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Mitochondrial Dysfunction and Changes of Myokine as a Mechanism of Age-related Sarcopenia

가령에 따른 근감소증의 기전으로서의 미토콘드리아 기능이상 및 마이오카인의 변화

2015 년 8 월

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Directed by Professor Sung-Hee Choi, M.D., Ph. D.

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Abstract

Mitochondrial Dysfunction and Changes of Myokine as a Mechanism of Age-related Sarcopenia

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Sarcopenia is a phenomenon that accompanies aging. It is defined as the progressive loss of skeletal muscle quantity and quality. Sarcopenia reduces physical performance and enhances physical frailty in the elderly. In this study, we explore the possible mechanisms of sarcopenia by assessing the hypothesis that age-related sarcopenia in humans may be associated with changes in muscle mitochondria or changes in the expression and/or secretion of muscle secretory proteins, or myokine.

We recruited young diseased (YD; 18 males and 11 females) and old diseased (OD; 5 males and 13 females) groups of both genders who underwent total hip replacement surgery due to osteoarthritis or avascular necrosis. Clinical characteristics were evaluated by assessing history, physical examination, body composition (determined by dual energy X-ray absorptiometry), and muscle strength (determined by isokinetic dynamometer). Serum concentrations of adipokines and myokines were measured by radioimmunoassay or quantitative enzyme immunoassay of fasting blood samples. Quantitative and qualitative evaluation of skeletal muscle mitochondria were determined by electron microscopy, light microscopy with immunohistochemical staining, spectrophotometric assay of mitochondrial respiratory chain activity, oxygen consumption measurement by high-resolution respirometry, and western blot analysis of mitochondrial complexes. Microarray analysis was performed to identify candidate mRNAs that encode proteins possibly involved in sarcopenia, and these candidates were subjected to real-time PCR. Finally, tissue expression profiles of interleukin (IL)-6 in young and old patients were compared in the skeletal muscle tissue by western blot analysis.

Average muscle mass in the unaffected leg of female subjects was significantly lower in OD than in YD. Muscle strength and quality were more significantly reduced in OD subjects than in YD subjects of both genders, but more strikingly in female subjects. Age was significantly correlated with

muscle strength and quality in both genders. Muscle strength and quality showed marginal to significant negative correlations with physical dysfunction. Morphological changes of mitochondria were not observed in OD skeletal muscle. However, the number of mitochondria and the amount of complex protein were significantly higher in the OD group. Maximum oxidative capacity measured by high-resolution respirometry was not significantly lower in OD subjects than in YD subjects, but tended to be lower in the low muscle quality group than in the high muscle quality group. Complex I activity was significantly higher in the low muscle quality group and the OD group. Immunohistochemical staining indicated abnormal mitochondrial proliferation in OD muscle. There was no significant difference in IL-6 levels in serum and muscle tissue of OD and YD groups; however, both were significantly correlated with muscle strength. High IL-6 expression in muscle tissue seemed to be related to lower maximal oxidative capacity and higher compensatory expression of complex protein in mitochondria.

In conclusion, qualitative muscle deterioration is the main characteristic of age-related sarcopenia. This qualitative change in skeletal muscle is likely to be associated with qualitative change of mitochondria. Quantitative changes in mitochondrial numbers and complex protein levels seemed to occur as a compensatory mechanism for the aging-related qualitative changes. Serum and tissue IL-6 levels tended to increase with aging, and IL-6 levels negatively correlated with muscle strength. Thus,

serum and tissue IL-6 might be involved in the development of age-related sarcopenia.

Key words: aging, muscle, sarcopenia, mitochondria, myokine

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I. Introduction

Aging is identified by a progressive decline in cellular structure and function resulting from the accumulation of multiple changes over time. Skeletal muscle accounts for <50% of body weight and 75% of the body's cell mass (1); it is the largest organ in the body, generating force and producing human movement. Therefore, understanding basic cellular mechanisms underlying the aging process of skeletal muscle may provide anti-aging solutions for humans.

Sarcopenia is defined as the progressive loss of skeletal muscle mass with advancing age (2). It is characterized by a deterioration of muscle strength and quality, and leads to a gradual decline in functional performance and an increase in physical frailty in aged individuals (3). Sarcopenia also is associated with insulin resistance (4), and it is an independent predictor of mortality in aged populations (5-6). Thus, understanding the mechanisms involved in sarcopenia is of great importance to promote health in the elderly. Multiple mechanisms have been proposed, such as altered motor unit organization, diminished levels of anabolic hormones (7), altered rates of specific muscle protein synthesis (8), and involvement of inflammatory mediators (9); however, the precise mechanisms remain unclear. Recent studies reported that age-related accumulation of mitochondrial abnormalities

and mitochondrial dysfunction (10) are attributable to sarcopenia. Cytokines produced by adipose tissue (adipokines) or skeletal muscle (myokines) are emerging candidates that may cause sarcopenia (11).

Mitochondria have a key role in cellular energy metabolism by producing adenosine triphosphate (ATP). Mitochondria also induce apoptosis by generating reactive oxygen species (ROS), which are side products of oxidative phosphorylation (12). Mitochondria are most abundant in skeletal muscle, because the energy demands of this organ are very high. For these reasons, mitochondrial dysfunctions such as reduced ATP synthesis and increased ROS production have been suggested as underlying mechanisms for aging of skeletal muscle cells and many other degenerative diseases including type 2 diabetes mellitus. However, the molecular details of muscular mitochondrial changes in older people and potential associations with sarcopenia are poorly understood.

Adipokines are produced by adipocytes and immune cells residing in adipose tissue (11). Adipokines mediate various metabolic effects caused by obesity. These effects are not confined to adipose tissue, and skeletal muscle is one of the target organs of adipokines. Adipokines may have a significant effect on skeletal muscle metabolism, but this has not been extensively explored (13).

Myokines are humoral factors that are produced and secreted from contracting myocytes, and have been reported to mediate major metabolic

effects specifically caused by exercise (14). They act in an autocrine, paracrine, or endocrine mechanism (14). Myokines regulate glucose and lipid homeostasis, muscle growth, and inflammatory responses (15), which could be involved in the development of sarcopenia. The potential role of myokines in the development of sarcopenia is drawing significant research attention. We hypothesized that a decline in mitochondrial function or changes in cytokine (adipokines or myokines) expression or secretion could be cellular mechanisms underlying the decline in muscle strength and quality in aged individuals.

II. Materials & Methods

2.1. Subjects

Patients who underwent total hip replacement arthroplasty (THRA) at Seoul National University Bundang Hospital (SNUBH) from February 2008 to November 2009 were eligible for this study. Patients diagnosed with primary muscle diseases, peripheral arterial disease, or chronic illness such as liver disease, renal failure, and cancer were excluded. Patients with diabetes mellitus, which can be complicated with diabetic neuropathy, also were excluded. We also excluded those who were taking corticosteroids, anabolic hormones, or other medications that might affect muscle mass and function. Patients who were not able to walk for a distance of at least 500 m or could not perform activities of daily living were excluded. Among patients who meet the above criteria, we further excluded patients who underwent orthopedic surgery with diagnoses other than avascular necrosis or osteoarthritis. These included Legg-Calve-Perthes disease, dysplastic hip, septic hip sequelae, developmental dysplasia of the hip, and intertrochanteric fracture. In these patients, severe joint deformity can influence muscle mass and strength, or severe pain may limit the accuracy of muscle strength evaluation. The remaining patients were classified into two age groups (young, $\leq$55 years; old, $\geq$65 years). The final study cohort included 29 young

patients (18 males and 11 females) and 18 old patients (5 males and 13 females). We designate these groups as young diseased (YD) and old diseased (OD) because the patients had pathological musculoskeletal conditions that led to the surgical procedure and their muscle mass and strength were not the same as those in normal populations. The study protocol was approved by the Institutional Review Board of SNUBH (IRB No. B-0710-050-009). Written informed consent was obtained from patients or their legal guardians.

2.2. Measurement of anthropometric parameters and body composition profile

Subject height and body weight were measured in hospital gown and bare feet, to the nearest 0.1 cm and 0.1 kg, respectively. Waist circumference (WC) was measured at the midpoint between the lower costal margin and the iliac crest while the patient was standing, and hip circumference (HC) was measured at the widest part of the hips. Body mass index (BMI) was calculated as weight divided by squared height (kg/m^2). Blood pressure was measured twice when subjects were in a relaxed state for at least 30 min; the mean of the two measurements was used for the analysis.

Body composition was measured using a dual-energy X-ray absorptiometry (DXA) whole-body scanner (Lunar Prodigy, GE Healthcare, Madison, WI, USA); enCORE 2004 software (GE Healthcare) was used to

estimate regional and total fat, lean soft tissue mass, and bone mineral content. Landmarks were set to differentiate appendages (upper and lower extremities) from the trunk. A line between the glenoid fossa of the scapula and the humeral head separates the arm from the trunk. The regions from the inferior border of the ischial tuberosity to the distal tip of the toes were considered as the leg tissue. Appendicular skeletal muscle mass (ASM) was calculated as total muscle mass in upper and lower extremities, which is directly correlated with total body skeletal muscle mass (16). We determined skeletal mass index as ASM in terms of body weight (ASM/Wt) or height (ASM/Ht^2). ASM/Wt was calculated as ASM divided by body weight and multiplied by 100 (%) (modified from a previous study (17)). ASM/Ht^2 was calculated as ASM divided by height squared (kg/m^2) because absolute muscle mass is correlated strongly with height (18). The study subjects had undergone THRA; therefore, muscle mass and strength in the two legs might differ. We classified legs into affected (that which underwent THRA) and unaffected (on the other side of the body) groups and analyzed them separately.

2.3. Measurements of biochemical parameters

Subjects were fasted for 14 h (overnight), and venous blood samples were drawn from the antecubital vein in the morning. Plasma was separated immediately by centrifugation at 2,000 rpm for 20 min at 4°C. Biochemical

parameters were measured immediately after sampling and plasma samples for adipokines and myokines were stored at −70°C until analyses were performed.

Fasting plasma glucose was measured using the glucose oxidase method. Total cholesterol, creatinine, AST, and ALT were measured using enzymatic methods with a Hitachi 747 chemistry analyzer (Hitachi, Tokyo, Japan). Hs-CRP concentration was measured by immunonephelometry (Dade Behring, Marburg, Germany). Hemoglobin A1c was measured by ion-exchange high performance liquid chromatography (VARIANT II, Bio-Rad Laboratories, CA).

The fasting plasma concentration of adiponectin, leptin, resistin, visfatin, tumor necrosis factor-alpha (TNF-α), and interleukin 6 (IL-6) was determined using a radioimmunoassay kit (LINCO kit, St. Charles, MO, USA) in the preliminary study. In the final experiment, quantitative sandwich enzyme immunoassay was used to measure serum IL-6 (R&D Systems, Inc. Minneapolis, MN, USA).

2.4. Muscle strength measurement

The muscle strength of knee extensor and knee flexor in both legs was tested using an isokinetic dynamometer (Biodex System 3 Pro, Biodex Inc., Shirley, NY, USA) at an angular velocity of 60 degree/s. Isokinetic strength is the

force generated by a muscle against resistance at a constant rate of movement (Adams GM. 1998. Exercise physiology: Laboratory manual. Boston, MA: WCB McGraw-Hill. American College of Sports Medicine. 1995. ACSM's guidelines for exercise testing and prescription. Baltimore: Williams & Wilkins.). Isokinetic strength testing provides an objective means of quantifying existing levels of muscular strength, and the measurements are highly reproducible (19). The knee is the most commonly tested joint. Knee extension is very important for body movements such as rising from a chair, climbing stairs, and jumping. Knee extension strength also provides the forward momentum needed for walking (20). Knee flexors and rotators are required for deceleration of limb motion during walking or running (21). Subjects performed five maximal voluntary contractions, and the peak torque was recorded. A measure of muscle quality was calculated as the ratio of muscle strength to the corresponding leg muscle mass.

2.5. Evaluation for physical dysfunction

To investigate the effect of reduced muscle strength and quality on physical impairment, we used the Western Ontario McMaster Osteoarthritis questionnaire (WOMAC) (22). This questionnaire assesses three dimensions: pain, stiffness, and physical function/dysfunction. The physical function/dysfunction category queries subjects to rate their ability to descend

stairs, ascend stairs, rise from sitting, standing, bending to floor, walking on level surfaces, get in or out of a car, go shopping, put on socks, rise from bed, take off socks, rest in bed, sit, get on or off of toilet, perform heavy domestic duties, and perform light domestic duties. Physical dysfunction was rated with a score between 0 and 4 (0=no dysfunction; 4=extreme dysfunction), and the sum (maximum 68) was presented as WOMAC dysfunction.

2.6. Comparison with functionally normal subjects

To compare muscle mass and strength in normal functioning subjects with those of our diseased subjects, we recruited two sets of reference cohorts whom we designated as young control (YC) and old control (OC).

