

Effects of local vibrations on skeletal muscle trophism in elderly people: Mechanical, cellular, and molecular events

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Received February 23, 2009; Accepted May 7, 2009

DOI: 10.3892/ijmm_00000259

Abstract. Several studies have examined the effects of vibrations on muscle mass and performance in young healthy people. We studied the effects of vibrations on muscles of elderly male and female volunteers (65-85 years of age) diagnosed with sarcopenia. We applied mechanical vibrations locally (local vibrational training) to the thigh muscles at 300 Hz for a period of 12 weeks, starting with a session of 15 min stimulation once a week and increasing to three sessions of 15 min per week. Treated muscles displayed enhanced maximal isometric strength and increased content of fast MyHC-2X myosin. Single muscle fiber analysis did not show any change in cross-sectional area or in specific tension. Analysis of transcriptional profiles by microarray revealed changes in gene expression after 12 weeks of local vibrational training. In particular, pathways related with energy metabolism, sarcomeric protein balance and oxidative stress response were affected. We conclude that vibration treatment is effective in counteracting the loss of muscular strength associated with sarcopenia and the mode of action of vibration is based on cellular and molecular changes which do not include increase in fiber or muscle size.

Introduction

Aging is associated with progressive loss of neuromuscular function. The term sarcopenia is commonly used to describe the loss of skeletal muscle mass and strength that occurs in connection with biological aging in the elderly. The onset of sarcopenia is generally assumed to occur around 60 years of

age, with atrophy being an important symptom. The progression of sarcopenia is influenced by several factors, including genetic components, lifestyle, age-related diseases, decreases in the levels of hormones (GH, testosterone, IGF-1), loss of motor units, and decreased regenerative capacity of skeletal muscle stem cells (1,2). Atrophic conditions in the elderly are exacerbated when diseases force them to bed. The main countermeasure is regular and moderate exercise, but, besides general advice to stay active, there are no definitive indications for optimal training and/or treatment of sarcopenia (3,4).

In recent years, mechanical muscle vibration received considerable attention as a useful method of muscle stimulation in clinical therapy and sports training, but the results remain controversial. Only a few studies described specific vibrational training protocols, and this lack of information generates uncertainties regarding the most effective vibration intensities, frequencies, and application protocols. When frequency is considered, the main question is whether vibrations are applied to the whole body or to specific muscles. The body tolerates a vibration frequency in the range 20-50 Hz, whereas the level for local application to specific skeletal muscles is in the range of 300-500 Hz.

In view of the diversity of applied treatments, a precise comparison of results reported in scientific literature is difficult, and for this reason we report data on different vibrational protocols without extensive discussion. For instance, Bosco and coworkers showed positive effects of passive whole-body vibration under different conditions, reporting significant increases in muscle strength and power (5), elevations in plasma concentrations of testosterone and growth hormone (6,7), and improvement in neuromuscular properties (8). Another study showed that maximal isometric voluntary contraction increased (in young men) only when vibration was combined with squat training (7). De Ruiter and colleagues studied the effects on young men over a period of 11 weeks of whole-body vibration at 30 Hz and found that neither the strength nor the contractile properties of the knee extensor muscle improved (9). It is also worth remembering that negative effects of whole-body vibration on health were reported. Hand-arm vibration syndrome and

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Key words: vibrations, skeletal muscle, single fibers, gene expression

Table I. Anthropometric parameters of study participants.

| | Age years | Height cm | Weight pre-training kg | Weight post-training kg | BMI pre-training kgm ⁻² | BMI post-training kgm ⁻² |
|--------|--------------|--------------|---------------------------|----------------------------|---------------------------------------|----------------------------------------|
| Male | 75.3±6.9 | 163.0±5.0 | 76.9±6.7 | 75.3±6.2 | 29.1±3.5 | 29.0±3.3 |
| Female | 71.0±5.7 | 159.0±5.0 | 71.6±18.8 | 66.4±15 | 28.3±8.1 | 28.2±7.6 |

Data reported in the Table are the anthropometric measurements of male and female elderly volunteers. The values are means ±SD.

vascular disorders (10,11), low back pain (12) and spinal health risks (13), were reported in vibration-exposed workers.

The possible involvement of the nervous system in modifications of muscle performance induced by vibration has been the focus of several studies (14). It was hypothesized that mechanical stimuli are transmitted to sensory receptors of muscles, most likely the spindles, and that receptor activation results in the reflex stimulation of motor units, as seen in the tonic vibration reflex (15). However, the tonic vibration stretch reflex was originally described as the result of a brief exposure to high-frequency stimulation applied directly to a tendon (16,17). Any activation prolonged for tens of seconds could induce a reduction in muscle spindle firing frequency and, as a consequence, a decrease in muscle activation (15). Moreover, muscle spindle firing induced by vibration excites not only motor neurons but also interneurons in the spinal cord, which reciprocally inhibit the motor neurons of antagonist muscles (18). Brunetti and coworkers studied posture stability after vibratory stimulation following anterior cruciate ligament reconstruction. These authors concluded that vibration leads to a faster and more complete recovery of equilibrium, confirming a role for vibration in proprioceptive stimulation (19). In conclusion, it remains difficult to find a rationale that is logically used to design vibration treatment and to predict treatment outcomes.

In this study, we aimed to determine whether a training program of passive muscle stimulation, in which local mechanical vibrations at high frequency (300 Hz) were applied to the lower limbs in the absence of any voluntary muscle contraction, induces an increase in muscle mass and strength in elderly subjects showing signs of sarcopenia. Isometric strength developed in maximal voluntary contractions by knee extensor muscles, and thigh circumference, were the parameters selected to evaluate the impact of local muscle mechanical vibrations in elderly male and female volunteers, and thigh circumference was used as an indicator of structural modifications. Additionally, using small fragments of tissue from needle biopsies of the *vastus lateralis* (VL) muscle (pre- and post-training), some cellular features (fiber types, fiber cross-sectional area, single fiber tension development) and gene expression profiles were analyzed.

