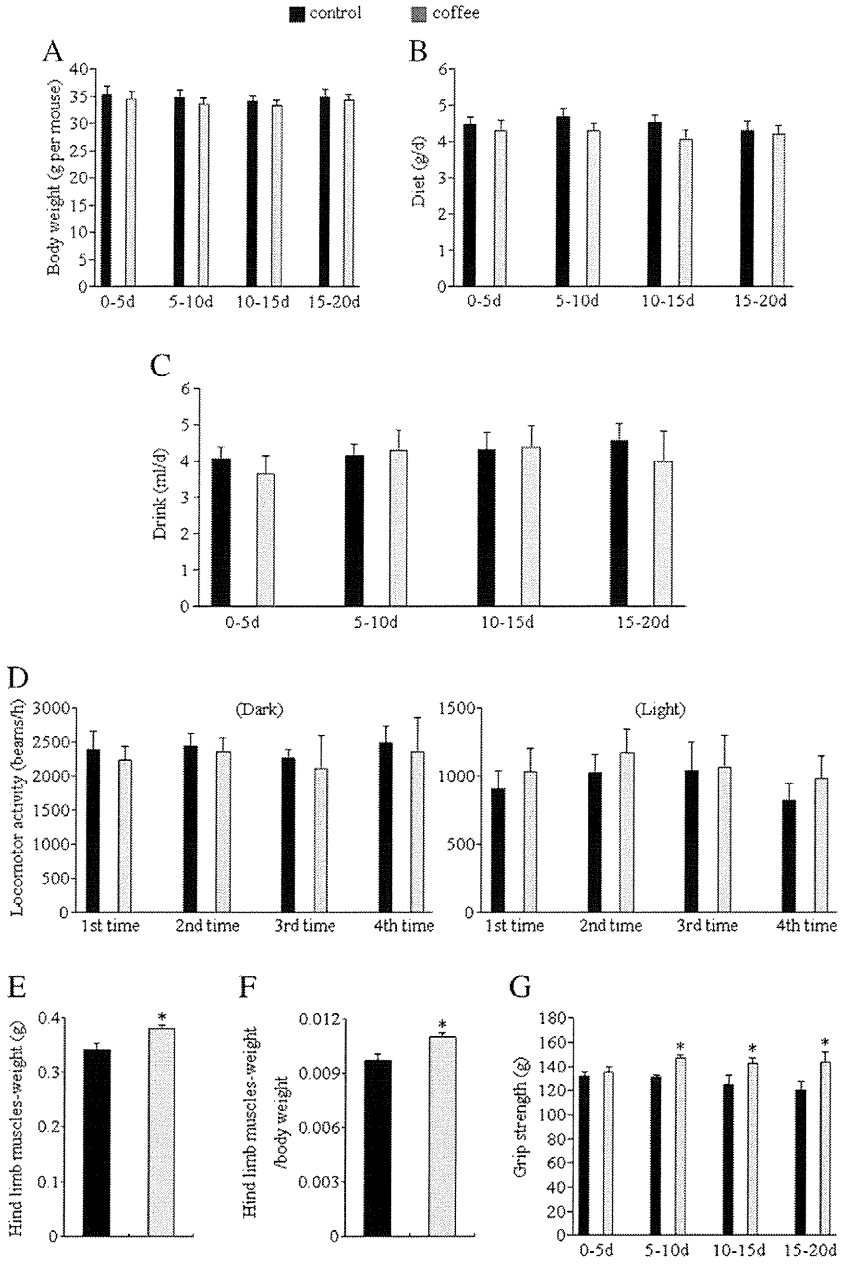

Fig. 1. Effects of coffee treatment on aged mice in vivo. Twenty-seven month-old mice were treated with coffee for 4 weeks. (A–C) Coffee treatment did not change body weight (A), the amount of the daily diet intake (B), or the volume of water intake (C). (D) Coffee did not change the daily locomotor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