Subjects from the community-based cohort study Korean Longitudinal Study of Health and Aging (KLoSHA) were used for the normal functioning OC group. Subjects in the OC group were matched for age and BMI (15 male and 26 female). Data for normal young subjects (16 male and 11 female) were obtained from the normal functioning study participants undergoing muscle biopsy (courtesy of Dr. Lim).

2.7 Muscle sample

On the pre-operation day, all patients had a standardized diet containing 10 kcal/kg (per meal), in which the proportions of carbohydrate,

fat, and protein were 50%, 30%, and 20%, respectively. All patients started fasting from midnight until the operation day. To rule out exercise effects on the structure and function of muscle mitochondria, patients were prohibited from excessive exercise for two days before surgery. A tissue sample was obtained from the gluteus maximus muscle, wrapped in saline-soaked gauze, and immediately delivered to the laboratory. A portion of the resected muscle tissue was frozen immediately in liquid nitrogen and stored at −70°C for measurement of enzyme activity. Another portion was used for mitochondrial isolation, immunohistochemical staining, and electron microscopic examination.

2.8. Evaluation of mitochondrial morphology and function

Mitochondrial function was estimated by measurement of oxygen consumption, mitochondrial respiratory chain (MRC) activity, and histochemical staining.

Isolation of mitochondria

All steps were performed at 4°C. Muscle tissue was cut into small pieces with a pair of scissors. These pieces were minced and homogenized in ice-cold mitochondria isolation buffer (MIB; 250 mM sucrose, 10 mM Tris,

and 1 mM Na_2-EDTA, pH 7.4) in a 1 ml tapered tissue grinder (Wheaton Industries, USA) using the Homogenizer T10 (IKA WERKE, Germany). The homogenate was cleared of nuclear and cellular debris by centrifuging at 3,000 rpm for 10 min. The supernatant was transferred to a new tube and centrifuged at 9,500 rpm for 10 min. To isolate mitochondrial fractions, the pellet was suspended in MIB and centrifuged at 6,500 rpm for 10 min. The mitochondrial pellet was washed in MIB three times using a Pasteur pipette, and then spun down at 6,500 rpm for 10 min. The mitochondrial pellet was resuspended in mitochondrial respiration medium [Mir05; 0.5 mM EGTA, 3 mM $MgCl_2 \cdot 6H_2O$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, and 0.1% BSA (essentially fatty acid free, fraction V), adjusted to pH 7.1 with KOH at 37°C). Protein content was measured in each sample, and equivalent protein contents were used to assay all respirometry samples. Mitochondrial suspensions were stored on ice for at least one hour before performing assays to permit mitochondrial membrane rearrangement.

High-resolution respirometry for measuring mitochondrial oxygen consumption

Mitochondrial oxygen consumption was measured using the high-resolution Oxygraph respirometer (Oroboros Instruments, Innsbruck,

Austria). This instrument has two chambers, which were set at 2 ml. The medium had been equilibrated with air in each chamber at 37°C and stirred at 750 rpm until a signal was stable at air saturation. The amount of total protein added to each chamber was 200 μg, so the final concentration used for measurements was 100 μg/ml. To measure different mitochondrial respiratory states, ADP, specific respiratory substrates, and specific respiratory inhibitors were injected sequentially. The substrates were glutamate and malate for complex I and succinate for complex II; the inhibitors were rotenone for complex I and antimycin A for complex III. The respiratory protocol was followed as described previously (23). DatLab 4 software (Oroboros Instruments) was used for data acquisition and analysis. When all substrates, cytochrome c, and ADP were added to the assay reaction mixture, the O_2 consumption rate reflected the maximal oxidative capacity (state 3 respiration).

Mitochondrial respiratory chain assay

A portion of frozen muscle tissue was prepared as previously described. The muscle tissue was homogenized, centrifuged, and the supernatant was filtered through layers of cheesecloth. The supernatant fractions contained nuclei and mitochondria, and centrifuged as before and filtered so that the remaining supernatant contained only mitochondria.

Enzyme activities were measured for citrate synthase (a nuclear DNA-encoded mitochondrial enzyme) and enzymes belonging to each MRC complex (I, II, III, and IV) using a spectrophotometer (DU-730, Beckman Coulter, Fullerton, CA). The MRC assay protocol was described previously (24). Enzyme activity was expressed with respect to that of citrate synthase, which is a marker of mitochondrial mass.

Immunohistochemical staining

A portion of muscle tissue sample was previously frozen in isopentane-cooled liquid nitrogen and stored at −70°C. This sample was serially cross-sectioned into 10-μm thick layers using a cryostat microtome maintained at −20°C and mounted on a coated microscope slide. The sections were stained with histochemical methods for the detection of oxidative enzyme activity as described previously (25).

Electron microscopy

Mitochondrial structure was evaluated by transmission electron microscopy. Muscle tissue was sliced into small pieces (1 × 1 mm) and placed in ice-cold fixative buffer (2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2). To minimize the oxygen exposure time, this procedure was performed immediately after muscle sample was dissected. Specimens were

postfixed for 1.5 h in 2% OsO_4 in 0.1 M phosphate buffer, washed briefly with deuterated H_2O, dehydrated through a graded ethanol series (50, 60, 70, 80, 90, 95, and 100%), and infiltrated with a mixture of propylene oxide (Acros Organics, USA) and EPON epoxy resin. Finally, samples were embedded with only epoxy resin. The samples were loaded into capsules and polymerized at 38°C for 12 h and 60°C for 48 h. Thin sections were made using an ultramicrotome (RMC MT-XL) and collected on copper grids. Ultrathin sections for electron microscopy were approximately 50 nm thick. The ultrathin sections were stained with 4% uranyl acetate and 4% lead citrate solution and examined using a JEM-1400 electron microscope at 80 kV. Transmission electron photomicrographs were obtained at ×10,000 and ×50,000 magnification.

2.9. Microarray

RNA integrity was analyzed using the Agilent 2100 Bioanalyzer, and RNA integrity numbers for all samples were >8.5. Microarray analysis was performed using Affymetrix chips (Affymetrix GeneChip Human or Rat Gene 1.0 ST Array) to identify gene expression profiles in our study groups. Gene expression data were analyzed using Expression Console software (Affymetrix) (26). The Robust Multichip Analysis tool was used to normalize expression data, and Quantile Normalization was used to remove technical

variability in the experiments. We calculated the log fold-change of each probe under different conditions and in independent samples. Student's *t*-test was performed to determine the statistical significance of differential expression using GenePattern software (27).

2.10. Quantitative real time PCR and western blot analysis

Total RNA was isolated from frozen muscle samples using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of RNA using Superscript II Reverse Transcriptase (Invitrogen). Quantitative RT-PCR was performed using SYBR Green PCR master mix and an ABI prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). The following PCR primers were used for each gene (5′→3′): human IL-6-F (forward), GGT ACA TCC TCG ACG GCA TCT; IL-6-R (reverse), GTG CCT CTT TGC TGC TTT CAC; TNF-α-F, CCC AGG GAC CTC TCT CTA ATC A; TNF-α-R, AGC TGC CCC TCA GCT TGA G; MSTN-F, TGG TCA TGA TCT TGC TGT AAC; MSTN-R, TGT CTG TTA CCT TGA CCT CTA; CAT-F, GAA CTG TCC CTA CCG TGC TCG A; CAT-R, CAA ACC TTG GTG AGA TCG AA; GPX1-F, GAG GTG GGA GGA CAG GAG TTC TT; GPX1-R, CTTCCTACCCTCAAGTATGTCCG; NAMPT-F, GCA GAA

GCC GAG TTC AAC ATC; NAMPT-R, TGC TTG TGT TGG GTG GAT ATT G; SIRT3-F, TCGCAACTATACCCAGAACATAGACA; SIRT3-R, CTG TTG CAA AGG AAC CAT GAC A; UCP2-F, CAT TCT GAC CAT GGT GCG TAC TGA; UCP2-R, GTT CAT GTA TCT CGT CTT GAC CAC; UCP3-F, GGA CAA CTC CAG CCT CAC TA; UCP3-R, CTG GGC CAC CAT CTT TAT CA; GAPDH-F, GAT CAT CAG CAA TGC CTC CT; GAPDH-R, TGT GGT CAT GAG TCC TTC CA.

The tissue lysates were subjected to SDS-PAGE and proteins were transferred to polyvinylidene difluoride (PVDF) membrane. Antibodies against IL-6 and GAPDH were purchased from Cell Signaling Technology (Danvers, MA). Immunodetection was performed by enhanced chemiluminescence (Pierce, Rockford, IL).

2.11. Statistical analysis

All continuous variables are presented as the mean ± standard error (±S.E.) or as a percentage. The number of subjects in each group was too limited for parametric analysis; therefore, we used non-parametric analytical methods. Mean values were compared between groups using the Mann-Whitney U test. Unaffected and affected legs were compared using Wilcoxon's signed rank test. Correlation analysis between variables was computed using Spearman's correlation. *P* value <0.05 was considered

statistically significant. All statistical analyses were performed using IBM SPSS Statistics 19 (SPSS Inc., Chicago, Illinois).

III. Results

3.1 Clinical characteristics of the study population

Clinical characteristics of the study population are presented in **Table 1**. Clinical characteristics were analyzed separately according to gender because average body composition and muscle strength differ in males and females. The average ages of male subjects were 39 and 72 years old, and the average ages of female subjects were 40 and 72 years old, in the YD and OD groups, respectively. No significant differences were observed in obesity indices of YD and OD subjects in both genders. However, male subjects in the OD group had significantly larger waist circumference than that of males in the YD group (YD, 84.0 ± 7.1; OD, 93.3 ± 6.7; p = 0.045). Systolic blood pressure in male subjects was significantly higher in the OD group than the YD group. Although patients with diabetes mellitus were excluded during enrollment for this study, fasting plasma glucose level in male subjects was marginally higher in OD than YD. However, the HbA1c level, which is a marker of average blood glucose levels over the previous months, was comparable in both groups. Glucose tolerance profiles in female subjects were not significantly different between YD and OD groups. Total levels of cholesterol, creatinine, liver enzymes, and hs-CRP did not differ significantly between the groups for both genders. Avascular necrosis was the most

Table 1. The anthropometric and biochemical characteristics of the subjects

	Male			Female		
	YD	OD	*P* value	YD	OD	*P* value
N	18	5		11	13	
Age	38.7±10.4	71.8±6.2	<0.001	39.8±13.3	71.8±2.5	<0.001
Body weight (kg)	69.8±6.9	69.4±13.9	1.000	64.3±19.6	56.6±8.6	0.691
Height (cm)	173.4±7.4	165.5±6.8	0.071	159.3±6.0	153.3±4.1	0.009
BMI (kg/m^2)	23.3±2.3	25.2±4.0	0.319	25.1±6.0	24.1±3.2	0.776
WC (cm)	84.0±7.1	93.3±6.7	0.045	82.3±16.3	80.2±7.2	0.887
HC (cm)	94.3±4.9	100.8±6.0	0.058	95.8±12.0	93.8±3.5	0.740
Waist hip ratio	0.89±0.05	0.93±0.02	0.262	0.85±0.07	0.85±0.07	1.000
SBP (mmHg)	120.8±9.3	133.1±5.1	0.009	114.9±12.0	123.0±14.1	0.150
DBP (mmHg)	73.2±9.2	76.1±8.8	0.493	71.0±10.1	71.3±11.5	0.865
FPG (mg/dl)	95.6±6.1	101.2±5.8	0.080	97.2±5.9	98.1±11.8	0.691
HbA1c (%)	5.6±0.3	5.6±0.3	0.774	5.6±0.3	5.7±0.4	0.649
TC (mg/dl)	184.4±27.4	211.8±86.3	0.745	189.8±38.2	202.7±31.0	0.494
Cr (mg/dl)	1.1±0.1	1.1±0.1	0.538	0.9±0.1	1.0±0.1	0.093
AST (IU/L)	23.2±7.6	29.2±8.9	0.130	24.9±14.9	19.8±2.6	0.494
ALT (IU/L)	26.5±14.5	39.8±34.2	0.538	27.1±17.3	21.2±14.9	0.252
Hs-CRP (mg/dl)	0.5±0.6	0.5±0.5	0.308	0.37±0.84	0.26±0.28	0.820
Indication of surgery	AVN, 16; DA, 2	AVN, 2; DA, 3	NA	AVN, 3; DA, 8	AVN, 3; DA, 10	NA

Data are means ± SD

BMI, body mass index; WC, waist circumference; HC, hip circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; TC, total cholesterol; CRP, c-reactive protein; TNF, tumor necrosis factor; WOMAC, Western Ontario and McMaster Universities Arthritis Index; AVN, avascular necrosis; DA: degenerative arthritis; LCPD, Legg-Calve-Perthes disease; DDH, developmental dysplasia of the hip

P value was obtained by Mann-Whitney U test

common cause of THRA in young male patients, whereas osteoarthritis was the most common cause in other groups.

