β -Hydroxy- β -Methylbutyrate Did Not Enhance High Intensity Resistance Training-Induced Improvements in Myofiber Dimensions and Myogenic Capacity in Aged Female Rats

Jeong-Su Kim^{1,2,*}, Young-Min Park¹, Sang-Rok Lee¹, Ihssan S. Masad³, Andy V. Khamoui^{1,2}, Edward Jo^{1,2}, Bong-Sup Park¹, Bahram H. Arjmandi^{1,2}, Lynn B. Panton^{1,2}, Won Jun Lee^{1,4}, and Samuel C. Grant^{2,3}

Older women exhibit blunted skeletal muscle hypertrophy following resistance training (RT) compared to other age and gender cohorts that is partially due to an impaired regenerative capacity. In the present study, we examined whether β -hydroxy- β -methylbutyrate (HMB) provision to aged female rodents would enhance regenerative mechanisms and facilitate RT-induced myofiber growth. Nineteen-month old female Sprague-Dawley rats were randomly divided into three groups: HMB (0.48 g/kg/d; n = 6), non-HMB (n = 6), and control (n = 4). HMB and non-HMB groups underwent RT every third day for 10 weeks using a ladder climbing apparatus. Whole body strength, grip strength, and body composition was evaluated before and after RT. The gastrocnemius and soleus muscles were analyzed using magnetic resonance diffusion tensor imaging, RT-PCR, and immunohistochemistry to determine myofiber dimensions, transcript expression, and satellite cells/myonuclei, respectively. ANOVAs were used with significance set at $p < 0.05$. There were significant time effects (pre vs. post) for whole body strength (+262%), grip strength (+17%), lean mass (+20%), and fat mass (-19%). Both RT groups exhibited significant increases in the mean myofiber cross-sectional area (CSA) in the gastrocnemius and soleus (+8–22%) compared to control. Moreover, both groups demonstrated significant increases in the numbers of satellite cells (+100–108%) and myonuclei (+32%) in the soleus but not the gastrocnemius. A significant *IGF-I* mRNA elevation was only observed in soleus of the HMB group (+33%) whereas *MGF* and *myogenin* increased significantly in both groups (+32–40%). Our findings suggest that HMB did not further enhance intense RT-mediated myogenic mechanisms and myofiber CSA in aged female rats.

INTRODUCTION

Sarcopenia refers to the degenerative loss of skeletal muscle mass and function with advancing age (Fielding et al., 2011). Current etiological frameworks suggest that the sarcopenic phenotype may be partially driven by a slowing of regenerative/recovery processes responsible for maintaining tissue integrity (e.g. myogenic program) (Barani et al., 2003; Gallegly et al., 2004; Marsh et al., 1997; Verdijk et al., 2007). In postnatal vertebrates, skeletal muscle regeneration occurs through the actions of satellite cells, a population of muscle stem cells (i.e. satellite cells) located between the basal lamina and sarcolemma (Mauro, 1961). Resident satellite cells normally exist in a mitotically quiescent state in adult muscle, but re-enter the myogenic program in response to regenerative cues such as mechanical loading, injury, or disease (Lepper et al., 2011; Sambasivan et al., 2011). Activated satellite cells may then proliferate nominally to repair small localized damage, fuse together to form myofibers in the face of considerable muscle injury, or provide additional nuclei to support myofiber expansion (Adams, 2006; Kook et al., 2006; Le Grand and Rudnicki, 2007). Because reduced basal satellite cell numbers have been observed in aged skeletal muscle (Day et al., 2010; Shefer et al., 2006; 2010), the ability to undergo the regenerative process during senescence may be delayed and/or impaired, possibly contributing to muscle loss.

In clinical terms, the consequences of sarcopenia include reduced mobility and susceptibility to injury, all of which impinge upon quality of life. Given the considerable public health relevance of sarcopenia, countermeasure strategies have been studied extensively, ranging from pharmacological-based approaches to nutrition and exercise. Amongst the exercise countermeasures, resistance training (RT) has demonstrated potency and effectiveness in stimulating muscle growth (Mayhew

¹Department of Nutrition, Food, and Exercise Sciences, The Florida State University, Tallahassee, FL, USA, ²The Center for Advancing Exercise and Nutrition Research on Aging, The Florida State University, Tallahassee, FL, USA, ³The National High Magnetic Field Laboratory and Department of Chemical and Biomedical Engineering, The Florida State University, Tallahassee, FL, USA, ⁴Department of Exercise Science, Ewha Womans University, Seoul 120-749, Korea