Materials and methods

Subjects. The study involved nine elderly people (four males and five females) with diagnoses of sarcopenia according to the criteria of the Centers for Disease Control and Prevention (CDCP). Anthropometric characteristics are summarized in

Table I. The study was approved by the local ethics committee, and was performed in accordance with the 1964 Declaration of Helsinki. All individuals provided written informed consent before participating in the study.

The inclusion criteria were as follows: diagnosis of sarcopenia; normal ECG and blood pressure; and absence of bone/joint, metabolic, or cardiovascular diseases. Exclusion criteria were the presence of metabolic and/or cardiovascular diseases, evidence of hereditary or acquired muscular disease, or diagnosis of respiratory or psychiatric disorders. No subject was under treatment with testosterone or other pharmacological interventions known to influence muscle mass.

Training protocol and experimental design. The conditioning protocol consisted of application of local high-intensity vibrations on the lower limbs using the VISS apparatus. The VISS device (Vissman, Rome, Italy) is a tool capable of producing acoustic waves of different frequencies without affecting the set width. The device is not an acoustic wave generator, but rather a flux modulator, and has two components. These are a compressor delivering pressure in the range 0-400 millibar and a modulator producing an oscillatory air flux to create acoustic waves through a two-way rotating valve. The transducer develops a time-modulated sinusoidal wave (300 Hz). During application of vibration, subjects were invited to avoid isometric contractions of the treated muscle. The experimental protocol required that local mechano-acoustic vibratory stimulation was applied on the skin of the distal part of the quadriceps close to the tendon insertions of the intermedius femoris, rectus femoris, vastus medialis, and vastus lateralis muscles. The entire treatment lasted 12 weeks. The duration of each application was 15 min and the frequency chosen was 300 Hz. From weeks 1 to 8 the subjects received one application per week, whereas from weeks 9 to 12 they received three applications per week. We refer to this type of stimulation protocol as 'local high-intensity vibrational training'.

Two weeks before the training period, enrolled subjects were familiarized with the test session protocol. Isometric tests were performed a week before (pre-session) and a week after the conditioning period (post-session), to measure the bilateral maximal isometric strength of the lower limbs, the body mass index (BMI), and thigh circumference, assessed with a measuring tape at two levels on the dominant leg of each standing subject. The circumferences measured were the maximal circumference at one-third of the distance between the trochanter and the tibial-femoral joint space, and the minimum circumference above the knee. Skinfold measurements were next recorded at the anterior mid-thigh using a skinfold caliper.



SPANDIDOS PUBLICATIONS n weeks after the end of the conditioning period of high-intensity vibrational training, the isometric test was repeated to monitor lower limb strength level.

Isometric strength measurement. Bilateral isometric torque developed by the knee extensor muscles was measured during maximal voluntary contractions using a Leg Extension machine (Panatta Sport; Apiro [MC], Italy) equipped with a load cell (Globus Italia, Codognè [TV], Italy). Participants were seated with the trunk-thigh and the knee joint angles at 90°. Subjects performed maximal voluntary isometric contractions of the knee extensors three times. Isometric contractions lasted for 5 sec, and were separated by 2 min rest intervals. The highest value of torque attained was taken as the isometric contraction strength. In each subject, the variation of knee extension isometric force was expressed as a percentage of the pre-training value.

Cellular and molecular analysis of muscle biopsies. Muscle biopsies were obtained using a semi-automatic needle (Precisa 13 Gauge; Hospital Service, Rome, Italy) from the VL muscle at a level corresponding to one-third of the distance from the upper margin of the patella to the anterior superior iliac spine, after local anesthesia with lidocaine (0.5%, w/v). In each subject, several samples were collected from the same needle insertion and were divided into three groups: (i) samples immersed in skinning solution (see below) were used for dissection of single muscle fibers; (ii) samples immersed in Laemmli buffer (see below) were used for gel electrophoresis; and, (iii) samples immersed in Trizol reagent (Invitrogen, Paisley, UK) were used for extraction of RNA for analysis of transcriptional profiles.

Muscle fibers, mechanical characterization. Muscle biopsy fragments for single fiber dissection were immersed in ice-cold skinning solution with 50% (v/v) glycerol. Skinning solution is a high-potassium, high-EGTA solution which depolarizes membranes, removes calcium, and induces a rigor state, thus ensuring optimal conditions for fiber preservation. The fragments were stored at -20°C and analyzed within 2 weeks of sampling. On the day of the experiment, the skinning-glycerol mixture was washed off and replaced with ice-cold skinning solution containing ATP, to induce fiber relaxation. Single fibers were manually dissected under a stereo-microscope (x10-60 magnification). Following dissection, fibers were bathed for 30 min in skinning solution containing 1% (v/v) Triton X-100 to ensure complete membrane solubilization. Fiber segments 1-2 mm in length were cut, and light aluminum clips were applied at both ends. Skinning, relaxing, pre-activating, and activating solutions employed for single fiber experiments were prepared as described previously (20). Fiber segments were transferred to the experimental apparatus, and cross-sectional area and tension development during maximal calcium-activated isometric contractions at 12°C were measured according to a previously described procedure (21).