3.2 Body composition and muscle strength/quality of the study population

Total body lean mass and ASM (a sum of muscle mass in upper and lower extremities) were not significantly lower in both genders of the OD group compared to the YD group. Female OD subjects were the only group that had significant changes in average muscle mass in the unaffected leg (YD, 6.2 ± 1.4 kg; OD, 5.1 ± 0.7 kg; p = 0.021). Muscle mass of affected legs in female subjects was significantly and marginally reduced in YD and OD groups, respectively, compared with that of unaffected legs. Indices of sarcopenia such as ASM/Wt and ASM/Ht^2 did not significantly differ in YD and OD groups in both genders (**Table 2**).

Age-related changes in muscle strength were more evident. In female subjects, peak torque in the OD group was significantly lower than that in the YD group in affected and unaffected legs for both flexion and extension. In male subjects, peak torque declined in affected and unaffected legs in the OD group; these changes were consistent with those of the female group, but they were less striking. Peak torque of knee flexion and extension in the unaffected

Table 2. Comparison of muscle mass by age group, stratified by sex

	Male			Female		
	Young diseased	Old diseased	*P* value	Young diseased	Old diseased	*P* value
Lean mass (kg)						
Total body	51.2±5.2	49.2±7.4	0.820	37.6±7.8	34.5±3.7	0.346
Leg-affected	8.1±1.2	7.3±1.3	0.249	5.7±1.2[a]	5.0±0.8[b]	0.093
Leg-unaffected	8.1±1.2	7.7±1.0	0.682	6.2±1.4	5.1±0.7	0.021
ASM (kg)	22.4±3.2	21.0±3.7	0.820	16.3±3.8	13.7±1.8	0.123
ASM/Wt (%)	73.0±6.6	72.8±9.7	0.682	58.8±6.8	61.8±7.1	0.674
ASM/Ht^2 (kg/m^2)	7.47±0.71	7.45±0.78	0.963	6.33±1.04	5.84±0.70	0.228

Data are means ± SD

ASM, Appendicular skeletal muscle (total muscle mass in upper- and lower-extremities)

Statistical significance test was done by Mann-Whitney U test (comparison between young and old diseased groups) or Wilcoxon signed rank test (between affected and unaffected legs)

a, $p < 0.05$ vs. unaffected leg; b, $p = 0.071$ vs. unaffected leg

legs of OD male subjects was approximately 70–80% of that in YD male subjects; for OD female subjects, it was approximately 55–64% of that in YD female subjects. Peak torque in the affected leg of both YD and OD female subjects was reduced, with a more significant difference in the YD group. In male subjects, only the YD group had significantly lower peak torque in the affected leg at flexion **(Table 3)**.

Muscle quality, which is defined as peak torque per kg lean leg mass, was significantly greater at both flexion and extension for both affected and unaffected legs in YD female subjects than in OD female subjects; for male subjects, significantly reduced muscle quality in the OD group was observed only at extension of the knee in the unaffected leg **(Table 3)**.

3.3. Correlations between age, muscle strength, and physical disability

Next, we performed correlation analysis between age, muscle mass, muscle quality, and muscle strength **(Table 4)**. Analyses were performed only for the unaffected leg because the affected leg may not accurately reflect age-related sarcopenia. In both genders, age was significantly negatively correlated with muscle strength. Correlation coefficients ranged from –0.53 to –0.66, indicating moderate to strong correlation between age and muscle strength. Muscle quality also was significantly negatively correlated with age

Table 3. Comparison of leg muscle strength/quality and severity of physical dysfunction by age group, stratified by sex

		Male			Female		
		Young diseased	Old diseased	*P* value	Young diseased	Old diseased	*P* value
Affected leg							
Peak torque (Nm)	Flexion	51.1±17.5[a]	34.5±13.9	0.152	32.4±9.3[a]	21.0±7.8[b]	0.006
	Extension	105.6±28.0	67.3±21.6	0.020	75.2±24.5[a]	41.6±13.4[a]	0.001
PT/lean mass (Nm/kg)	Flexion	6.3±1.4[a]	4.8±2.1	0.277	5.8±1.7[a]	4.4±1.7[b]	0.061
	Extension	13.3±2.9	9.4±4.4	0.158	13.6±5.0	8.5±2.3[a]	0.024
Unaffected leg							
Peak torque (Nm)	Flexion	55.2±18.2	43.2±17.7	0.249	41.4±12.4	26.4±6.6	0.002
	Extension	109.1±39.7	74.3±24.0	0.148	94.6±28.8	51.7±17.9	0.002
PT/lean mass	Flexion	6.9±1.5	5.5±2.0	0.185	6.7±1.3	5.3±1.5	0.043
(Nm/kg)	Extension	13.7±3.8	9.5±2.2	0.027	15.3±4.2	10.2±3.1	0.008
Physical dysfunction							
WOMAC dysfunction		34.5±10.7	41.5±11.9	0.477	39.5±11.9	49.7±19.3	0.181

Data are means ± SD.

WOMAC, Western Ontario and McMaster Universities Arthritis Index

Statistical significance test was done by Mann-Whitney U test (comparison between young and old diseased groups) or Wilcoxon signed rank test (between affected and unaffected legs)

a, $p < 0.05$ vs. unaffected leg; b, $p < 0.10$ vs. unaffected leg

Table 4. Relationship of age to the muscle mass and strength in unaffected legs

	Age	
	Male	Female
	r	*r*
Leg muscle mass	-0.363	-0.477*
Peak Torque Flexion	-0.585**	-0.575**
Peak Torque Extension	-0.527*	-0.662**
Muscle quality Flexion	-0.657**	-0.480*
Muscle quality Extension	-0.580**	-0.624**

Correlation coefficient and *P* value were evaluated by spearman's correlation test

* $p < 0.05$; ** $p < 0.01$

in both genders. The correlation between muscle mass and age was not significant in male subjects ($r = –0.363$, $p = 0.116$), whereas it was significant in female subjects ($r = –0.477$, $p = 0.025$).

Age was marginally correlated with WOMAC dysfunction score ($r = 0.341$, $p = 0.052$). WOMAC dysfunction score was significantly correlated with lower leg muscle strength. Muscle strength in the affected leg was more strongly correlated with physical dysfunction. Declining muscle quality appeared to have a greater contribution to physical dysfunction than declining muscle mass **(Table 5)**.

In both genders of the OD group, the reduction in muscle strength was more striking than the reduction in muscle mass. This suggests that muscle strength per unit muscle mass (muscle quality) significantly declines with aging. We hypothesized that this decrease in muscle quality could be caused by aging-related quantitative or qualitative changes in mitochondrial function. Therefore, we examined quantitative changes in muscle mitochondria in YD and OD groups.

3.4 Quantitative change in muscle mitochondria

Electron microscopy results were available for 6 YD and OD subjects. We did not observe any significant differences in mitochondrial size, morphology, or organization in YD and OD skeletal muscle. Mitochondrial

Table 5. Relationship of physical disability to age, muscle mass, strength and quality

	WOMAC Dysfunction	
	r	*P* value
Age	0.341	0.052
Unaffected leg		
Leg muscle mass	-0.194	0.323
Peak Torque Flexion	-0.378	0.043
Peak Torque Extension	-0.321	0.090
Peak torque/muscle mass Flexion	-0.353	0.071
Peak torque/muscle mass Extension	-0.274	0.166
Affected leg		
Leg muscle mass	-0.124	0.531
Peak Torque Flexion	-0.559	0.002
Peak Torque Extension	-0.523	0.004
Peak torque/muscle mass Flexion	-0.627	0.001
Peak torque/muscle mass Extension	-0.571	0.002

Correlation coefficient and *P* value were evaluated by spearman's correlation test

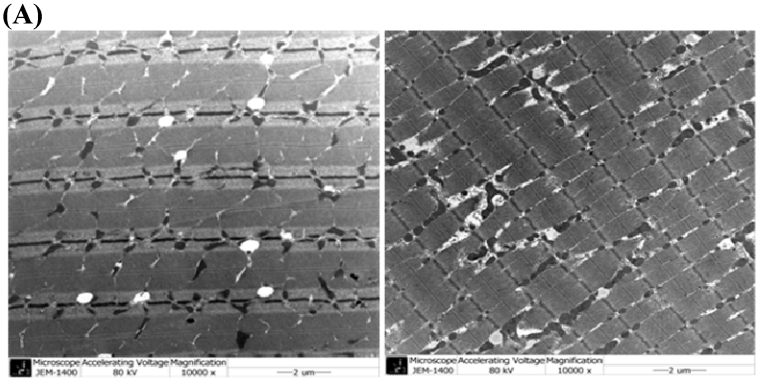
numbers were significantly higher in OD muscle samples **(Figure 1)**. Western blot analysis of mitochondria indicated that the content of mitochondrial complexes was significantly higher in OD muscle samples **(Figure 2)**.

3.5. Mitochondrial function in skeletal muscle

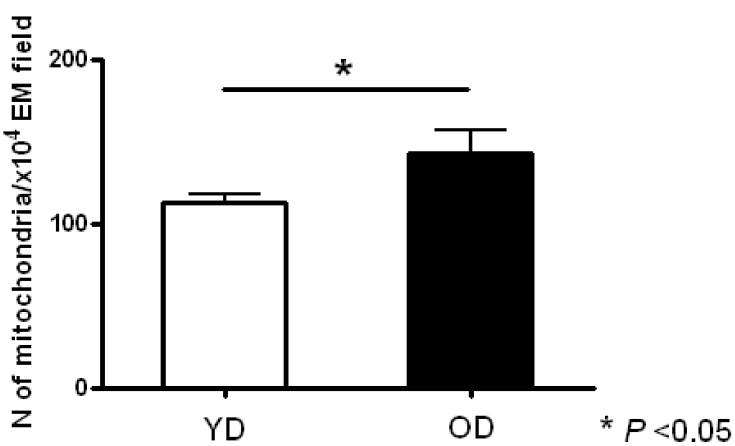
Enzyme activities involved in mitochondrial respiratory chain complexes were analyzed by performing MRC assays for 20 (14 male and 6 female) YD subjects and 9 (2 male and 7 female) OD subjects. Oxygen consumption rate per unit mitochondria was measured by high-resolution respirometry in 19 (14 male and 5 female) YD subjects and 10 (3 male and 7 female) OD subjects. Maximal O_2 consumption capacity could not be obtained in 3 YD and 2 OD subjects. We anticipated that O_2 consumption capacity per unit mitochondria would be lower in OD muscle samples than in YD samples. However, the results showed that it was not significantly different between YD and OD, but tended to be insignificantly higher in the OD group **(Figure 3A)**. Cytochrome c oxidase (complex IV) activity was similarly reduced than the normal range in YD and OD muscle samples (YD, 45.8%; OD, 36.4%; $p = 0.501$). NADH dehydrogenase (complex I) activity was significantly higher in OD muscle samples (81.1%) than in YD samples (47.4%; $p = 0.002$) **(Figure 3B)**.

Figure 1. Electron microscopic examination of the morphology and the number of mitochondria

(A)



(B)

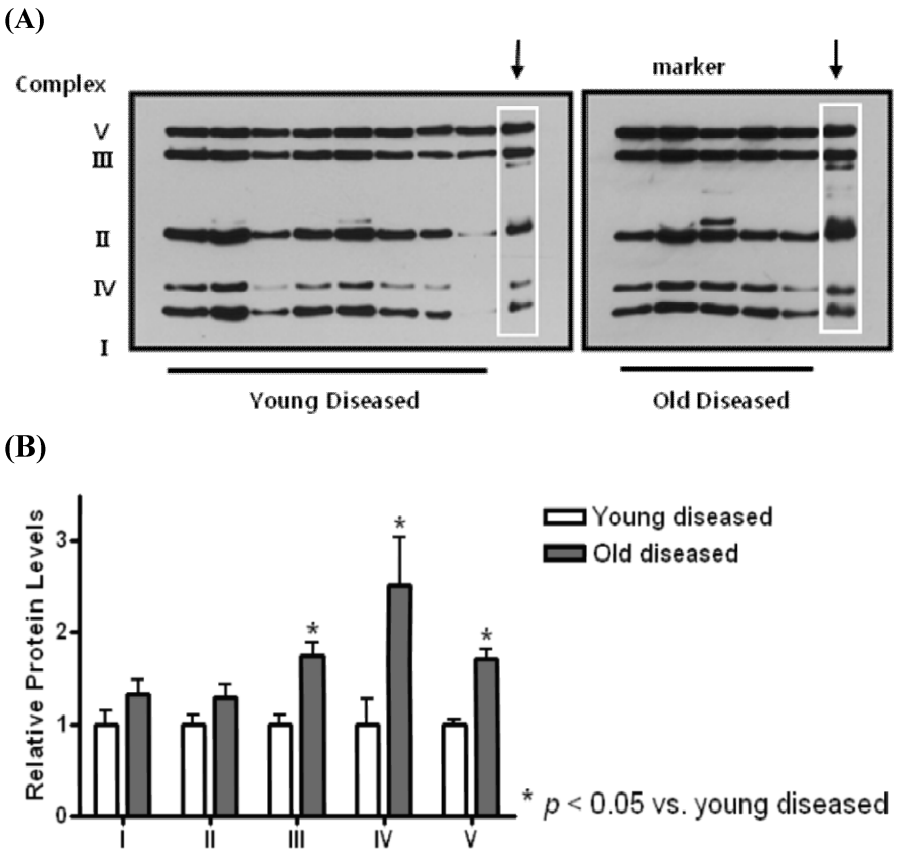


(A) Representative transmission electron microscope image of young and old diseased skeletal muscle (x 10000).

