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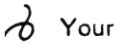
	TAKE OUT CHARACTER INDICATED	 Your proof.
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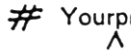
	INSERT SPACE	 Yourproof.
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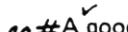
	EVEN SPACE	 A good proof.
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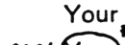
	CAPITALS	Your proof.  Your proof.
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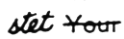
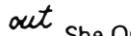
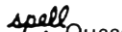
	DELETE, SEE COPY	 She Our proof.
	SPELL OUT	 Queen Eliz.
	START PARAGRAPH	 read. Your
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HIGHLIGHTED TOPIC | *Biomechanics and Mechanotransduction in Cells and Tissues*

High-frequency, low-magnitude vibrations suppress the number of blood vessels per muscle fiber in mouse soleus muscle

AQ: 1

Walter L. Murfee,[1] Laura A. Hammett,[1] Caroline Evans,[1] Liqin Xie,[2] Maria Squire,[2] Clinton Rubin,[2] Stefan Judex,[2] and Thomas C. Skalak[1]

[1]*Department of Biomedical Engineering, University of Virginia Health Sciences Center, Charlottesville, Virginia; and* [2]*Department of Biomedical Engineering, State University of New York at Stony Brook, Stony Brook, New York*

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Murfee, Walter L., Laura A. Hammett, Caroline Evans, Liqin Xie, Maria Squire, Clinton Rubin, Stefan Judex, and Thomas C. Skalak. High-frequency, low-magnitude vibrations suppress the number of blood vessels per muscle fiber in mouse soleus muscle. *J Appl Physiol* 98: 000–000, 2005. First published January 27, 2005; doi:10.1152/japplphysiol.01135.2004.—Extremely low-magnitude (0.3 g), high-frequency (30–90 Hz), whole body vibrations can stimulate bone formation and are hypothesized to provide a surrogate for the oscillations of muscle during contraction. Little is known, however, about the potential of these mechanical signals to stimulate adaptive responses in other tissues. The objective of this study was to determine whether low-level mechanical signals produce structural adaptations in the vasculature of skeletal muscle. Eight-week-old male BALB/cByJ (BALB) mice were divided into two experimental groups: mice subjected to low-level, whole body vibrations (45 Hz, 0.3 g) superimposed on normal cage activities for 15 min/day ($n = 6$), and age-matched controls ($n = 7$). After the 6-wk experimental protocol, sections from end and mid regions of the soleus muscles were stained with BSI lectin, an endothelial cell marker, and smooth muscle (SM) α-actin, a perivascular cell marker. Six weeks of this low-level vibration caused a 29% decrease in the number of lectin-positive vessels per muscle fiber in the end region of the soleus muscle, indicating a significant reduction in the number of capillaries per muscle fibers. Similarly, these vibrations caused a 36% reduction in SM α-actin-positive vessels per muscle fiber, indicating a reduction in the number of arterioles and venules. The decreases in lectin- and SM α-actin-positive vessels per muscle fiber ratios were not significant in the mid muscle sections. These results demonstrate the sensitivity of the vasculature in mouse skeletal muscle to whole body, low-level mechanical signals.

AQ: 2

AQ: 3 microcirculation; angiogenesis; musculoskeletal adaptation

MECHANICAL SIGNALS ARE CRITICAL to the regulation of a wide spectra of vertebrate systems, including gene expression, cell growth, tissue architecture, organ morphology and even function of the organism. However, the critical frequency and amplitude ranges for influencing the plasticity of the response remains elusive. Recent evidence has indicated that extremely low-level mechanical signals, delivered to the weight-bearing skeleton in the form of vibration, are anabolic to bone (30, 31). This provides evidence that bone tissue, often thought to be anabolically responsive only to those strains generated during extreme physical activity (2,000–3,000 microstrain), may in fact be sensitive to the extremely low-level, high-frequency mechanical signals that arise through normal daily activities. More specifically, 30- to 90-Hz vibrations applied for as little as 10 min/day at one-third of the Earth's acceleration (0.3 g) induce bone strains two orders of magnitude less than peak strains experienced during normal locomotion, yet still lead to increased bone formation, reduced bone loss, and enhanced mechanical properties in various species including sheep, mice, rats, and humans (17, 29–33, 39). Similarly, whole body vibration can be used to improve muscular performance, balance, and neurosensing (6, 27, 34, 36, 38). Despite the clinical potential of low-magnitude, whole body vibration to provide a nonpharmacological intervention for diseases such as osteoporosis, the impact of these mechanical signals on the metabolism and morphology of other tissues remains unknown.