Electrophoretic separation and quantification of myosin heavy chain (MyHC) isoforms. MyHC isoform composition was determined in biopsy samples. Muscle biopsy fragments were stored in Laemmli solution (Tris 62.5 mM, Glycerol 10% [v/v], SDS 2.3% [w/v], β -mercaptoethanol 5% [v/v], with

E-64 0.1% [w/v] and leupeptin 0.1% [w/v] as anti-proteolytic factors; pH 6.8). After heating for 5 min at 80°C appropriate amounts of the protein suspension were loaded onto polyacrylamide gels (~1 μ g of total protein/lane). Separation of MyHC isoforms was achieved on 8% (w/v) gels (18 cm x 16 cm x 1 mm) at 70 V for 1.5 h and at 230 V for a further time according to the guidelines of Talmadge and Roy (22). After silver staining, three separate bands were detected in the 200-kDa region, corresponding to MyHC-1, -2A, and -2X, in order of migration from fastest to slowest. To quantify myosin isoform distribution, densitometric analyses of silver-stained bands were performed on at least two independent electrophoretic runs of each biopsy sample fragment. The mean values represent the measurements presented. Gel patterns were digitized with an EPSON 1650 scanner at a resolution of 1,200 dpi. Each band was characterized by a value of the Brightness-Area Product (BAP), using a constant threshold after black/white inversion employing Adobe Photoshop software. From each gel, BAP values for the bands identified as MyHC isoforms were summed and the BAP value for each isoform was expressed as a percentage of the total. The reproducibility of the procedure was confirmed by calculating isoform ratios of selected samples from gels loaded with different amounts of such samples.

Gene expression profile. A high-density oligonucleotide microarray technique was used to identify variations in gene expression induced by mechanical vibration. This technique involves RNA isolation, amplification, and labeling. The human oligonucleotide gene set consisting of 21,329 (70-mer) oligonucleotides (Operon version 2.0), designed on the basis of the Human Unigene clusters, was employed. RNA was extracted from the biopsy samples of three different elderly subjects before training, and at the end of the vibrational training period. Very pure RNA samples from each subject were amplified and labeled. Microarray co-hybridization involved simultaneous hybridization with pre-training and post-training samples and dye-swap duplicated microarrays were analyzed for each subject. Arrays were scanned, and recorded fluorescence intensities were subjected to Lowess normalization (23). The expression of each gene was defined as the log base-2 of the ratio between the intensity of cyanine-coupled aaRNA from post-training samples and that of cyanine-coupled aaRNA from pre-training samples ($\log_2 I_{\text{post-training}}/I_{\text{pre-training}}$). Differentially expressed genes were selected using a per-mutation test procedure known as the 'Significance Analysis of Microarrays' (SAM), which defines genes with a computed score larger than the threshold value as showing significant variation. The false discovery rate (FDR) associated with the given threshold was additionally calculated from permutation data.

Statistical analysis. Data were expressed as means \pm SE (Figs.) or SD (Tables). Statistical significance was set at $p < 0.05$ and was calculated using the unpaired Student's t-test, with Welch's correction. Prism5 GraphPad software (Abacus Concepts GraphPad Software, San Diego, CA) was employed for statistical analyses. Gene expression analysis was based on SAM data and variations are expressed as means with SD.

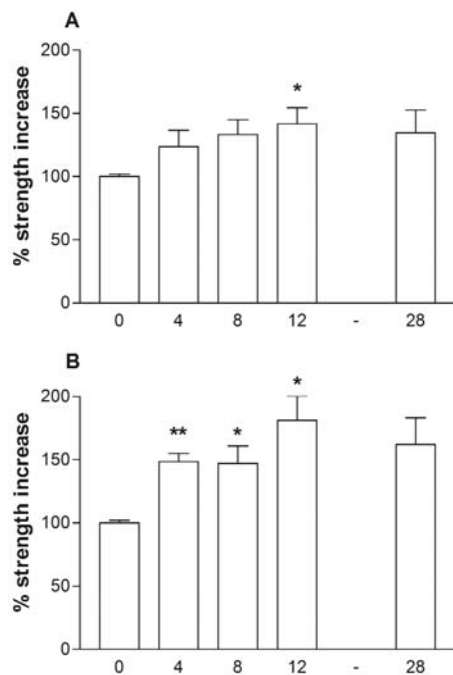


Figure 1. Variations in bilateral isometric strength of leg extensor muscles treated with local muscle vibration at 300 Hz in nine elderly male (A) and female (B) subjects. The histograms show the percentage increases in bilateral isometric strength, based on leg extension, at different times during the period of stimulation. 0 week, pre-training; 4, 8, and 12 weeks, intervals during the stimulation period when strength was measured. During the first 8 weeks, treatment was based on a single vibration application (300 Hz) of 15 min per week. From weeks 8 to 12, treatment was increased to three applications of 15 min per week (300 Hz). Treatment was interrupted after 12 weeks, and strength was measured again at week 28. Isometric strength measurements from the pre-training sessions are taken as 100%. Significant variations, * $p < 0.05$, ** $p < 0.01$.

Results

Maximal isometric strength of knee extensor muscles. Isometric strength of leg extensor muscles was measured before, and after 4, 8, and 12 weeks of vibrational training. The application of local vibration to the lower part of the thighs improved isometric strength (Fig. 1). In both male and female subjects (anthropometric characteristic in Table I), the increase was appreciable, beginning at 4 weeks (females) and 8 weeks (males) of vibration training. Importantly, during the first 8 weeks of the procedure, stimulation was scheduled only once a week. This increase in strength remained at a plateau up to week 12, even though training frequency was increased to three times per week from week 8 (see Training protocol). In general, the response was better in female than in male subjects. In male subjects, isometric strength increased by ~25% at weeks 4 and 8, with a significant increase at week 12 of training ($141.7 \pm 12.7\%$, $n=4$, $p < 0.05$). Follow-up measurements after 28 weeks revealed that the increase in strength was consistently maintained ($134.5 \pm 18.0\%$) (Fig. 1A). In female subjects, the increase in bilateral isometric limb strength was higher than in males, commencing at week 4 ($148.5 \pm 6.5\%$, $n=5$, $p \leq 0.001$), and remained significant until the end of the training period ($181.2 \pm 19.3\%$, $n=4$, $p \leq 0.001$). The increase in strength at 12 weeks of stimulation was not significantly different between female and male subjects.