(B) The number of mitochondria was significantly increased in the old diseased than in the young diseased subjects.

YD, young diseased group; OD, old diseased group

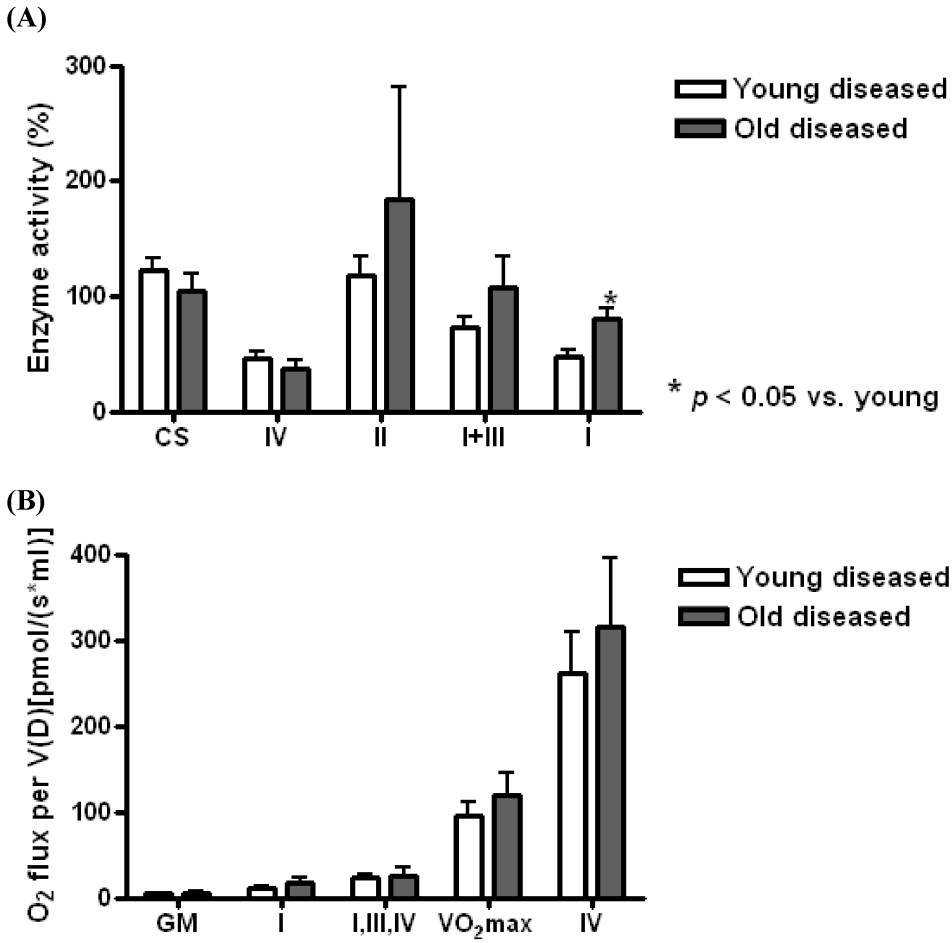
Figure 2. Complex western blot in young and old diseased muscle



(A) Representative western blot images of mitochondrial complex proteins

(B) Protein expressions of mitochondrial complex III, IV and V were significantly increased in the old diseased group.

Figure 3. Comparison of mitochondrial function between young and old diseased group



(A) Enzyme activity of mitochondrial complex protein (Mitochondrial respiratory chain assay)

(B) O_2 consumption measurement by high resolution respirometry.

CS, citrate synthase; GM, glutamate and malate; VO_2max, maximum O_2 consumption capacity

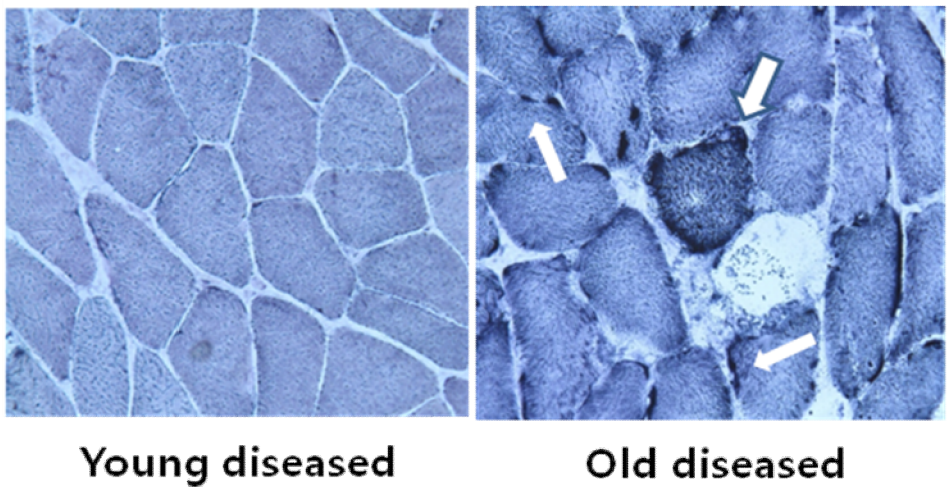
3.6 Immunohistochemistry staining of mitochondrial protein in muscles

We performed immunohistochemical staining with succinate dehydrogenase (SDH) to assess MRC complex II. OD muscle tended to show more evidence of subsarcolemmal mitochondrial aggregation and SDH blue fibers than YD muscle, suggesting that OD muscle had abnormal mitochondrial proliferation **(Figure 4)**. These results may provide evidence of declining mitochondrial quality in OD muscle.

3.7. Quantitative and qualitative features of mitochondria with respect to muscle strength

The observation of aging-related quantitative and qualitative changes of mitochondria in skeletal muscle was somewhat unexpected. The participants in our study were surgical candidates who had varying degrees of muscle dysfunction, and muscle mass and strength were highly variable even within the same age groups. Therefore, it was possible that muscle quality changes resulted from the underlying disease rather than aging. To obtain a better understanding of the relationship between sarcopenia and mitochondrial

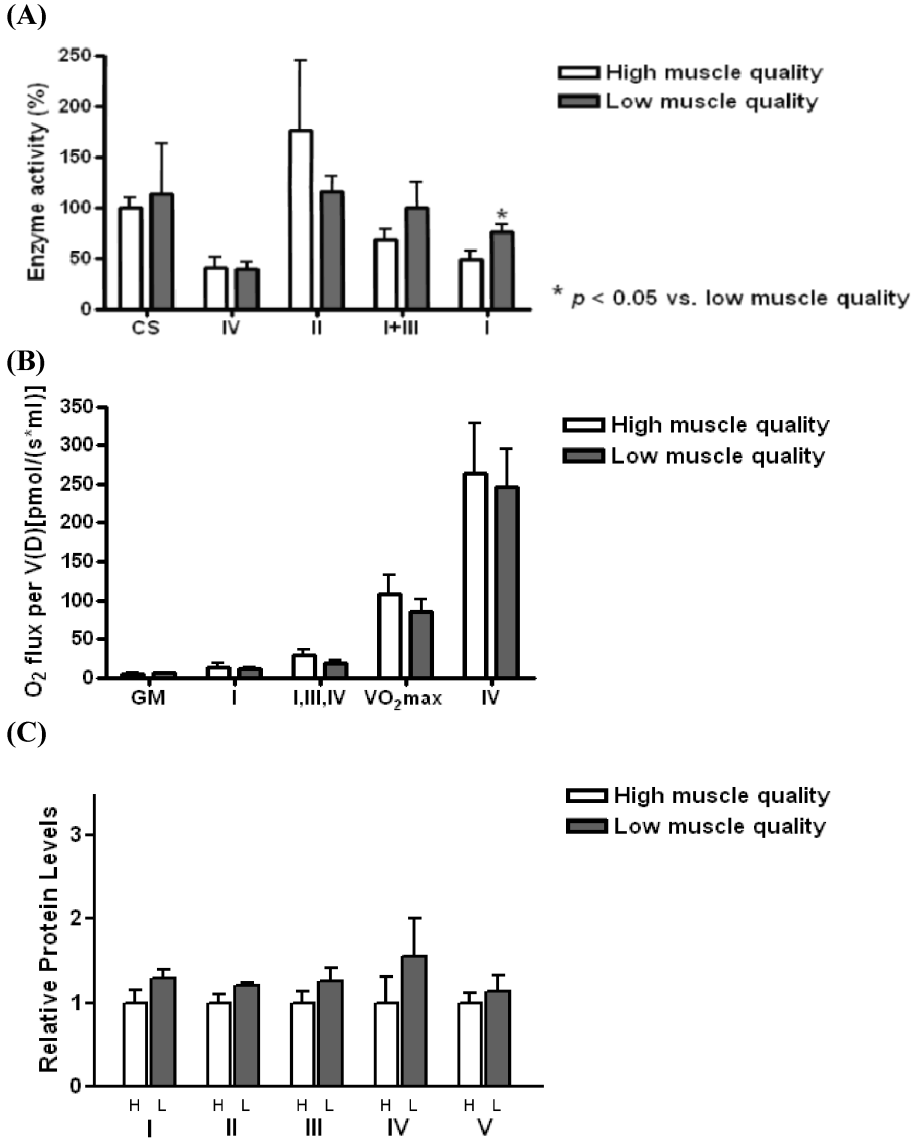
Figure 4. SDH staining of young and old diseased muscle



Thick arrow, succinate dehydrogenase (SDH) blue fiber; thin arrow, subsarcolemal mitochondrial proliferation

changes, we classified our study subjects into two groups according to muscle quality, and compared MRC complex activity, oxidative capacity, and MRC complex protein expression. As the muscle quality differs with the gender, we defined the group with high and low muscle quality separately in men and women. O_2 consumption capacity per unit mitochondria tended to be lower in the low muscle quality group than in the high muscle quality group, although these data were not statistically significant. This result differed from the previous analysis of O_2 consumption in YD and OD groups **(Figure 5A)**. NADH dehydrogenase (complex I) activity was significantly higher in the low muscle quality group than in the high muscle quality group, which was consistent with the observation of high complex I activity in the OD group **(Figure 5B)**. Therefore, the observation of high O_2 consumption capacity in OD skeletal muscle was probably confounded by the underlying muscle dysfunction in the YD group. Complex I activity was significantly lower in the YD group and the high muscle quality group, which suggested that high complex I activity could enhance sarcopenia. The amount of mitochondrial complex protein expression was increased in the low muscle quality group, which is thought to be consistent with the finding observed between the YD and OD groups **(Figure 5C)**.

Figure 5. Mitochondrial changes according to the muscle quality



(A) Enzyme activity of mitochondrial complex protein

(B) O_2 consumption measurement by high resolution respirometry.