Muscle is also known to experience relatively high-frequency mechanical stimuli. During muscle fiber contraction, there is strong evidence of mechanical energy in the frequency domain of 10–100 Hz. This same frequency domain is also evident in the functional strain history of the skeleton (10), with a considerable portion of the mechanical regulatory information that a bone is subject to derived from these persistent mechanical signals. In addition to bone's sensitivity to mechanical signals in this range, muscle spindle primary endings have been shown to be sensitive to high-frequency vibrations (3, 4, 22). High-frequency, low-amplitude (0.3 mm) tendon vibration for less than 4 min/day can attenuate hindlimb unloading effects in rat soleus muscle (8).

The effects of mechanical stimuli on the musculature can also produce changes in the vasculature. Externally applied mechanical forces elicited by stretching, electrical stimulation of muscles, or internally generated forces by exercise are associated with vascular adaptations at the arteriole and capillary level (1, 2, 7, 13, 26). Depending on the type and magnitude of the stimulus, hemodynamic alterations can cause vascular cell remodeling, including differentiation and proliferation, leading to network growth and in some cases rarefaction (2, 13, 23, 24, 25, 37), and indicate the ability of adult microvasculature to adapt to imposed epigenetic strains of magnitudes and frequencies in the range of 1–20% strain and 1–10 Hz (5, 19, 35). However, the ability of the microvasculature to respond to mechanical signals outside these ranges is

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essentially unknown. Recognizing that whole body vibrations are transduced from the skeleton to attached postural muscles and muscle is able to sense vibration, we postulated that whole body vibration could have an effect on the vasculature in muscle. Such an effect might be mediated via direct physical transduction of muscle strains to the vascular cells or via an anabolic/phenotypic adaptation of the muscle that leads to alterations in blood supply.

On the basis of the use of the number of blood vessels per muscle fiber as a metric of microvascular remodeling in skeletal muscle in response to mechanical stimulation (1, 2, 7, 13), the objective of this study was to assess the effect of high-frequency, low-magnitude vibrations on the number of capillaries and arterioles per muscle fiber. BSI lectin was used to identify endothelial cells lining microvessels, whereas an antibody against smooth muscle (SM) α-actin was used to label perivascular cells, defined as vascular mural cells wrapping around or elongated along the endothelium, along arterioles and venules. We hypothesized that high-frequency, low-magnitude, whole body vibrations, known to be anabolic to bone, could increase the number of vessels per muscle fiber in skeletal muscle. Our findings for the first time suggest that low-level, whole body vibrations are capable of affecting the microvasculature within skeletal muscle.

METHODS

Experimental protocol. All experimental procedures were performed in accordance with the guidelines of the State University of New York at Stony Brook Animal Care and Use Committee. Representing a model for the young adult musculoskeletal system, eight wk-old male BALB/cByJ (BALB) mice (Jackson Laboratory, Bar Harbor, ME) were divided into two experimental groups: age-matched control (AC; $n = 10$) and mechanically stimulated (MS; $n = 10$). Inbred BALB mice were chosen for this study because of sensitivity of their skeleton to whole body vibration (17, 18). Each mouse was housed in an individual plastic cage. For mice in the MS group, whole body vibrations were applied by placing the individual plastic cages on a plate, which was vertically oscillating in a sinusoidal fashion at 45 Hz, producing peak accelerations of 0.3 g (Refs. 9, 17; 1.0 g = Earth's gravitational field or 9.8 ms^{-2}). Vertical peak-to-peak displacement of the cage amounted to 74 μm, making the displacement barely perceptible to human touch. Each mouse was allowed to freely move within its cage at all times, including during the 15-min vibration session, thus superimposing the vibratory external mechanical signal onto normal cage activity. After the 6-wk protocol, mice were euthanized by carbon dioxide inhalation, and the right soleus muscle was harvested from each animal, sectioned, and stained for BSI lectin and SM α-actin.