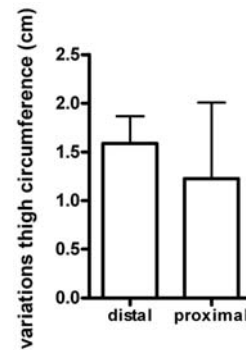


Figure 2. Variations in thigh circumference. Two thigh circumferences, one distal (just above the knee) and one proximal (at 2/3 of the knee-trochanter distance, to avoid the adductor muscles) were measured before the beginning of treatment (pre-training) and after 12 weeks of treatment (post-training). Changes are expressed in cm. As average, the distal and proximal circumferences showed small and insignificant increases after vibrational training in both male and female subjects.

Variations of leg extensor muscle mass were evaluated from the measurements of two thigh circumferences, one distal (just above the knee), and one proximal (at 2/3 of the knee-trochanter distance), to avoid the influence of adductor muscles. After vibrational training the distal circumferences showed small and insignificant increases (0.5-3 cm) in both male and female subjects, whereas, with proximal circumferences, variations ranged from a slight decrease in two female subjects to an increase (0.5-6 cm) in other subjects (Fig. 2). Moreover, skinfold measurements at the end of the training period were not significantly different from those measured before training (data not shown). In conclusion, no significant variations in muscle mass accompanied increases in muscle strength.

Muscle fiber cross-sectional area and specific tension. Single muscle fibers were isolated from biopsy fragments of the VL muscle obtained before and after vibrational training. From each biopsy, 8-9 fibers were analyzed, to yield data on a total of 80 fibers both before and after training. The average cross-sectional area (CSA) was $3667.0 \pm 310.7 \mu\text{m}^2$ in pre-training samples, and $4238.0 \pm 357.4 \mu\text{m}^2$ in post-training samples, thus, vibrational training did not significantly modify the CSA of single fibers (Fig. 3A). In specific tension measurements, the isometric strength per unit of fiber area was $177.0 \pm 14.6 \text{ mN mm}^{-2}$ in pre-training samples, and $164.0 \pm 17.4 \text{ mN mm}^{-2}$ in post-training specimens. These values were not significantly different (Fig. 3B).

Myosin isoform composition of biopsy samples. The fiber type composition of the VL muscle was determined by analyzing the proportion of slow (MyHC-1) and fast (MyHC-2A and -2X) myosin heavy chain isoforms in biopsy samples taken before and after vibrational training. Myosin isoforms can be considered as molecular markers of fiber types (24). The average values for myosin isoform distribution in males (Fig. 4A) and females (Fig. 4B) are shown. In both groups, fast MyHC-2X levels were increased after vibration training, whereas slow MyHC-1 proportions were significantly lower in post-training compared to pre-training samples. The shift in myosin isoform expression might indicate either fiber

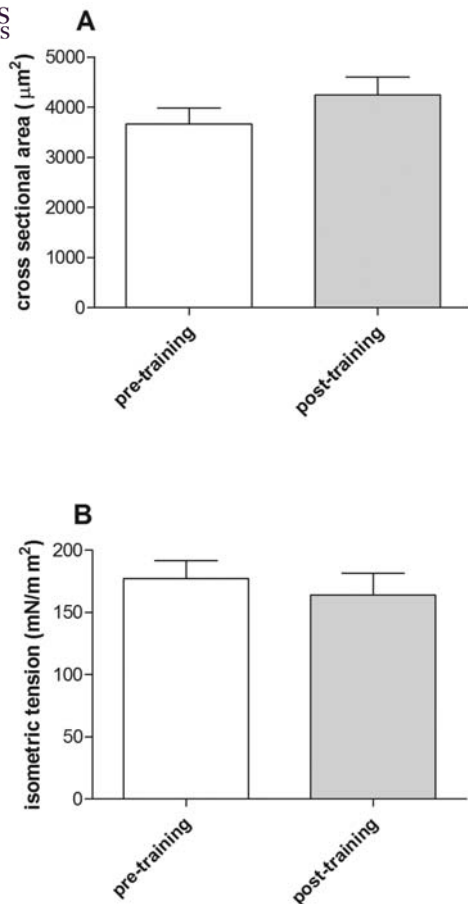


Figure 3. Single-fiber analysis, cross sectional area (A) and specific isometric tension (B). (A) Cross-sectional area (CSA) of single muscle fibers isolated from the biopsy samples from the vastus lateralis muscle before and after vibrational training of 12 weeks. (B) Measurements of specific isometric tension (force/CSA) developed in maximal calcium-activated contraction by the same fibers. In both cases, no significant difference was detected between pre- and post-training samples. For each measurement, a total of 80 fibers were analyzed.

transition (from slow to fast; 2X) or differential growth (a specific increase in the size of 2X fibers). The lack of any significant increase in fiber thickness after vibrational training (see Fig. 3) suggests that the change in myosin isoform distribution are caused by a fiber-type transition.

Gene expression profiles. Gene expression profiles from oligonucleotide microarrays were obtained from biopsy samples of three subjects before and after vibrational training. We listed the genes found significantly regulated after the vibrational training on each subject and we assessed possible changes in gene transcription analyzing these regulated genes. Genes that appeared to be significantly affected were further evaluated to elucidate the mechanisms by which vibration-trained muscles showed performance enhancement. Some genes with well-known muscle activities received particular attention, and were classified according to function. Such categories included, specifically, (i) genes of energy metabolism (Table II); (ii) genes involved in sarcomeric protein synthesis, protein degradation, and calcium homeostasis (Table III); and, (iii) genes dealing with oxidative stress (Table IV).