(C) Mitochondrial complex protein expression

CS, citrate synthase; GM, glutamate and malate; VO_2max, maximum O_2 consumption capacity

3.8. Microarray analysis of gene expression profiles

We performed microarray analysis in 5 YD and 5 OD subjects to examine gene expression profiles associated with sarcopenia. The microarrays showed that expression of 473 genes were upregulated and 180 genes were downregulated in OD skeletal muscle. We targeted mitochondrial-related proteins, adipokines, and myokines as candidate genes involved in age-related sarcopenia. The microarray analysis results are presented in **Table 6**. IL-6 expression was significantly higher in OD skeletal muscle (fold-change = 1.516, $p < 0.05$). Expression of uncoupling protein 2 (UCP2), catalase, sirtuin3 (Sirt3), and nicotinamide phosphoribosyltransferase (Nampt, visfatin) was elevated in OD skeletal muscle, but this was not statistically significant. Expression of uncoupling protein 3 (UCP3) and myostatin was significantly reduced in OD skeletal muscle (fold-changes for UCP3 and myostatin were 1.789 and 1.632, respectively; $p < 0.05$). Expression of adiponectin was marginally reduced in OD skeletal muscle (fold-change = 1.482, $p = 0.050$). Expression of C1q tumor necrosis factor–related protein 5, klotho beta, PR domain containing 16, glutathione peroxidase 1 (GPX1), secreted frizzled–related protein 5, resistin, fibroblast growth factor 21, leptin, fibroblast growth factor receptor 1, and tumor necrosis factor were reduced in OD skeletal muscle, but these differences were not statistically significant. We selected IL-6, TNF-α, myostatin, catalase, GPX1, Nampt, Sirt3, UCP2,

Table 6. Results of microarray analysis: Changes of mitochondrial, adipokine and myokine genes in old diseased muscle

Gene Symbol	Gene Description	T score	Fold changes	*P* for T score	*P* for fold change
Up-regulated genes in old age					
IL6	interleukin 6 (interferon, beta 2)	1.729	1.516	0.098	0.040
UCP2	Uncoupling protein 2	0.505	1.204	0.617	0.221
CAT	Catalase	0.722	1.138	0.477	0.341
SIRT3	Sirtuin 3	-0.570	1.053	0.576	0.632
NAMPT	Pre-B-cell colony enhancing factor 1	0.908	1.003	0.374	0.956
Down-regulated genes in old age					
UCP3	uncoupling protein 3	-2.481	1.789	0.023	0.013
MSTN	Myostatin	-2.341	1.632	0.031	0.024
ADIPOQ	adiponectin, C1Q and collagen domain containing	-0.422	1.482	0.679	0.050
C1QTNF5	C1q and tumor necrosis factor related protein 5	-1.535	1.306	0.138	0.127
KLB	Klotho beta	-0.656	1.144	0.521	0.341
PRDM16	PR domain containing 16	-1.277	1.075	0.215	0.547
GPX1	Glutathione peroxidase 1, transcript variant 2	0.114	1.062	0.909	0.605
SFRP5	Secreted frizzled-related protein 5	-0.429	1.055	0.674	0.635
RETN	Resistin	-0.609	1.040	0.550	0.715
FGF21	Fibroblast growth factor 21	-1.242	1.024	0.227	0.812
LEP	Leptin	-0.061	1.023	0.954	0.815
FGFR1	Fibroblast growth factor receptor 1	0.259	1.016	0.731	0.863
TNF	Tumor necrosis factor	-1.042	1.011	0.309	0.899

and UCP3 for real-time PCR analysis.

3.9. Evaluation of serum adipokines and myokines

Adiponectin, leptin, resistin, Nampt, visfatin, TNF-α, and IL-6 are representative adipokines currently under investigation as potential contributors to sarcopenia and obesity (11). Resistin, Nampt, TNF-α and IL-6 also are secreted from skeletal muscle. We measured and compared several serum adipokines and myokines in YD and OD female subjects as a pilot study to identify possible contributors to age-related sarcopenia (n = 4 in each group). Serum adiponectin, resistin, leptin, visfatin, and TNF-α levels were not significantly different in the groups. By contrast, serum IL-6 level was marginally and statistically higher in OD female subjects than in YD female subjects **(Table 7)**.

3.10. Expression of mitochondrial proteins and myokines transcripts in skeletal muscles

IL-6 mRNA expression was reduced in OD skeletal muscle. Expression of TNF-α, myostatin, catalase, GPX1, Nampt, and Sirt3 did not significantly

Table 7. Comparison of serum adipokine and myokine levels in the female young and old diseased groups (pilot study)

	Female		
	Young diseased	Old diseased	*P* value
Adiponectin (pg/ml)	3.0±1.4	3.6±1.0	0.686
Leptin (pg/ml)	29.4±25.0	39.0±33.0	0.486
IL-6 (pg/ml)	1.59±0.97	9.65±9.38	0.057
Resistin (pg/ml)	4.7±2.9	6.1±4.5	0.686
Visfatin (ng/ml)	0.9±0.4	4.6±6.5	1.000
TNF-α (pg/ml)	4.7±2.2	5.8±2.4	0.686

IL-6, interleukin-6; TNF, tumor necrosis factor

P value was obtained by Mann-Whitney U test

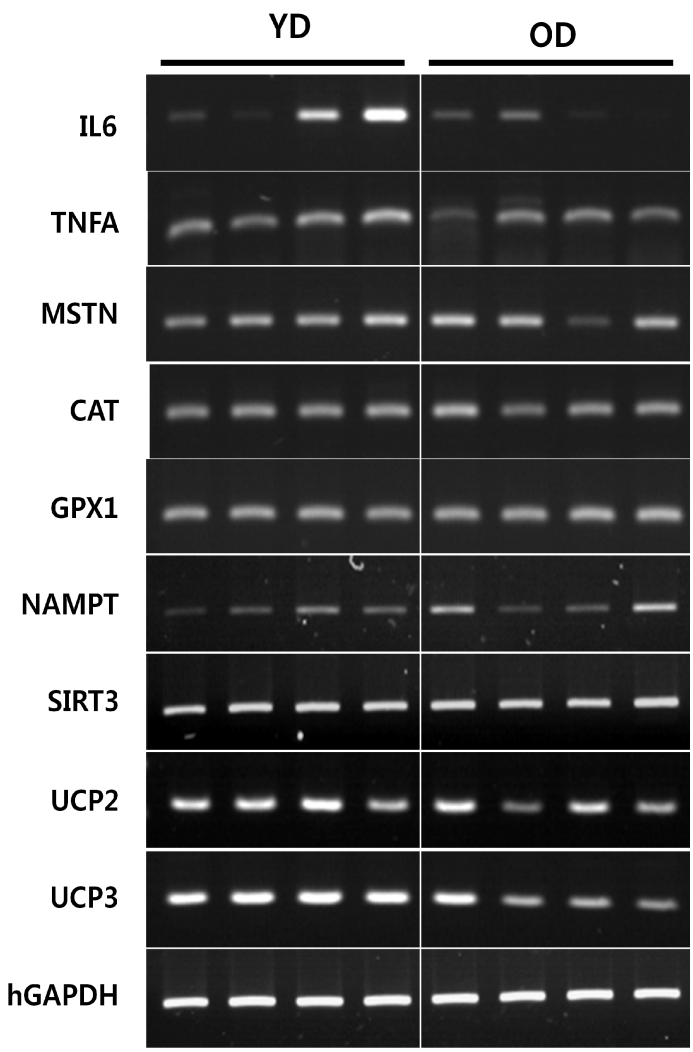
differ in YD and OD skeletal muscle. UCP2 and UCP3 were slightly reduced in OD skeletal muscle **(Figure 6)**.

3.11. Evaluation of the role of IL-6 in sarcopenia

The results showed that IL-6 levels increased in serum, whereas IL-6 transcript levels declined in muscle tissue of OD subjects. We hypothesized that serum and tissue IL-6 may have different roles in age-related sarcopenia. Therefore, we measured serum IL-6 in a larger number of patients and performed a western blot analysis to confirm the results of the pilot blood test and RT-PCR analysis.

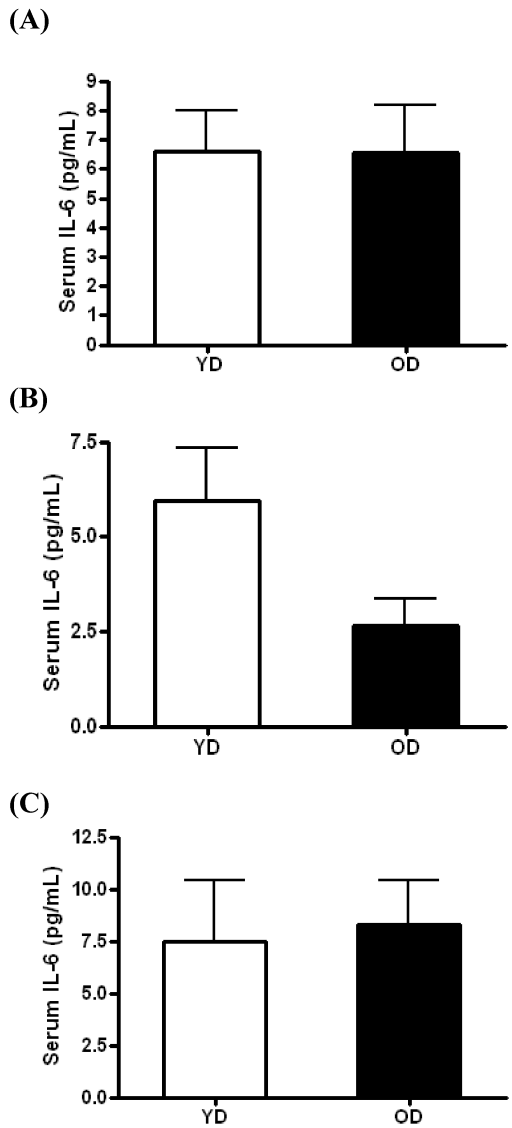
Serum IL-6 was measured in 27 YD (16 male and 11 female) and 13 OD (4 male and 9 female) subjects. Serum IL-6 levels did not significantly differ in YD and OD groups. A gender difference was suspected, with higher serum IL-6 level in the YD male group and higher serum IL-6 level in the OD female group **(Figure 7)**. Western blot analysis was done in 23 YD (14 male and 9 female) and 8 OD (2 male and 6 female) subjects. IL-6 protein expression levels in skeletal muscle tended to be higher in the OD group, particularly in female subjects ($p = 0.066$, **Figure 8**). Serum IL-6 level was negatively correlated with muscle strength ($r = –0.333$, $p = 0.047$ versus peak torque extension in the unaffected leg; $r = –0.297$, $p = 0.079$ versus peak

Figure 6. RT-PCR results of target protein in young and old diseased muscles



YD, young diseased; OD, old diseased; IL6, interleukin-6; TNFA, tumor necrosis factor-α; MSTN, myostatin; CAT, catalase; GPX1, glutathione peroxidase 1; NAMPT, nicotinamide phosphoribosyltransferase; SIRT3, sirtuin 3; UCP2, uncoupling protein 2; UCP3, uncoupling protein 3

Figure 7. Comparison of serum IL-6 level between the young and old diseased groups



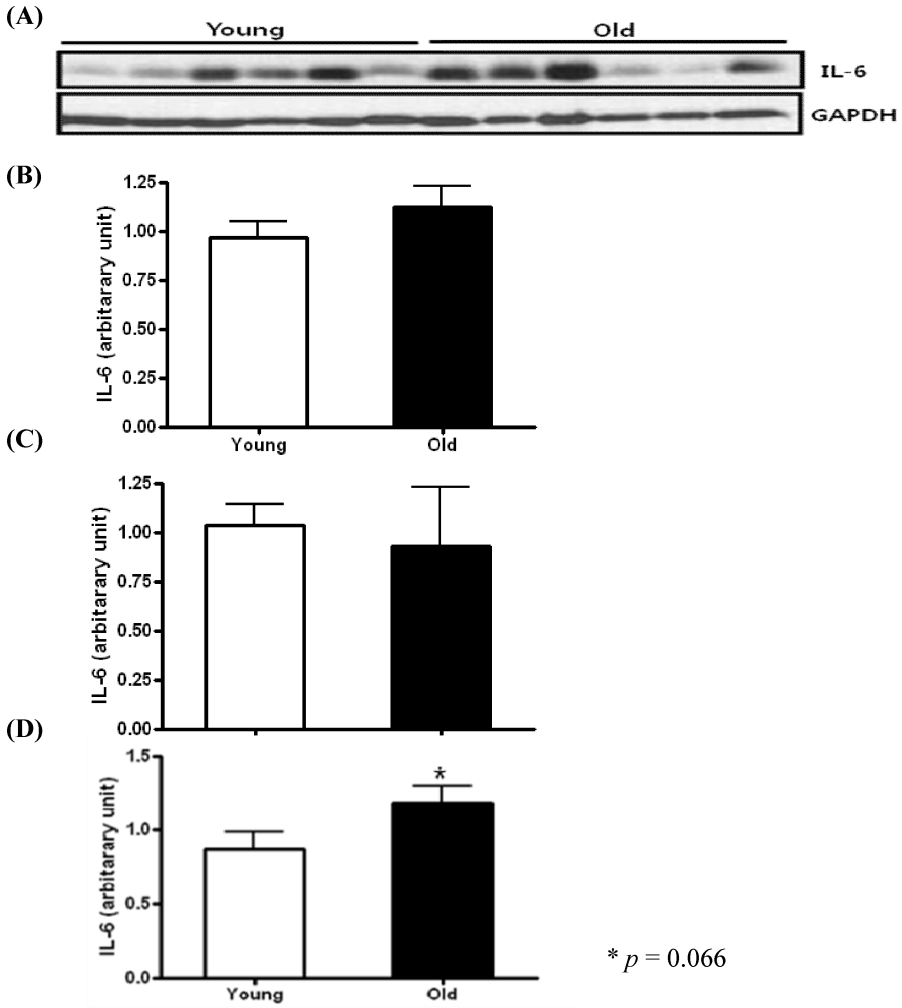
(A) Serum IL-6 level in total subjects.