Tissue harvesting and sectioning. The soleus muscle from the right limb was dissected from the posterior surface of the tibia and pinned to approximately retain the original in vivo muscle length. The pinned muscle specimens were embedded in TBS tissue freezing medium (Triangle Biomedical Sciences), frozen in dry ice-cooled isopentane, and stored in an isopentane-filled Eppendorf tube at −80°C until sectioning. Before being sectioned, each specimen was embedded and re-snap frozen in Tissue-Tek OCT. Each soleus muscle was oriented to ensure that sectioning would begin at the distal end. Multiple frozen sections were cut from the end (1,000 μm into the muscle) and mid region (4,500 μm into the muscle) of each muscle specimen. Sections were mounted onto gelatin-coated slides and fixed in 100% methanol for 30 min at −20°C.

Immunochemistry. The number of lectin-positive and SM α-actin-positive vessels per muscle fiber, the number of muscle fibers per area, and the total muscle cross-sectional area were evaluated in both the end and mid-region sections of the muscle. Acceptable specimens were determined by satisfaction of two criteria: *1*) positive vascular staining of BSI lectin and SM α-actin across the entire cross section of the muscle and *2*) muscle sections were obtained from both the end and mid regions of the muscle. Samples that failed to satisfy these two criteria were not included for analysis. Consequently, three samples from the control group and four samples from the mechanically stimulated group were excluded, resulting in $n = 7$ for control (AC) and $n = 6$ for MS. BSI lectin was used to identify endothelial cells lining microvessels, whereas an antibody against SM α-actin was used to label perivascular cells, defined as vascular mural cells wrapping around or elongated along the endothelium. This includes pericytes and mature and immature SM cells. BSI lectin was previously characterized as a marker of capillaries in sectioned muscle (14). In our experience, BSI lectin labels all capillaries including angiogenic sprouts in skeletal muscle and rat mesentery. In contrast, SM α-actin labels SM cells wrapping around arterioles and venules and is also expressed by a population of pericytes found elongated along capillaries. SM α-actin is not expressed by pericytes along every capillary and thus has been used as a marker of arterioles, venules, and capillaries with differentiated pericytes (13, 37). Additionally, end soleus muscle sections from control and mechanically stimulated mice were assessed for myocyte myosin ATPase activity (pH 4.3) to determine muscle fiber type ($n = 3$ for AC group, $n = 4$ for MS group).

After tissue harvesting and sectioning, muscle sections were washed in PBS for 30 min and incubated in 1:20 BSI lectin-FITC (Sigma), 1:200 IA4-CY3 (Sigma) in antibody buffer (PBS with 0.1% saponin, 2% bovine serum albumin) for 1 h at room temperature. Sections were then washed every 15 min for 30 min in PBS containing 0.1% saponin. After tissue staining, sections were examined with a Nikon TE 300 microscope equipped with confocal accessories (Bio-Rad μ-Radiance) using a ×20 Nikon water/oil immersion objective. Sections with positive BSI lectin and SM α-actin staining and void of tissue folds from each region of the muscle were imaged and montaged using CorelbDRAW 9 (Fig. 1). Each image montage was imported into Scion Image, and the total number of lectin-positive capillary vessels (determined by excluding any vessel with a lectin-labeled lumen diameter >10 μm), the total number of SM α-actin vessels (primarily arterioles and venules), the total number of muscle fibers, and total muscle cross-sectional area were measured for each cross section. The observer making the counts was blinded to the identity of the specimen. For soleus muscle end sections processed for myosin ATPase activity, entire cross sections were imaged with an Olympus BX51 microscope equipped with a DP70 digital camera using a ×4 dry objective. To evaluate the percentage of type I slow oxidative muscle fibers, the number of dark muscle fibers was divided by the total number of muscle fibers.

Statistical analysis. Two-tailed Student's *t*-tests were used to compare histological parameters between control and mechanically stimulated groups per muscle region. Spatial differences in the microvascular response to mechanical vibration were based on assessing the interaction between region (end vs. mid) and the level of mechanical stimulation (control vs. vibration) with a two-factor ANOVA. Statistical significance was accepted for $P < 0.05$. All statistical analyses were performed using SigmaStat, and values are presented as means and SD.

RESULTS

Six weeks of extremely low-level, whole body vibrations for 15 min/day stimulated vascular remodeling in the soleus muscle. More specifically, in the end region of the mouse soleus muscle, high-frequency, low-magnitude vibrations superimposed on normal cage activity reduced the number of lectin-positive vessels per muscle fiber by 29% ($P = 0.02$; Fig. 2*A*) and SM α-actin-positive vessels per muscle fiber by 36% ($P = 0.05$; Fig. 3*B*) in the end region of the mouse soleus muscle.