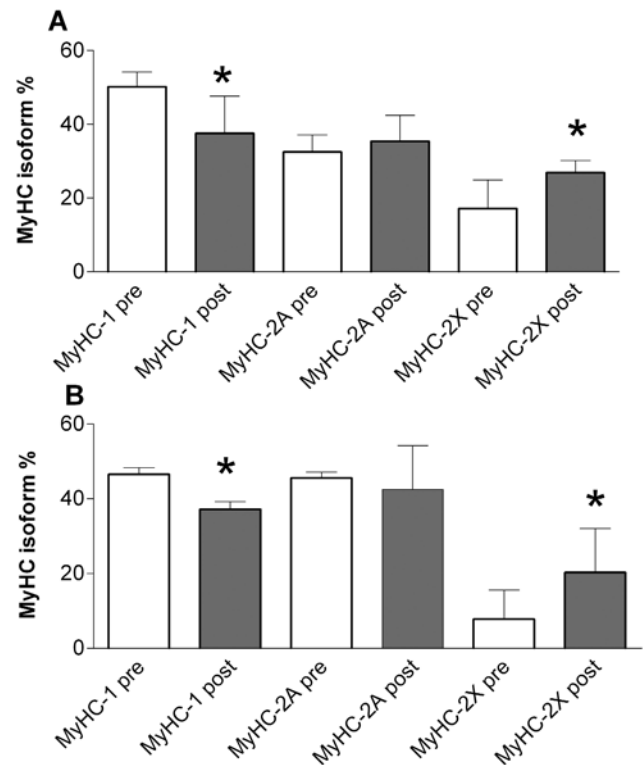


Figure 4. MyHC isoform distribution in biopsy samples collected before and after 12 weeks of vibrational training in nine elderly subjects. MyHC isoform distribution was determined by electrophoretic separation and densitometric analysis of proteins of biopsy samples from the vastus lateralis muscle. A and B show the percentage distributions of MyHC isoforms (1, 2A, and 2X) in male and female subjects, respectively. The grey columns represent the percentages of the MyHC isoform distribution post-training, and the white columns represent pre-training percentages. (A) The mean percentage of MyHC-1 decreased by 13%, whereas that of MyHC-2X increased by 12%, in elderly male subjects. (B) The mean percentage of MyHC-1 decreased by 11%, whereas that of MyHC-2X increased by 12%, in female subjects. * $p \leq 0.05$.

Genes involved in energy metabolism. Several enzymes involved in glucose and glycogen metabolism were upregulated after vibration therapy (Table II). We observed increased expression of the *phosphoinositide-3-kinase, regulatory subunit, polypeptide 3* (PIK3R3) gene that encodes a binding protein in the insulin-dependent pathway leading to inhibition of glycogen synthase kinase-3 (GSK-3), and that thus mediates net dephosphorylation of glycogen synthase (GS), with concomitant activation of the glycogen pathway (25). It is important to remember that PI3kinase functions upstream to Akt, a signaling factor very relevant to muscle hypertrophy mechanisms (26). Moreover, *protein phosphatase regulatory subunits 3A and 3C* (PPP1R 3A and 3C), genes encoding key enzymes for glycogen conservation in muscle (25), were upregulated. Another gene showing enhanced expression was *dihydrolipoamide dehydrogenase, E3 complex of pyruvate dehydrogenase complex* (DLD), a protein of the pyruvate dehydrogenase complex that transforms pyruvate into acetyl-coenzyme A. In particular, the E3 component reduces NAD^+ to NADH (27). Yet another up-regulated gene of interest was *fatty acid CoA ligase* that is important for fatty acid activation in the cytoplasm prior to β -oxidation. *Glucan (1,4- α -), branching enzyme 1* (glycogen branching enzyme, [relevant

Table II. Changes in energy metabolism genes.

| Subject | Gene name | Mean $\log_2 I_{\text{post-training}}/I_{\text{pre-training}}$ \pm SD | GB accession | UniGene ID | Gene symbol |
|---------|--------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------|--------------|------------|-------------|
| 1 | Phosphoinositide-3-kinase, regulatory subunit, polypeptide 3 (p55, γ) | 0.86 \pm 0.39 | NM_003629 | 88051 | PIK3R3 |
| 2 | Protein phosphatase 1, regulatory (inhibitor) subunit 3A (glycogen and sarcoplasmic reticulum binding | 1.08 \pm 0.13 | NM_002711 | 127614 | PPP1R3A |
| 2 | Protein phosphatase 1, regulatory (inhibitor) subunit 3C | 0.99 \pm 0.17 | BC012625 | 303090 | PPP1R3C |
| 2 | Dihydrolipoamide dehydrogenase (E3 component of pyruvate dehydrogenase complex, 2-oxo-glutarate complex) | 0.85 \pm 0.16 | NM_000108 | 74635 | DLD |
| 2 | Fatty-acid-coenzyme A ligase, long-chain 2 | 0.91 \pm 0.09 | NM_021122 | 154890 | FACL2 |
| 1 | Solute carrier family 16 (monocarboxylic acid transporters), member 4 | 0.84 \pm 0.32 | NM_004696 | 23590 | SLC16A4 |
| 1 | Solute carrier family 21 (organic anion transporter), member 11 | 0.65 \pm 0.12 | NM_013272 | 14805 | SLC21A11 |
| 1 | Solute carrier family 6 (neurotransmitter transporter), member 14 | 0.80 \pm 0.32 | NM_007231 | 162211 | SLC6A14 |
| 1 | Ubiquinol-cytochrome c reductase core protein II | 0.69 \pm 0.16 | NM_003366 | 173554 | UQCRC2 |
| 5 | Glucan (1,4- α -), branching enzyme 1 (glycogen branching enzyme, Andersen disease, glycogen storage) | -1.16 \pm 0.15 | NM_000158 | 1691 | GBE1 |

The first column specifies in which of the three different elderly subjects (individuals 1, 2, or 5) the change in expression was detected. The second column lists the common names of the different genes up- or down-regulated in the vastus lateralis muscle, when post- and pre-training expression levels were compared. The third column reports changes in expression levels of genes as means of the log base-2 of the ratios ($\log_2 I_{\text{post-training}}/I_{\text{pre-training}}$), with SD. The fourth column lists Gene Bank Accession numbers, the fifth the UniGene Identification numbers, and the sixth specific gene symbols. Genes involved in energy metabolism were classified as upregulated (positive values) or downregulated (negative values).

to Andersen's disease and glycogen storage disease]) (GBE1), which participates in glycogen synthesis, was downregulated.