(B) Serum IL-6 level in male subjects

(C) Serum IL-6 level in female subjects

YD, young diseased; OD, old diseased

Figure 8. Comparison of IL-6 expression in skeletal muscle between the young and old diseased groups



(A) Representative western blot image of tissue IL-6 expression

(B) Tissue IL-6 expression in total subjects.

(C) Tissue IL-6 expression in male subjects

(D) Tissue IL-6 expression in female subjects

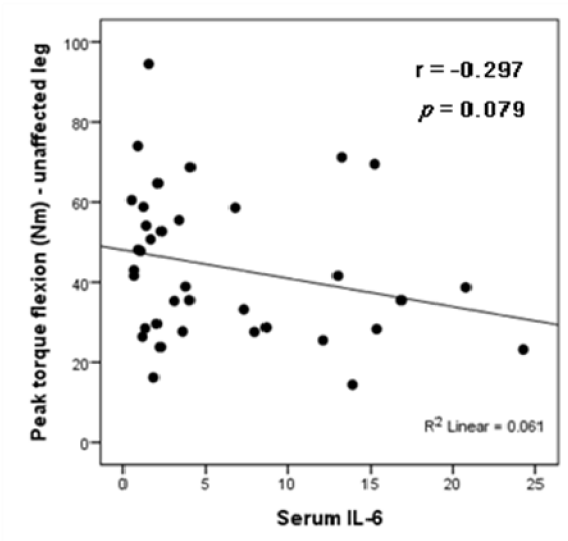
torque flexion in the unaffected leg, **Figure 9**). IL-6 protein expression levels in skeletal muscle were negatively correlated with muscle quality (r = –0.434, p = 0.027 versus peak torque extension/muscle mass in the unaffected leg; r = –0.337, p = 0.092 versus peak torque flexion/muscle mass in the unaffected leg, **Figure 10**).

3.12. Serum and tissue IL-6 levels negatively correlate with muscle strength

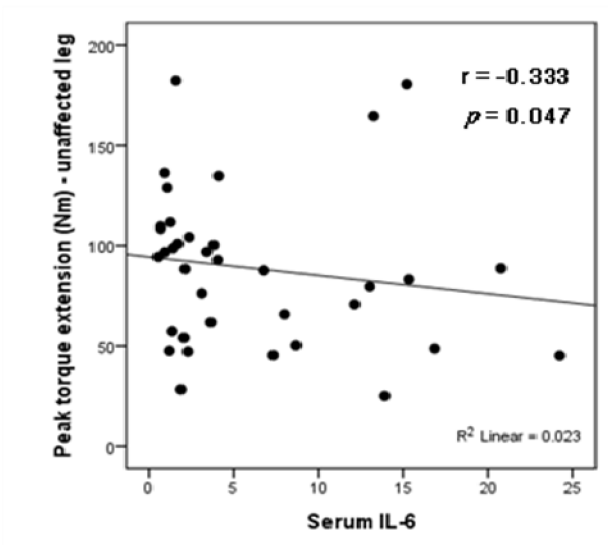
We analyzed whether serum and tissue IL-6 levels differ according to muscle quality. Serum IL-6 levels did not differ between the age groups, but was marginally higher in the low-quality muscle group **(Figure 11).** Similar trends were observed in both genders when assessing muscle quality, although gender trends differed when assessing age groups. Tissue IL-6 expression levels tended to increase in the low-quality muscle group, although this was not statistically significant. Similar traits were observed in both genders **(Figure 12)**. These results suggest that both serum and tissue IL-6 content could affect muscle quality.

Figure 9. Relationship between serum IL-6 level and muscle strength

(A)



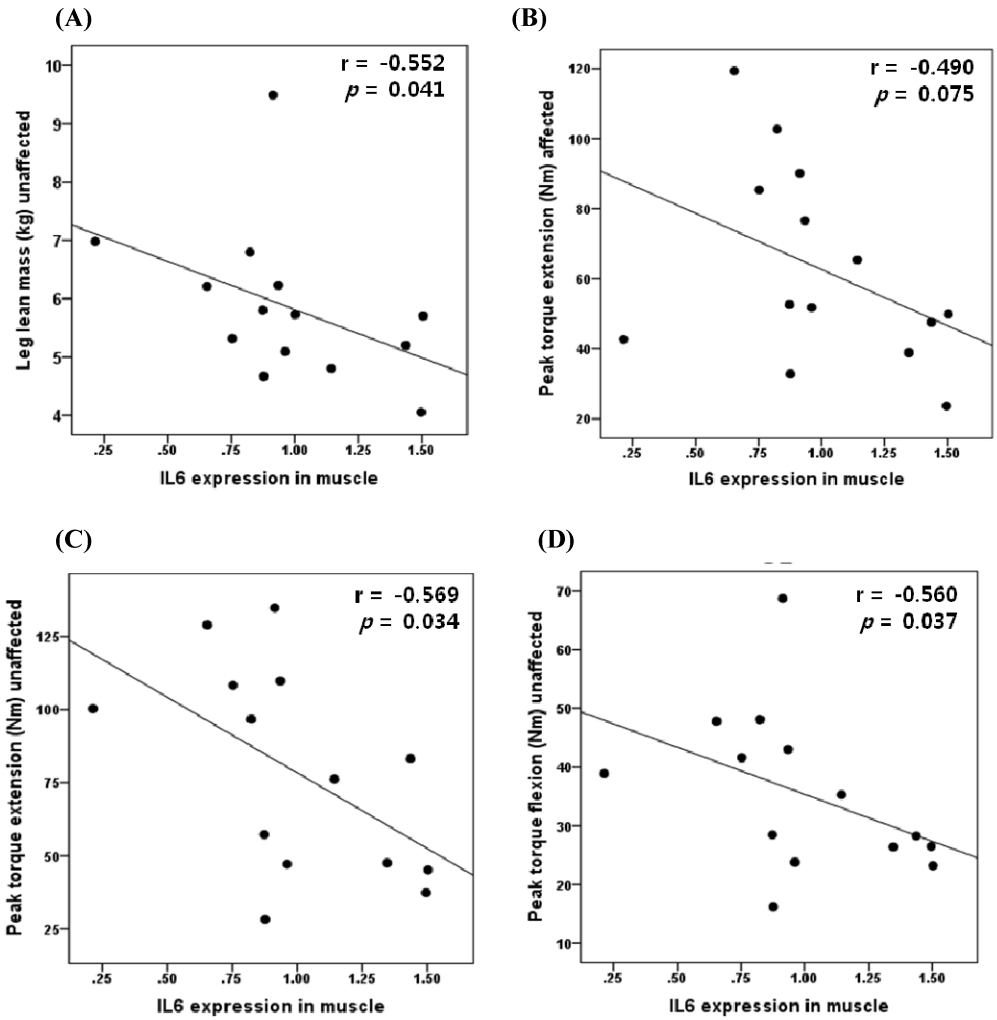
(B)



(A) Correlation between serum IL-6 and peak torque at flexion (unaffected leg)

(B) Correlation between serum IL-6 and peak torque at extension (unaffected leg)

Figure 10. Comparison of IL-6 expression in skeletal muscle between the young and old diseased groups



(A) Correlation between tissue IL-6 and leg muscle mass (unaffected leg)

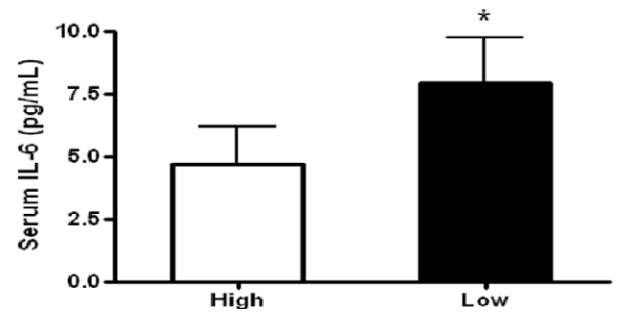
(B) Correlation between tissue IL-6 and peak torque at extension (affected leg)

(C) Correlation between tissue IL-6 and peak torque at extension (unaffected leg)

(D) Correlation between tissue IL-6 and peak torque at flexion (unaffected leg)

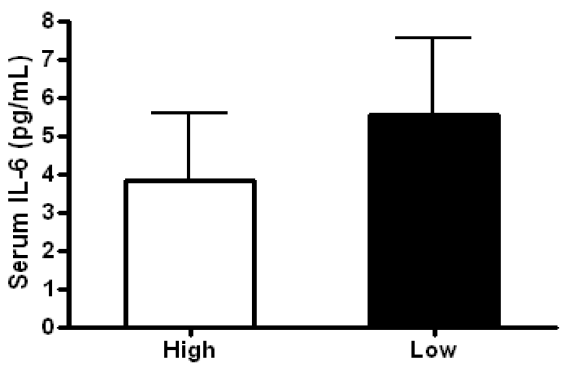
Figure 11. Serum IL-6 level according to muscle quality

(A)

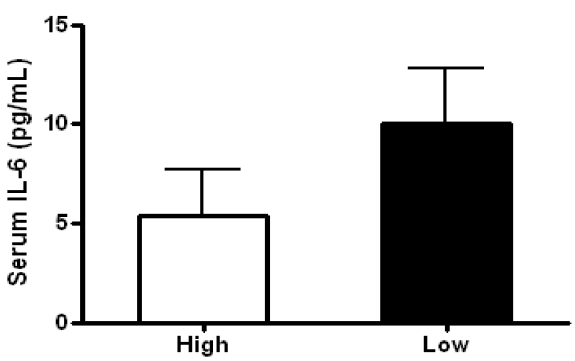


* $p = 0.067$

(B)



(C)

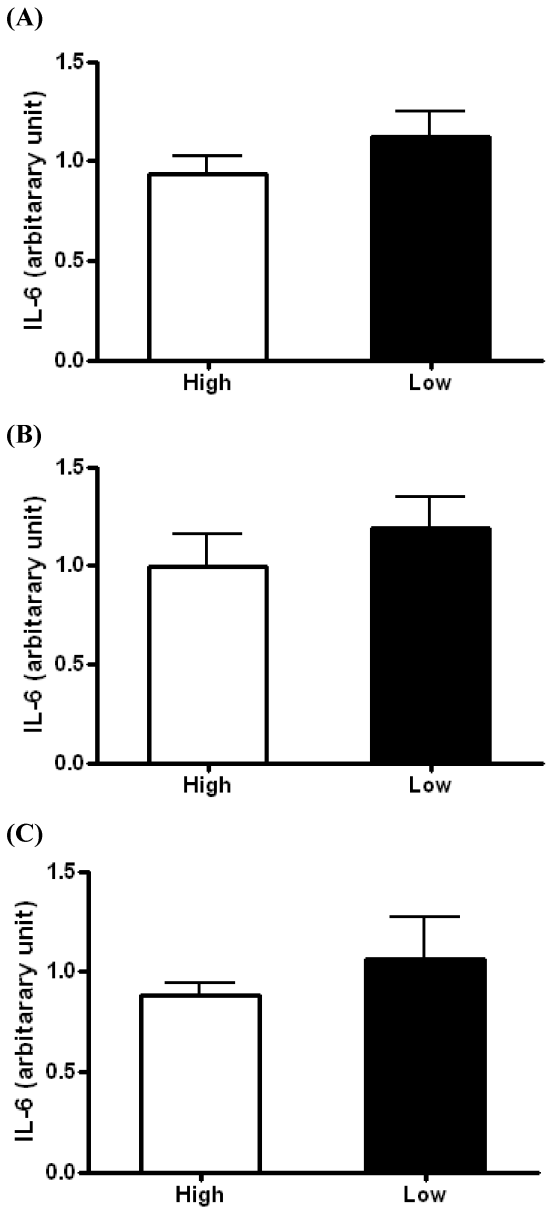


(A) Serum IL-6 level in total subjects. High, high muscle quality; Low, low muscle quality

(B) Serum IL-6 level in male subjects

(C) Serum IL-6 level in female subjects

Figure 12. IL-6 expression in the skeletal muscle according to muscle quality



(A) Tissue IL-6 expression in total subjects. High, high muscle quality; Low, low muscle quality

(B) Tissue IL-6 expression in male subjects

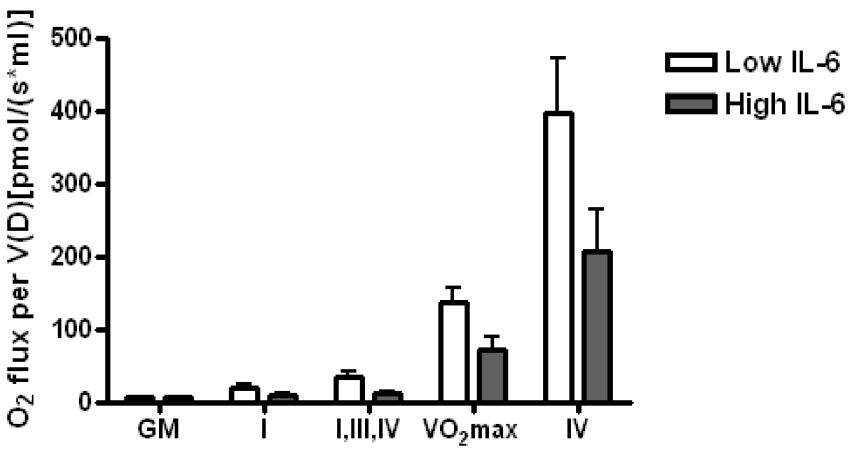
(C) Tissue IL-6 expression in female subjects