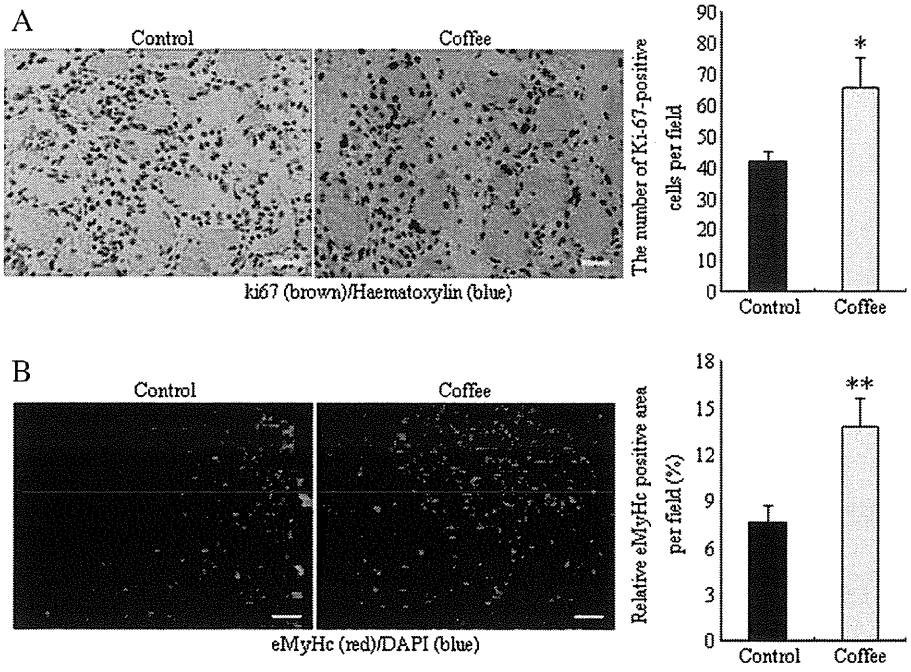

Fig. 2. Coffee treatment accelerated the regeneration of the injured skeletal muscles in aged mice. After 4 weeks of coffee treatment, we injected cardiotoxin into the tibialis anterior muscles of the mice to injure the muscles and isolated them 3 days later for (A) or 5 days later for (B). (A) The left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

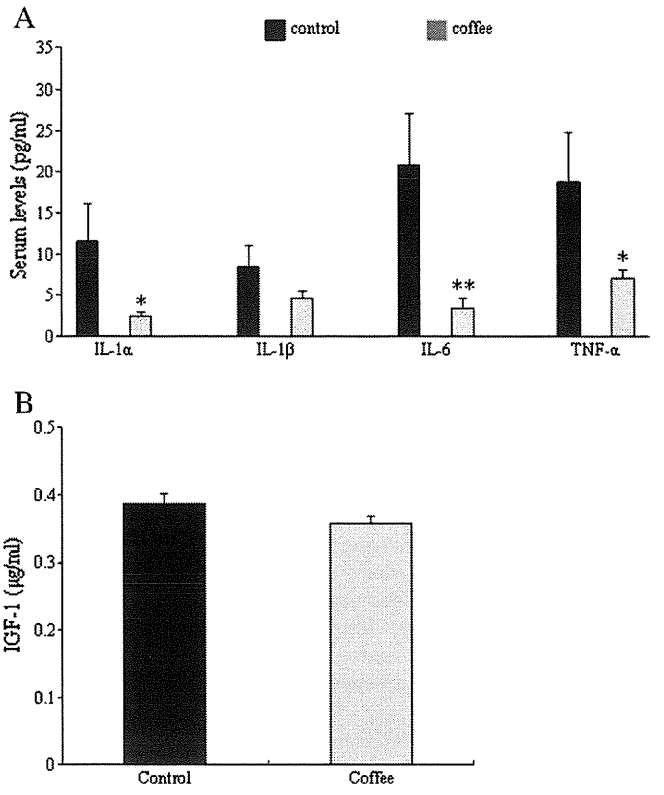

Fig. 3. Coffee treatment decreased the serum pro-inflammatory mediator levels, but did not affect the serum IGF-1 levels. ELISA determined serum levels of pro-inflammatory mediators and IGF-1 in aged mice treated with coffee for 4 weeks. (A) Coffee treatment significantly decreased the serum levels of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