Expression of specific genes encoding several transporters was enhanced, including genes encoding *solute carrier family 16 (monocarboxylic acid transporters), member 4* (SLC16A4) (28), a transport system implicated in both intracellular pH regulation during muscle contraction and lactate removal. The gene *solute carrier family 21 (organic anion transporter), member 11* (SLC21A11), functioning in the transport of organic anions and thyroid hormones, and in modulation of glucose phosphorylation (29), was also up-regulated. Moreover, the expression of *solute carrier family 6 (neurotransmitter transporter), member 14* (SLC6A14), a gene encoding an amino acid transporter implicated in the utilization of non-glucidic substrates for plastic cellular reorganization and as an energy source (30), was increased. Notably, we observed elevated expression of *ubiquinol-cytochrome c reductase core protein II* (UQCRC2), encoding a cytochrome reductase of the mitochondrial complex III (31).

Genes involved in sarcomeric protein synthesis and degradation, and calcium homeostasis. Several genes encoding sarcomeric proteins were upregulated (Table III). These included *actin $\alpha 2$* (ACTA2), one of the six actin isoforms of thin filament proteins, *destrin* (DSTN), a key enzyme in actin polymerization (32), and *myosin, light polypeptide, regulatory, non-sarcomeric* (MLCB), together with *chaperonin ADP-*

ribosylation factor-like 5 (ARL5A), and *short coiled-coil protein* (SCOCO), linked to myosin folding and myofibrillogenesis (33). In the context of protein turnover, we observed upregulation of *ubiquitin-specific protease 15* (USP15), involved in the removal of damaged proteins, and down-regulation of *ubiquitin-conjugating enzyme E2G 1* (UBE2G1), encoding a protease, and an enzyme that conjugates target proteins with ubiquitin, respectively. Moreover, expression of *calpain 3* (p94), specifically expressed in skeletal muscle, was enhanced. Calpain 3 mediates muscle remodeling by cleavage and release of myofibrillar proteins, targeting these proteins for ubiquitination and proteasomal degradation (34,35). The genes *α -actinin-2-associated LIM protein* (ALP), and *Leman coiled-coil protein* or *angiomotin-like protein 2* (LCCP), encoding proteins important for sarcomeric structure (in particular, the Z line), and *Titin immunoglobulin domain protein* (TTID), encoding a big, elastic protein that stabilizes sarcomeric filaments (36-38), were additionally upregulated. Another interesting gene displaying increased expression was *Popeye protein 3* (POP3). POP genes are expressed in cardiac and skeletal muscles (39), and participate in the synthesis and stabilization of mRNA encoding tubulin (40). Genes encoding *ryanodine type 3* (RyR3), a receptor of the sarcoplasmic reticulum, and *calcium homeostasis endoplasmic reticulum protein* (CHERP), an intracellular calcium-mobilizing agent (41), were additionally upregulated.



| Subject | Gene ID | Mean $\log_2 I_{\text{post-training}}/I_{\text{pre-training}}$ \pm SD | GB accession | UniGene ID | Gene symbol |
|---------|---------------------------------------------------------------|-------------------------------------------------------------------------------|--------------|------------|-------------|
| 2 | Actin, α 2, smooth muscle, aorta | 1.29 \pm 0.37 | NM_001613 | 195851 | ACTA2 |
| 2 | Destrin (actin depolymerizing factor) | 0.83 \pm 0.13 | NM_006870 | 82306 | DSTN |
| 1 | Myosin, light polypeptide, regulatory, non-sarcomeric (20 kD) | 0.80 \pm 0.32 | NM_006471 | 233936 | MLCB |
| 2 | ADP-ribosylation factor-like 5 | 1.09 \pm 0.20 | NM_012097 | 342849 | ARL5 |
| 2 | Short coiled-coil protein | 1.16 \pm 0.65 | NM_032547 | 286013 | HRIHFB2072 |
| 2 | Titin immunoglobulin domain protein (myotilin) | 1.07 \pm 0.26 | NM_006790 | 84665 | TTID |
| 2 | α -actinin-2-associated LIM protein | 1.35 \pm 0.70 | BC001017 | 135281 | ALP |
| 2 | Leman coiled-coil protein | 1.05 \pm 0.25 | NM_016201 | 92186 | LCCP |
| 2 | Ryanodine receptor 3 | 1.40 \pm 0.63 | NM_001036 | 9349 | RYR3 |
| 1 | Calcium homeostasis endoplasmic reticulum protein | 1.04 \pm 0.64 | NM_006387 | 6430 | CHERP |
| 1 | Popeye protein 3 | 0.89 \pm 0.18 | AK055600 | 303154 | POP3 |
| 2 | Ubiquitin-specific protease 15 | 0.92 \pm 0.18 | NM_006313 | 23168 | USP15 |
| 5 | Ubiquitin-conjugating enzyme E2G 1 (UBC7 homolog, C. elegans) | -2.23 \pm 0.41 | NM_003342 | 78563 | UBE2G1 |
| 1 | Calpain 3, (p94) | 0.84 \pm 0.22 | NM_000070 | 40300 | CAPN3 |

The first column specifies in which of the three different elderly subjects (individuals 1, 2, or 5) the change in expression was detected. The second column lists the common names of the different genes up- or down-regulated in the vastus lateralis muscle, when post- and pre-training expression levels were compared. The third column reports changes in expression levels of genes as means of the log base-2 of the ratios ($\log_2 I_{\text{post-training}}/I_{\text{pre-training}}$) with SD. The fourth column lists Gene Bank Accession numbers, the fifth lists the UniGene Identification numbers, and the sixth, specific gene symbols. Genes involved in energy metabolism were classified as upregulated (positive values) or downregulated (negative values).