3.13. Relationship of tissue IL-6 expression with mitochondrial function and content

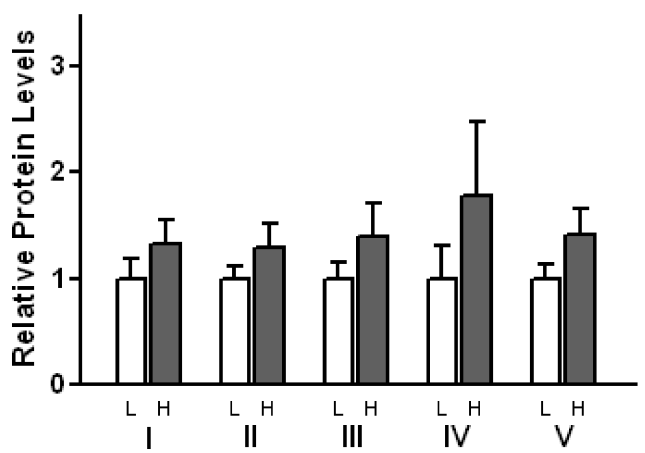
We investigated the relationship between IL-6 expression and mitochondrial function in skeletal muscle, which were both likely to influence the development of sarcopenia. The study subjects were classified into two groups with high or low IL-6 expression in skeletal muscle; we compared mitochondrial oxidative capacity and the amount of complex protein expression in the two groups. We observed higher maximal oxidative capacity in the high IL-6 expression group (70.5 ± 59.2 $pmol \cdot s^{-1} \cdot cm^{-3}$) than in the low expression group (136.4 ± 66.4 $pmol \cdot s^{-1} \cdot cm^{-3}$), and this result was marginally significant ($p = 0.065$). There was no significant difference in mitochondrial complex amount between the two groups, but the high IL-6 expression groups tended to have higher levels of complex protein expression **(Figure 13).**

Figure 13. Relationship between IL-6 expression in the skeletal muscle and mitochondrial changes

(A)



(B)



(A) O_2 consumption measurement by high resolution respirometry.

(B) Mitochondrial complex protein expression

GM, glutamate and malate; VO_2max, maximum O_2 consumption capacity

IV. Discussion

In this study, we observed that qualitative changes were more prominent than quantitative changes during aging of human skeletal muscle. To understand possible mechanisms of age-related sarcopenia, our main interests were to investigate changes in mitochondrial mass/function and adipokine/myokine expression. Aged human muscle showed evidence of abnormal mitochondria, but had approximately comparable levels of mitochondrial function as that in younger subjects due to elevated mitochondrial proliferation in the older subjects. IL-6 levels in serum and skeletal muscle were not significantly different in the young and old groups, but they did show mild negative correlations with muscle strength or quality.

4.1. The characteristics of sarcopenia

The current study found that aging was accompanied by some reduction in muscle mass, but the reduction in muscle strength was much greater. This result is consistent with that reported by a previous large-scale cohort study (28). Our observation that physical performance was more strongly correlated with muscle strength than with muscle mass also is consistent with a previous report (29). Muscle strength is a function of muscle

mass and muscle quality. Therefore, we speculated that a change in muscle quality, which is defined as muscle strength per unit muscle fiber, occurs with aging. We found that age-related changes in muscle mass and strength were more striking in women than men. However, it has been suggested that these age-related changes in muscle were more prominent in men than women (28, 30). This inconsistency is thought to be due to characteristics of the selected study populations. To better understand the characteristics of our population, we compared our population with a normal-functioning, non-surgical population. We used data from the Korean Longitudinal Study on Health and Aging cohort study, and compared the clinical characteristics of our elderly population with age- and BMI-matched normal-functioning controls. Muscle mass and strength of the elderly normal-functioning control and THRA subjects were not significantly different in both genders. We were unable to perform age- and BMI-matched comparisons of our YD population with data from the KLoSHA cohort study due to the limited number of control subjects. Although the younger female subjects in the current study did not exhibit significant reductions in muscle mass and strength compared with that of control subjects, younger male subjects had reduced muscle mass and strength **(Table 8)**. Even in the subgroup of our younger male surgical patients that could be matched by age and BMI with non-surgical patients (n = 8), muscle strength was significantly lower than in non-surgical patients (data not shown). Thus, disease-related changes in muscle mass and function observed in each

Table 8. Comparison of characteristics between diseased and normal control subjects

	Old patients						Young patients					
	Male			Female			Male			Female		
	Diseased	Control	*P* value	Diseased	Control	*P* value	Diseased	Control	*P* value	Diseased	Control	*P* value
N	5	15		13	26		18	16		11	11	
Age	71.8±6.2	72.1±5.3	0.735	71.8±2.5	71.3±3.6	0.401	38.7±10.4	29.3±4.7	0.003	39.8±13.3	29.6±5.0	0.047
BMI (kg/m^2)	25.2±4.0	24.6±3.8	0.933	24.1±3.2	23.6±3.1	0.435	23.3±2.3	24.4±4.5	1.000	25.1±5.9	22.1±3.9	0.243
FP (%)	25.8±4.1	22.7±6.5	0.262	35.3±7.3	34.4±6.2	0.447	22.8±6.5	19.8±1.9	0.335	37.6±7.0	34.1±7.0	0.387
LM (kg)	49.2±7.4	50.0±7.1	0.961	34.5±3.7	33.4±3.5	0.466	51.2±5.2	54.8±6.8	0.178	37.6±7.8	35.5±5.7	0.512
Leg LM (kg)	7.7±1.0	7.9±1.3	0.810	5.1±0.7	5.1±0.6	0.816	8.1±1.2	9.3±1.0	0.050	6.2±1.4	6.0±1.2	0.605
PTF (Nm)	43.2±17.7	43.6±19.3	0.940	43.2±17.7	44.9±23.1	0.810	55.2±18.2	93.7±17.7	< 0.001	41.4±12.4	44.0±9.1	0.512
PTE (Nm)	74.3±24.0	86.7±25.8	0.531	74.3±24.0	82.0±29.6	0.810	109.1±39.7	183.6±36.3	< 0.001	94.6±28.8	81.7±19.3	0.809

BMI, body mass index; FP, fat percent; LM, lean mass; PTF, peak torque at flexion; PTE, peak torque at extension

P value was obtained by Mann-Whitney U test

group of the current study probably affected the characteristics of sarcopenia.

4.2. Age-related mitochondrial changes in human skeletal muscle

Muscle contraction result from cyclic interactions between the contractile proteins myosin and actin, which is driven by the turnover of adenosine triphosphate (31). Mitochondria produce ATP to meet the cellular energy demand for contractile force in skeletal muscle (32). We postulated that quantitative or qualitative mitochondrial dysfunction and reduction in skeletal muscle oxidative capacity could be one of the mechanisms causing sarcopenia.

Electron microscopic examination revealed increased numbers of mitochondria in aged human skeletal muscle. This change was accompanied by quantitative changes in the levels of respiratory complex proteins. Oxidative capacity per unit mitochondria, which was evaluated by measuring oxygen consumption rate with the addition of substrates of mitochondrial respiration, was not reduced with aging. In the MRC assay, complex IV activity was reduced in both YD and OD subjects, and complex I activity was significantly lower in the YD group. Light microscopic examination with immunohistochemical staining revealed SDH blue fibers and subsarcolemmal aggregation, which are markers for abnormal mitochondrial proliferation, in at

least some of the OD skeletal muscle. In the analysis comparing high and low muscle quality groups, maximal oxidative capacity was reduced and mitochondrial protein complex expression increased in the low muscle quality group. Based on these results, we speculated that mitochondrial quality deteriorated with aging, and this change could explain, at least partly, the underlying mechanism of sarcopenia.

Some previous studies reported that mitochondrial content decreased in aged muscle (33), whereas others reported no change (34). Mitochondrial proliferation has been observed in patients with mitochondrial disease (35). Our observations of increased mitochondrial proliferation and increased MRC complex protein levels suggest that these change in aged muscle. Although our study subjects had some functional limitations that were indicated for surgery, their physical activity was sufficient to enable activities of daily living and low-intensity exercise. If the functional limitation progresses to a bed-ridden state, compensatory mitochondrial proliferation may not occur as observed in our study subjects, and severe mitochondrial dysfunction at the tissue level may occur.

Aging-related changes in mitochondrial oxidative capacity are controversial in previous studies (36-41). In our study, mitochondrial oxidative capacity was not significantly reduced in OD skeletal muscle. However, this result could have been complicated by the highly variable

muscle quality within each age group, which became evident during further analysis of high and low muscle quality groups.

Several points should be considered in the interpretation of this study. Inconsistencies in previous reports could be due in part to differences in the methods used to assess mitochondrial performance (32). Mitochondria isolated from relatively small samples may not accurately represent the total collective function of all mitochondria in intact tissue. Disruption of mitochondrial structural integrity during sample extraction may affect mitochondrial function, and the selection of mitochondria through differential centrifugation may bias the results (42). Another factor that may affect the study results is the muscle-type change in the aged population. Type II muscle fiber, which is a fast-twitching, glycolytic fiber, is more susceptible to aging (43) than type I fiber , which is a slow-twitching, oxidative fiber with abundant mitochondria that has higher oxidative enzyme capacity in aged skeletal muscle (44). We could not differentiate muscle fiber type in each experiment. The higher proportion of type I fiber in aged muscle might have influenced the quantitative and qualitative mitochondrial features observed in this study.

The MRC assay indicated that COX activity decreased in both young and old groups. This study enrolled patients who underwent orthopedic surgery (THRA) and could have varying degrees of physical dysfunction and varying muscle tissue changes; the skeletal muscle tissue samples were

obtained from these patients. The observed COX activity changes in both YD and OD groups in our study could have been influenced by patient immobilization.

Mitochondrial NADH dehydrogenase activity was significantly lower in YD subjects than in OD subjects. Generally, enzyme activity > 60–70% is considered normal, so no significant reduction in NADH dehydrogenase activity was observed in older subjects. We initially assumed that reduced NADH dehydrogenase activity in the YD group was caused by the observed variations in muscle quality in our subjects. However, NADH dehydrogenase activity was consistently lower in the high-quality muscle group. The cause of reduced NADH dehydrogenase activity in young patients is not clear in this study. However, complex I (NADH dehydrogenase) is one of the most important ROS production site (45), and the association between increased complex I activity and increased ROS production was reported previously (46-47). Therefore, reduced complex I activity in YD muscle could be a compensatory mechanism to reduce oxidative stress. Further study will be required to determine the exact relationship between muscle quality and regulation of complex I activity.

In summary, the present study showed that qualitative mitochondrial changes were associated with qualitative changes of skeletal muscle, whereas quantitative mitochondrial changes seemed occur for compensation in aging

skeletal muscle tissue.

4.3. Role of serum and tissue IL-6 in sarcopenia

IL-6 is an interleukin that acts as both a pro-inflammatory cytokine and an anti-inflammatory myokine (48). In a preliminary pilot study of older patients, we observed high IL-6 levels in serum and low IL-6 mRNA expression levels in muscle tissue. Our original hypothesis was that serum IL-6 (as a pro-inflammatory cytokine) and tissue IL-6 (as an essential regulator of satellite cell–mediated skeletal muscle hypertrophy, (49)) would have different effects on sarcopenia. However, our current cohort study, serum and tissue levels of IL-6 were both negatively correlated with muscle mass, strength, and quality.

Chronically elevated serum IL-6 results in reduced body mass, hyperinsulinemia, and impaired insulin-stimulated glucose uptake by skeletal muscles (50). In the Health ABC Study, higher IL-6 plasma levels were associated with lower muscle mass and strength in older men and women (51). With aging, IL-6 becomes chronically elevated and promotes muscle catabolism most likely via suppression of cytokine signaling proteins (52). Oxidative stress could stimulate IL-6 release in myocyte (53), which could be part of the reason that increases in IL-6 expression and secretion occur with aging.