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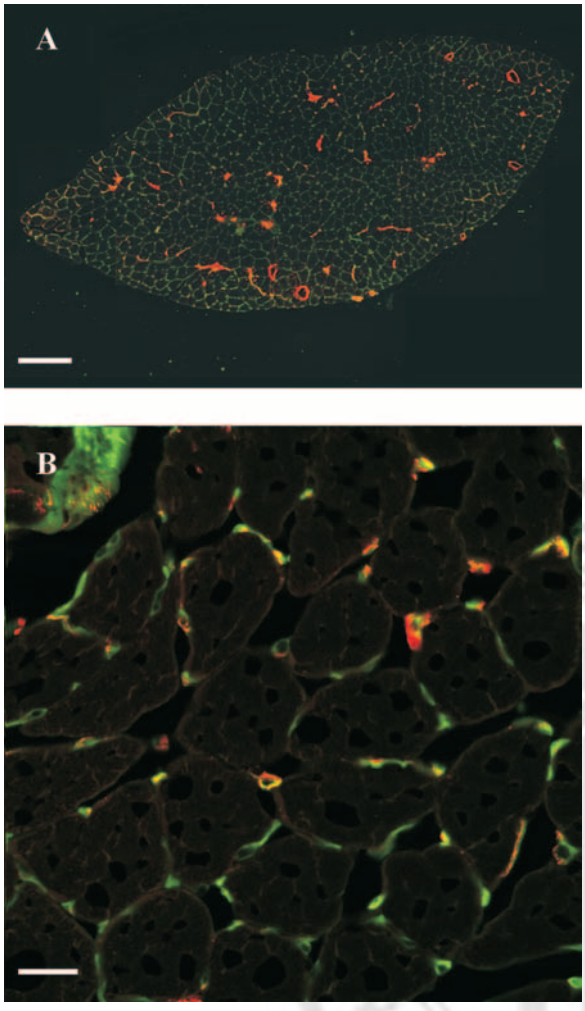

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Fig. 1. *A*: representative montage of a mouse soleus muscle cross section stained for BSI-lectin (green) and smooth muscle (SM) α-actin (red). Scale bar = 200 μm. *B*: high-magnification image of soleus muscle stained for BSI-lectin (green) and SM α-actin (red). Scale bar = 20 μm.

Although a similar trend was observed in the mid region of the soleus muscle, the vascular remodeling was not significant (Fig. 2, *A* and *B*). A two-way ANOVA revealed that, after allowing for effects of differences in region across end and mid regions, whole body mechanical vibration significantly reduced the number of lectin-positive vessels ($P = 0.012$) and SM α-actin-positive vessels ($P = 0.04$) in the soleus muscle. Mechanical vibrations did not alter muscle fibers per area or total area in either the end or mid regions of the muscle (Fig. 2, *C* and *D*). Quantification of the percentage of type I slow oxidative fibers in the end region of the mouse soleus muscle revealed that the whole body vibration stimulus did not induce slow-to-fast muscle fiber type transformation (Fig. 3).

DISCUSSION

The primary finding from this study is that brief exposure (15 min/day) of high-frequency (45 Hz), low-magnitude (0.3 g) mechanical vibration stimulates a reduction in the number of blood vessels per muscle fiber in mouse soleus muscle. Low level mechanical loading has been previously described to alter bone morphology and promote cellular growth in various animals, including mice (17, 31, 33), and we report for the first time that this extremely small stimulus applied for as little as 15 min/day can produce microvascular alterations in mouse skeletal muscle.

Low-level vibrations decreased the number of lectin-positive vessels per muscle fiber in the end region of the muscle, yet did not influence muscle fibers per area or total cross-sectional area, thus indicating that these mechanical signals reduced the number of capillaries for a given muscle group. Again in the end region of the soleus muscle, the vibration decreased the ratio of SM α-actin-positive vessels per muscle fiber, which can be explained by the loss of lectin positive capillaries lined with SM α-actin-positive pericytes, the loss of existing arterioles and venules, or the dedifferentiation or loss of SM α-actin-positive perivascular cells along existing vessels. The microvascular adaptations, which could indicate a reduction in blood supply to the musculature responsible for posture, is an apparent paradox, given that this loading regimen has been shown to be anabolic in a range of species, including mice (17, 31, 33). The concept that the adaptive response of the muscle was more pronounced in the distal segment as contrasted to the midsection, although surprising, is consistent with evidence of stretch preferentially inducing new sacromere growth near the muscle fiber end regions closest to the muscle-tendon joints and that the combination of stretch with electrical stimulation of the same fast contracting muscle causes slow-to-fast fiber type remodeling in the end region compared with the mid region (41).