Table IV. Genes involved in oxidative stress.

| Subject | Gene ID | Mean $\log_2 I_{\text{post-training}}/I_{\text{post-training}}$ \pm SD | GB accession | UniGene ID | Gene symbol |
|---------|------------------------------------|--------------------------------------------------------------------------------|--------------|------------|-------------|
| 2 | Nitric oxide synthase 1 (neuronal) | -1.18 \pm 0.53 | NM_000620 | 46752 | NOS1 |
| 2 | DNA polymerase epsilon p12 subunit | -1.14 \pm 0.31 | NM_019896 | 19980 | P12 |
| 2 | Peroxiredoxin 3 | -1.12 \pm 0.35 | NM_006793 | 75454 | PRDX3 |
| 2 | Thioredoxin | -1.11 \pm 0.47 | NM_003329 | 76136 | TXN |
| 2 | Methionine sulfoxide reductase A | -0.93 \pm 0.47 | NM_014772 | 64096 | MSRA |

The first column specifies in which of the three different elderly subjects (individuals 1, 2, or 5) the change in expression was detected. The second column lists the common names of the different genes up- or downregulated in the vastus lateralis muscle, when post- and pre-training expression levels were compared. The third column reports changes in expression levels of genes as means of the log base-2 of the ratios ($\log_2 I_{\text{post-training}}/I_{\text{post-training}}$), with SD. The fourth column lists Gene Bank Accession numbers, the fifth the UniGene Identification numbers, and the sixth specific gene symbols. Genes involved in energy metabolism were classified as upregulated (positive values) or downregulated (negative values).

Genes involved in oxidative stress. Oxidation of biological substrates, such as DNA, proteins, and lipids, in the elderly, is well-recognized, as oxidants are generated in the mitochondrial respiratory chain, and the deleterious function of such oxidants is particularly important in muscle fibers (1). Vibrational training caused down-regulation of the polymerase ϵ (P12) gene, encoding a low-affinity enzyme

involved in DNA duplication and repair (42) and cell cycle control (Table IV). This enzyme, an important contributor to DNA repair, is involved in the rescue of oxidized DNA, although enzyme activity is less efficient than that of other polymerases. Furthermore, expression of the *peroxiredoxin* (PRDX3) gene was decreased. PRDX3 is a specific anti-oxidant enzyme able to remove endogenous cytokine-induced

H₂O₂ (43), using electrons donated by thioredoxin (TXN), the gene for which was also downregulated. PRDX3 encodes a protein that acts as an antioxidant by facilitating the reduction of other proteins via cysteine thiol-disulfide exchange. Another gene displaying diminished expression was methionine sulfoxide reductase (MSRA), the protein product of which repairs oxidized methionine (44), one of the most important targets of protein oxidation. Moreover, downregulation of the constitutive nitric oxide synthase (NOS1) was also observed. This gene encodes a constitutive form of the enzyme that synthesizes nitric oxide from L-arginine in the presence of NADPH and O₂, and downregulation may be linked to an altered nitric oxide signaling mechanism in aged skeletal muscle (45).

Discussion

Muscle isometric strength is defined as the maximum force exerted against resistance, and decreases significantly in humans with age (46). Healthy elderly subjects typically display diminished muscle mass, reduced movement velocity and skeletal muscle strength, combining to form the diagnostic characteristics of the condition termed sarcopenia (1).

In recent years, novel 'passive training' methods were proposed for clinical treatment of muscle atrophy and in sport training, consisting of mechanical vibrations applied to specific muscles or over the entire body. A number of investigators showed that such treatments enhance muscle strength and power (8,47), plasma concentrations of testosterone and growth hormone (5), and neuromuscular functions (6). Other studies, however, have drawn opposite conclusions, claiming that no improvement of muscle performance was obtained after vibration treatment (9). In addition, negative effects of whole-body vibration on health were documented. Workers exposed to daily vibration display vascular disorders (10), increased levels of lumbar prolapse, and lower back pain (12).

In this study, the measurements of knee extensor muscle isometric torque, obtained during pre-, during (weeks 4 and 8), and at the end of training, confirmed that local high-intensity vibration increases overall muscle strength in elderly male and female subjects. Specifically, isometric strength was elevated by the fourth week of stimulation, remained high during the training period, and also for at least several weeks after cessation of training. Indeed, follow-up measurements at 16 weeks after the end of training revealed consistently high values of muscle strength, similar to the levels recorded at the end of the vibrational protocol, for both female and male volunteers, although some inter-individual variability was noted.