IL-6 concentrations in serum increase during exercise (54). In resting human skeletal muscle, IL-6 mRNA levels are very low. Exercise-induced plasma IL-6 concentrations increase in an approximately exponential manner. The peak IL-6 level is reached at the end of exercise or shortly thereafter, followed by a rapid decline to pre-exercise levels (14). Contracting skeletal muscle is the primary source of IL-6 in the circulation in response to exercise (55). Elevated serum IL-6 levels stimulate the production of the classical anti-inflammatory cytokines (56) thought to mediate the beneficial anti-inflammatory effect of exercise. IL-6 effects appear to strongly depend on the tissue, dose, and time. A low but chronic IL-6 exposure may not be as beneficial as acute exposure to rapidly elevating levels induced by exercise.

Whether serum or tissue IL-6 is more important for the development of sarcopenia is unclear. Comparison of YD and OD groups suggested that tissue IL-6 had a stronger association with sarcopenia than serum IL-6. However, analysis of high and low muscle quality groups revealed that serum IL-6 level might be related to muscle quality. Our study participants had lower extremity pain and limited motion, and were quite heterogeneous in terms of muscle mass and strength even within the same age and gender groups. This high variability limits our ability to perform more statistically rigorous experiments. Our study also was limited in the number of serum and muscle samples. Therefore, it was difficult to examine the pure effect of aging on the levels of serum and tissue IL-6.

IL-6 is secreted from organs other than skeletal muscle including adipocytes, vascular smooth muscle cells, endothelial cells, connective tissue, and brain (13, 57-59). Serum IL-6 concentration is affected by chronic illness, acute infection, and exercise (60). Thus, the relationship between serum IL-6 levels and sarcopenia might have differed among our study subjects.

Oxidative stress induces IL-6 expression in skeletal muscle, and insulin resistance is associated with IL-6 expression. Therefore, we examined the possible relationship between muscle IL-6 expression and mitochondrial dysfunction. Our analysis revealed that the group with high IL-6 expression in skeletal muscle had marginally reduced maximal oxidative capacity and elevated levels of mitochondrial complex protein. A previous study reported that treatment of cultured adipocytes with IL-6 reduced maximal respiratory capacity of mitochondria (61). IL-6 activated 5′ AMP-activated protein kinase (AMPK; a mediator of mitochondrial biogenesis) in cultured adipose tissue, and could regulate adipose tissue mitochondrial content (62). Muscle cytokine (IL-6) level was reported to be elevated in patients with mitochondrial myopathies (63). In the cancer cachexia mouse model, IL-6 reduced the expression of proteins regulating mitochondrial biogenesis and increased fission protein expression, which could contribute to mitochondrial proliferation (64). Thus, our observed association between IL-6 expression in skeletal muscle and mitochondrial function/biogenesis is plausible, and more

extensive research will be necessary in the future to identify this mechanism.

4.4. Other candidate proteins that may be involved in sarcopenia

Our study selected several target proteins by microarray analysis, and these candidate genes were further analyzed by RT-PCR. UCP2 and UCP3 expression was reduced in the OD group. UCP 2 and UCP3 regulate the generation of ROS by mitochondria (65) and regulate fat metabolism (66-67). UCP2 and UCP3 are expected to promote lifespan and may be appropriate therapeutic targets to restrict aging-related processes (67-68). The reduced UCP2 and UCP3 mRNA expression levels in the OD group may indicate more ROS-induced damage in aged skeletal muscle tissue. UCP2 and UCP3 were not the primary focus of the current study, and future research will be required to investigate their role in sarcopenia.

4.5. Discussion of methods

Selecting the study population

We enrolled surgical patients who were able to perform activities of daily living; however, these patients may have reduced muscle mass, strength, and physical performance compared to normal healthy subjects. We found considerable variation in muscle mass and strength even within the same age

group. This could bias the study and complicate interpretations of the study results. The age of our YD group was relatively high with respect to the age of the OD group, which could mitigate differences between the two groups. Future studies to investigate the mechanism of age-related sarcopenia should enroll a greater number of normal functioning subjects and maximize age differences between young and old groups.

Location of muscle sampling site

The rectus femoris, vastus medialis, vastus lateralis, and vastus intermedius are needed for knee extension. Muscles required for knee flexion and rotation are the hamstring muscles (biceps femoris, semitendinosus, and semimembranosus), gastrocnemius, plantaris, popliteus, adductor gracilis, and sartorius. We obtained excised skeletal muscle tissue samples from the gluteus maximus during the surgical procedure. However, the gluteus maximus is not directly involved in knee movement. Therefore, it is possible that the quantitative and qualitative mitochondrial features in the gluteus maximus muscle may not be strongly correlated with the muscle strength and quality measured in the knee joint. Generally, muscle biopsy is performed in the vastus lateralis muscle.

Evaluation of mitochondrial oxidative capacity

The yield during mitochondrial isolation is low (20–40%) (69), and isolated mitochondria may not represent the whole mitochondrial population in a cell, potentially introducing bias (70-72). Picard et al. reported that mitochondrial functional impairment with aging was exaggerated in isolated mitochondria compared to permeabilized myofibers. For this reason, experiments using permeabilized myofiber are considered as a gold standard to measure mitochondrial oxidative capacity. We recommend that future studies perform experiments using permeabilized muscle fiber and isolated mitochondria.

Evaluation of mitochondrial biogenesis

The number of mitochondria varies with the type of muscle fiber. It is difficult to distinguish the fiber type in electron micrographs. In addition, there are subsarcolemmal and intermyofibillar mitochondrial fractions; changes in mitochondrial content and function may differ according to the subcellular fraction other (73). Also, each subfraction may have a different role in the pathogenesis of certain disease conditions (74-75). With the limited number of images obtained, it was impossible to evaluate changes in each compartment. To overcome this limitation, it would be necessary to examine multiple fields in multiple slides for each individual sample, and then interpret them as an average of changes. Measurement of mitochondrial density rather

than only counting the number of mitochondria would be more useful to determine size changes of mitochondria, and gain a better understanding of the nature of mitochondrial biogenesis with aging.

4.6 Further Investigations

Some aspects of mitochondrial function were not investigated in this study. ROS production, mitochondrial biogenesis and turnover, energy sensing, apoptosis, senescence, and calcium dynamics are important for mitochondrial functions that may change in age-related diseases (76). The current study focused only on mitochondrial oxidative capacity. To comprehensively explore the mitochondrial contribution to age-related sarcopenia, a multifaceted approach to assessing mitochondrial function will be needed in future studies.

This study found marginally elevated IL-6 protein expression levels in old skeletal muscle and an inverse relationship with muscle mass and strength. Future research should elucidate the causal relationship and molecular mechanisms involved in this interaction, discover a way to inhibit the detrimental effect of IL-6 on sarcopenia, and determine the relationship

between IL-6 protein expression in skeletal muscle and mitochondrial function/content.

4.7. Limitations

The subjects in this study underwent total hip replacement arthroplasty and had varying degrees of physical dysfunction, especially in the YD male group, which limits the generalizability of the study result. Some of the results, such as mitochondrial function and changes in IL-6 levels observed in the study subjects, might have been influenced by the disease that led to surgery and not by age-related sarcopenia. Experiments were performed for a limited number of heterogeneous patients, which made the results more difficult to interpret.

There were some confounders that were not adequately controlled or adjusted. Age-induced changes in sex hormone concentrations is one of the mechanisms of age-related changes in muscle mass and strength (77). Hormone replacement therapy may prevent muscle strength decline in postmenopausal women (78) or elderly men (79). This study did not investigate menopausal status history, presence of hypogonadism, hormone replacement therapy, or blood concentration of sex hormone. Physical inactivity is associated with a decline in mitochondrial content and function (80), and exercise training is an effective way to improve muscle oxidative

capacity (81). A recent study suggested that mitochondrial dysfunction was not induced by aging per se, and reduced physical activity might play an important role in age-related mitochondrial changes (82). In the current study, evaluation of the subjects' physical activity levels was limited and possible effects of mitochondrial properties on sarcopenia were not controlled.

V. Conclusion

Qualitative deterioration of skeletal muscle is the main characteristic of age-related sarcopenia. This qualitative change in skeletal muscle is likely to be associated with qualitative change of mitochondria. Quantitative changes in the number of mitochondria and the levels of MRC complex proteins seemed to occur as a compensatory mechanism for these age-related qualitative changes. IL-6 levels in serum and skeletal muscle tissue tended to increase with aging, and IL-6 levels negatively correlated with muscle strength. Thus, serum and tissue IL-6 might be involved in the development of age-related sarcopenia.

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국문 초록

가령에 따른 근감소증의 기전으로서의 미토콘드리아 기능이상 및 마이오카인의 변화

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근감소증은 노화에 수반되는 현상 중 하나로, 점진적인 근육의 양적, 질적 저하를 뜻한다. 근감소증은 노인에서 신체 수행도를 감소시킨다. 본 연구에서는 노화관련 근감소증이 근육의 미토콘드리아의 변화 또는 근육 분비 단백인 마이오카인의 발현 및 분비 변화와 관련이 있을 것이라는 가설을 평가함으로써 근감소증의 기전을 밝히고자 하였다.

골관절염이나 대퇴무혈성괴사로 인해 고관절치환술을 받는 젊은 연령의 환자(YD; 남자 18명, 여자 11명)와 노령의 환자(OD; 남자

5명, 여자 13명)를 연구 대상으로 하였다. 연구 대상자의 특성을 파악하기 위해 병력청취 및 신체진찰을 시행하였고, 이중에너지X-선 흡수계측법을 이용하여 체성분을 분석하였으며, 등속성 근력계를 이용하여 근력을 측정하였다. 혈청 아디포카인 및 마이오카인 농도는 방사면역측정법 또는 정량적 효소면역분석법을 이용하여 공복 혈액 샘플에서 측정하였다. 또한 전자현미경 관찰, 면역화학염색을 이용한 광학현미경적 관찰, 분광광도법을 이용한 미토콘드리아 호흡사슬 활성도 측정, 고해상도 호흡측정계를 이용한 산소소모율 측정, 그리고 미토콘드리아 복합체 단백에 대한 웨스턴블롯분석을 통해 골격근 미토콘드리아의 양적, 질적 평가를 하였다. 그 외에 근감소증의 발생에 관여할 가능성이 있는 후보단백의 mRNA를 찾아내기 위해 마이크로어레이 분석을 시행하였고, 이들 후보 단백들에 대한 실시간중합효소연쇄반응검사를 시행하였다. 마지막으로, 젊은 연령과 노령의 환자에서 골격근 조직에서의 인터루킨-6 발현을 웨스턴블롯으로 분석하여 비교하였다.

여자에서 수술을 받은 쪽 하지와 반대쪽 하지의 평균 근육량은 OD군에서 YD군에 비해 유의하게 적었다. 남녀 모두에서 YD군에 비한 OD군의 근력 및 근육의 질 감소가 더 현격하게 나타났고, 이러한 경향은 여성에서 더 뚜렷하였다. 나이는 남녀 모두에서 근력

및 근육의 질과 유의한 상관관계를 보였다. 근력과 근육의 질은 신체기능이상의 정도와 경계성의 음의 상관관계를 보였다. OD군의 골격근에서 미토콘드리아의 형태학적인 변화는 관찰되지 않았다. 그러나 미토콘드리아 개수와 미토콘드리아 복합체 단백의 양은 유의하게 OD군에서 높았다. 고해상도 호흡측정계를 이용하여 측정한 최대 산소소모량은 OD군에서 YD군에 비해 낮지 않았으나, 근육의 질이 나쁜 군에서는 좋은 군에 비해 낮은 경향이 있었다. 복합체 I의 활성도는 OD군과 근육의 질이 나쁜 군에서 모두 유의하게 증가되어 있었다. SDH 면역화학염색 결과 OD군의 골격근에서 비정상적인 미토콘드리아의 증식이 관찰되었다. 혈청과 근육조직에서의 인터루킨-6 농도는 OD군과 YD군 사이에 의미 있는 차이가 없었다. 그러나 혈청 및 근육조직 인터루킨-6 농도는 모두 근력과는 유의한 상관관계를 보였다. 근육 조직에서의 인터루킨-6 발현 증가는 낮은 최대 산소소모량 및 보상적인 미토콘드리아 복합체 단백 발현 증가와 관련이 되어 있는 것으로 보인다.

근육의 질적인 악화는 노화와 관련된 근감소증의 주된 특징으로 생각된다. 이러한 골격근의 질적인 변화는 미토콘드리아의 질적인 변화와 관련이 있을 가능성이 있다. 미토콘드리아 개수와 미토콘드리아 복합체 단백의 양적인 변화는 노화와 관련된 미토콘드리아의

질적인 변화를 보상하기 위해 발생하는 것으로 추정된다. 혈청과 근육의 인터루킨-6 농도는 노화와 관련하여 증가하는 경향이 있으며, 근력과는 약한 음의 상관관계를 보인다. 이것으로 볼 때 혈청 및 근육 인터루킨-6 또한 노화 관련 근감소증의 발생에 기여할 가능성이 있을 것으로 생각된다.

주요어: 노화, 근육, 근감소증, 미토콘드리아, 마이오카인