High-frequency, low-magnitude vibrations in the same range as reported here can increase osteoblastic activity in adult animals, implicating this type of loading stimulation as a therapy for osteoporosis (29, 30, 33). In postmenopausal women and children with cerebral palsy, exposure of the skeleton to similar vibrations attenuates bone loss and increases bone mineral density (29, 34, 39). In this same group of mice, these mechanical signals served to enhance the trabecular bone morphology in the tibial metaphysis and epiphysis (42). Although the mechanisms by which bone cells sense such low-magnitude stimuli are not well understood, the data presented here indicate that the vasculature in a tissue functionally coupled to bone displays a similar sensitivity to this mechanical stimulus. The novelty of our result is that whole body vibration has the capability to affect the microvasculature and not solely that the number of vessels per muscle fiber is reduced in skeletal muscle for this particular application. Although other magnitudes, frequencies, and durations of vibration may cause different remodeling responses, this approach has indicated a mechanical means of modulating the microvasculature in muscle and may provide insight into the nature of the musculoskeletal "system." The significance of the current finding is that the highly noninvasive means of external whole body vibration has the potential to be used therapeutically or to help our understanding of vascular adaptations to mechanical loading regimens. AQ: 6

The direction of the reported microvasculature adaptations is surprising, given that this loading regimen has been shown to be anabolic to bone in a range of species, including mice (17, 31, 33). The reported reduction in the number of vessels per muscle fiber contradicts our original hypothesis. However, the directional effect of a mechanical stimulus depends on the resulting local stress alterations. For example, an increase in

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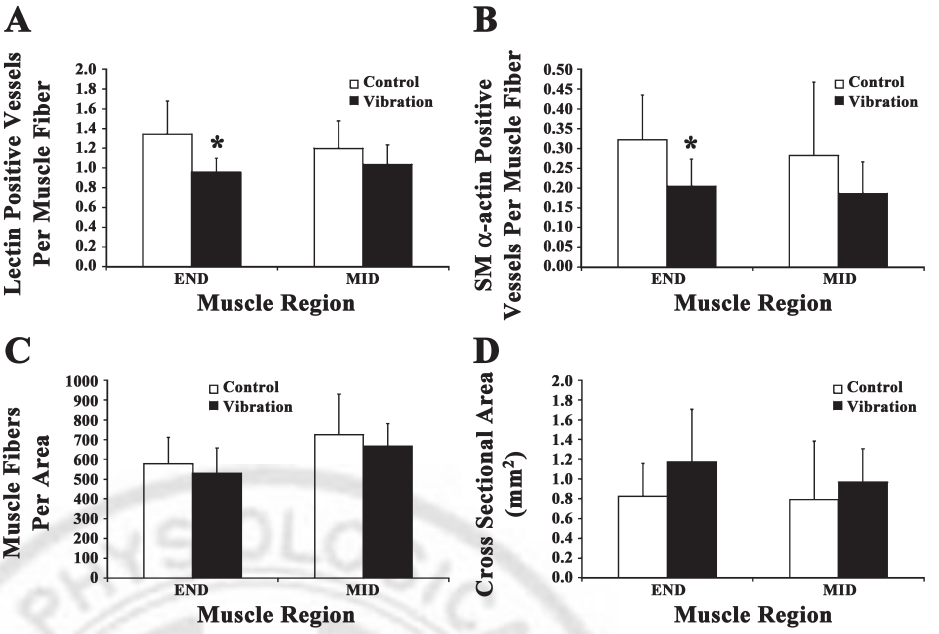

Fig. 2. Region-specific effects of whole body high-frequency, low-magnitude vibrations on soleus muscle: vibration effect on lectin-positive vessels per muscle fiber (*A*), vibration effect on SM α-actin-positive vessels per muscle fiber (*B*), vibration effect on muscle fibers per area (*C*), vibration effect on total cross sectional area (*D*). *Significant difference ($P < 0.05$) from age-matched control (AC) group using a Student's *t*-test [$n = 7$ for AC group, $n = 6$ for mechanically stimulated (MS) group]. Values are means and SD.

pressure in the case of hypertension might be thought to cause a positive vascular effect, but the pressure increase in smaller arterioles is accompanied by lumen reduction and an increase in wall thickness resulting in a reduction in wall stress. This local hemodynamic alteration is associated with microvascular rarefaction (23), demonstrating the need to understand the transduction of high-frequency, low-magnitude vibrations to the local stress level in blood vessels.