The sustained increase in lower limb strength suggests modifications in the properties of skeletal muscle. Measurements of thigh circumferences and skinfolds showed that the force increase was not accompanied by an increase in muscle mass. We therefore examined the mechanical properties of single fibers to determine whether the changes can be explained at the cellular level. After 12 weeks of local high-intensity vibration, neither the cross-sectional area nor the specific tension of the VL muscle fibers was changed, compared to pre-training data. Thus, the change in force of the whole extensor muscle group is not based on an alteration

in the force-developing ability of single fibers. Previous studies have shown that muscle disuse in the elderly causes a decrease in specific tension (48), whereas resistance training in body builders is associated with increased specific tension (49). Apparently, the improved strength of leg extensor muscles after vibrational training is not related to an increase in muscle fiber-specific tension. Biopsy examination, however, yielded a valuable and unexpected result. The fiber phenotype distribution of the skeletal muscle was altered. The proportion of the fast MyHC-2X isoform was increased, and the proportion of the slow MyHC-1 isoform was significantly lower, in post-training biopsies compared to pre-training biopsies. This shift in myosin isoform expression is a clear indication of changes in fiber type distribution (49). Fiber type transition from slow to 2X, as suggested by the shift in myosin isoforms, should correspond to changes in the proportions of oxidative and glycolytic fibers, because, in human muscles, slow fibers are mainly oxidative whereas 2X fibers are principally glycolytic (24). Importantly, expression profile analysis validated this metabolic feature. In fact, indications for changes in fiber type distribution were seen in the transcriptional profile. In particular, changes in two of three functional categories (metabolic genes and those encoding sarcomeric proteins) of differentially regulated genes were identified in all screened elderly subjects. Glycolytic and glycogen-dependent metabolism appeared to be stimulated in trained subjects. In this study, personal transcriptional profiles, including data on specific differentially regulated genes, were generated for each of three subjects. Initially, the results appeared inconsistent, but, when the experimental plan was considered, and, particularly, when the long-term nature of the treatment was properly weighted, the transcriptional profiles of the three elderly subjects can be best regarded as specific temporal windows on genes functioning after consolidated or stabilized metabolic (in the wide sense) modifications. We employed a microarray technique to simultaneously screen all pathways activated at a given time-point, and we were able to detect compensatory pathways activated in the cells. Metabolic genes, either up- or down-regulated, were indeed distinct between subjects, but converged into enhancement of the glycogenosynthesis/glycolysis pathway. Glycogenosynthesis is considered as a compensator for increased glucose consumption during training. This hypothesis is consistent with the increased proportion of (specifically) fast MyHC-2X fibers as these fibers employ glycolytic metabolism.

The upregulation of genes, such as actin, desmin, titin and angiomotin, encoding sarcomeric proteins, is consistent with an increase in regeneration that recruits satellite cells to proliferate and fuse into new differentiated myotubes, that are comparable to *in vivo* primary fibers. We hypothesize that new fibers are generated during the first 4 weeks of local high-intensity vibrational stimulation, based on data showing that at week 4, the bilateral isometric strength of lower limbs was significantly increased. We used a biopsy fragment from one subject to collect and grow satellite cells (50), to study whether myogenesis properties *in vitro* are influenced by local high-intensity vibrational training. We induced the differentiation program in these cells, and, after 7 days of differentiation, we used an antibody MF20 against myosins



the proportion of myotubes expressing the myosin chain proteins. The preliminary data reveal that, after vibrational training, the proportion of MF20-positive myotubes increased by 11.8% (from 41.3% pre-training to 53.1% post-training). These data further support the hypothesis that novel fibers stimulated by local high-intensity vibration are induced to differentiate into new, mature, fast MyHC-2X fibers.

Sarcopenic skeletal muscle shows reduced metabolic power because of several factors, such as decreased blood perfusion, increased fibrosis, and development of the atrophic state. Skeletal muscle tissue undergoes constant oxidation, and the main source of reactive oxygen species is the mitochondrion. A previous study by our group showed consistent oxidative damage in aged skeletal muscle tissue (1). Altered metabolism impairs the balance between oxidative stress and scavenger activity. In fact, several antioxidant genes were downregulated in the elderly, suggesting that ROS scavenger activity is insufficient. Peroxiredoxin is an important antioxidant enzyme that counteracts lipid peroxidation, protecting cells by removing H_2O_2 (51). Thioredoxin plays a key role in oxidation-reduction reactions (43). Methionine sulfoxide reductase A counteracts methionine oxidation of proteins (44), whereas polymerase ϵ is able to repair oxidized DNA, albeit at low efficiency (42). The downregulated constitutive NO synthase (NOS) could be responsible for lowered nitric oxide production and may disrupt nitric oxide signaling in skeletal muscle arterioles, thus impairing vasodilatation in old age (45). Also, in a study of atrophy induction by horizontal bed rest in young males, it was observed that sarcolemmal NOS1 immunofluorescence in the vastus lateralis muscle increased (52). These opposite effects may be explained by age-related variations in muscle conditioning. In fact, during aging, the bioavailability of substrates and cofactors important for NOS activity decreases (45), and this was not reversed by high intensity vibrational training applied to elderly subjects.

Only one elderly subject (subject 2, Table IV) showed downregulation of antioxidant genes mentioned above. This observation suggests that local high-intensity vibration exacerbate existing physiological and personal gene regulation conditions.

Overall, the results of our study indicate that vibrational training was accompanied by several changes at the muscle molecular level. However, the lack of correlation between marked increases in the force developed during maximal voluntary contraction on the one hand, and muscle mass, single fiber thickness, or specific tension, on the other, suggests that nervous control of contraction must also be considered as possibly influenced by our training protocol. Improved recruitment or better activation of motor units might also be invoked to explain the observed increases in muscle force. A recent study by Fattorini *et al* (14) provided evidence of long-lasting changes in proprioceptive motor control after vibrational training at 100 Hz, and such changes may be partly responsible for the improved contractile performance observed here.

In conclusion, our data validate the effectiveness of the local high-intensity vibrational training procedure to counteract sarcopenia. The technique is easy to use, requires little patient commitment or time, and can be employed on patients with joint and/or neuro-muscular disorders.

Acknowledgements

We thank Dr Bruno Loffredo for cardiological consultations and Professor Enzo Ballone for assistance with statistical analysis. This study was supported by research grants awarded to Professors R. Saggini, G. Fanò, and S. Fulle from the University 'G. d'Annunzio' of Chieti-Pescara.

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