The cause and functional consequence of a vibration-induced reduction in the number of vessels per muscle fiber on blood supply or other physiological outcomes are unclear and could additionally be attributed to muscle fiber type remodeling or vibration-induced changes in the local extracellular environment. Previous work has linked high-frequency contractile stimuli with slow-to-fast fiber type remodeling (11, 20, 21, 40), suggesting an oxidative-to-glycolytic transformation. The present study suggests that low-level vibration loading does not affect muscle fiber number per area, muscle fiber size, or type I-to-type II transformation. However, the decrease in vessels per muscle fiber could still be, in part, explained by a transformation of fast oxidative to fast glycolytic fiber types. The resulting glycolytic fibers would require only a reduced number of capillaries, a finding consistent with the current data. A single cycle of tendon vibration for 15 min at 150 Hz (1 mm displacement) of rat soleus muscle to decrease the secretion of bioassayable growth hormone secretion (12). Hudlicka et al. (15) suggest that electrical stimulation of afferent fibers in skeletal muscle leads directly to release of humoral factors, which then affect capillary growth. Finally, electrical stimulation producing contraction induced hyperfusion caused a threefold increase in VEGF mRNA abundance compared with passive hyperfusion (28). Thus the high-frequency, low-magnitude mechanical signals examined in this study could directly alter the local biochemical environment surrounding individual microvessels in the soleus muscle, which consists predominantly of oxidative fibers, causing a reduction in the number of vessels per muscle fiber.

Recent evidence showed that low-level mechanical signals can stimulate an anabolic response in bone (16, 30, 31, 33). The ability of whole body and tendon vibration to stimulate skeletal muscle to adapt (3, 12, 34, 38) further supports that muscle fibers are also sensitive to these vibratory signals. This report demonstrates the ability of extremely low-level vibrations applied for as little as 15 min/day to affect the microvascular architecture within skeletal muscle, but the functional outcome of these adaptations remains unclear. Our findings highlight the sensitivity of the microvasculature to subtle mechanical stimulation and the need to understand the potential system

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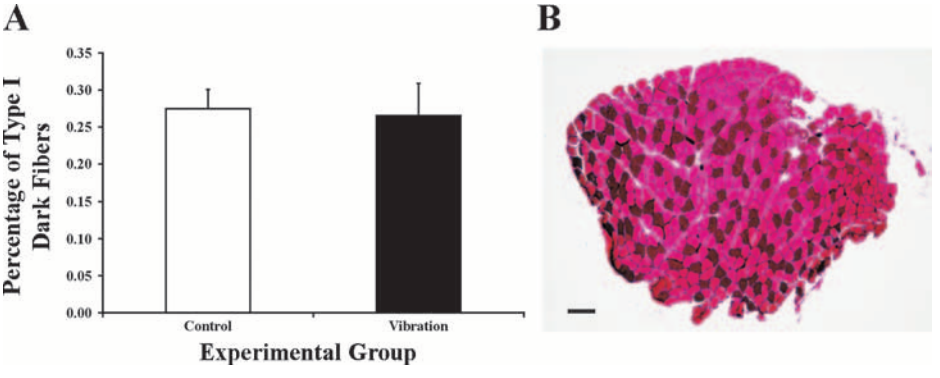

AQ: 7 Fig. 3. Effects of whole body, high-frequency, low-magnitude vibrations on end region muscle fiber type remodeling in mouse soleus muscle. *A*: percentage of type I muscle fibers in end region muscle sections indicated by a 4.3 pH myosin ATPase assay. *B*: representative image of a soleus end region cross section stained for myosin ATPase activity at 4.3 pH and counterstained with hematoxylin and eosin. Scale bar = 100 μm.

level effects of whole body vibration. The current work suggests the possibility of developing noninvasive methods for manipulating the microvascular architecture in skeletal muscle.

GRANTS

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AUTHOR QUERIES

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AQ5— Are 9 and 17 reference nos. in sentence beginning “For mice in the MS group”?

AQ6— Sentence beginning “The significance of the current finding” edited for clarity; meaning intact?

AQ7— H&E written out okay in Fig. 3 legend?