*Correspondence: jkim6@fsu.edu

Received August 2, 2012; revised August 30, 2012; accepted September 20, 2012; published online November 6, 2012

Keywords: aging, HMB, ladder climbing, magnetic resonance, satellite cell, sarcopenia

et al., 2009). Even frail elderly individuals beyond the seventh decade of life have shown increases in muscle protein synthesis and myosin heavy chain content following RT (Fiatarone et al., 1994; Yarasheski et al., 1999). Despite its general effectiveness, the degree of RT-induced muscle hypertrophy varies across different age and gender groups (Kosek et al., 2006; Tracy et al., 1999; Welle et al., 1996). We and others have previously observed a blunted hypertrophic response in older females following long-term RT (Bamman et al., 2003; Ivey et al., 2000; Kosek et al., 2006). Although the underlying mechanism has not been well-defined, the divergent extent of hypertrophy in old females may occur in part from reduced myogenic aptitude (Gallegly et al., 2004; Leiter et al., 2011), which could conceivably impair the regenerative processes activated in response to mechanical loading.

With respect to ergogenic strategies, β -hydroxy- β -methylbutyrate (HMB) has received interest as a countermeasure for muscle atrophy (Zanchi et al., 2011). HMB is formed from the metabolism of the essential branched chain amino acid leucine (Van Koeveering and Nissen, 1992). Evidence suggests that HMB exerts diverse effects on pathways implicated in the control of muscle mass such as regeneration (Komasio et al., 2009), protein synthesis (Pimentel et al., 2011), protein degradation (Smith et al., 2005), and apoptosis (Hao et al., 2011). For instance, Eley et al. (2008) evaluated the degree to which HMB antagonized the catabolic effects of lipopolysaccharide, tumor necrosis factor- α , and angiotensin II in murine myotubes. As expected, protein synthesis decreased significantly by 30-50% in the presence of all three catabolic agents; however, HMB treatment effectively abolished these reductions. There is also evidence that HMB can attenuate the increase in proteasome activity generated by proteolysis-inducing factor, a tumor-specific product implicated in the development of cancer cachexia (Smith et al., 2004). Moreover, Komasio and colleagues (2009) reported that myoblasts incubated with HMB showed increased cell number, *MyoD* and *myogenin* expression, and myosin heavy chain content compared to untreated conditions. These observed increases in cell mass, myogenic regulatory factors, and a skeletal muscle-specific protein point to an enhancement of myogenic events (e.g. proliferation and differentiation). Since HMB treatment displayed the potential to promote myogenic activity, its administration may be of value in circumstances of impaired regeneration such as the aforementioned blunted hypertrophic response in old females following RT. Therefore, the purpose of this study was to test the hypothesis that HMB provision to aged female rats would enhance the satellite cell myogenic program and facilitate RT-induced myofiber growth.

MATERIALS AND METHODS

Animals and experimental protocol

Aged female Sprague Dawley rats (19 months old; $n = 16$) were obtained from Harlan Laboratories (USA) and randomly divided into three groups: control ($n = 4$), HMB ($n = 6$), and non-HMB ($n = 6$). The control group was sacrificed for pre-training muscle tissue collection. The HMB and non-HMB groups then underwent pre-training assessment of whole body strength, grip strength, sensorimotor coordination, and body composition. Indices of body composition included lean and fat mass, which were determined using dual energy X-ray absorptiometry (DXA). After the pre-training assessments, both groups were familiarized with ladder climbing resistance exercise every third day for two weeks using a load equivalent to 30-50% of their own body weight. Upon completion of the familiarization period, the 10-wk

resistance training (RT) program was administered, which consisted of 24 total sessions (trained every third day). Whole body strength, grip strength, sensorimotor coordination, and body composition were assessed again after the 10-wk training (post-training assessment).

Within 18 h after the completion of the last RT session, animals were deeply anesthetized using 4.0-4.5% v/v isoflurane gas in medical grade oxygen. The gastrocnemius and soleus muscles of the right hindlimb were then isolated, weighed, and immediately frozen in liquid nitrogen for subsequent mRNA analyses. Muscle tissues were also mounted cross-sectionally in tragacanth gum and frozen in liquid nitrogen-cooled isopentane for subsequent satellite cell and myonuclei quantitation. Immediately after the muscle isolation, cardiac perfusion was performed to remove circulating blood and introduce a fixative solution in preparation for magnetic resonance (MR) imaging analysis. A perfusion needle was inserted into the left ventricle to circulate 200 ml of saline and 400 ml of 4% formaldehyde to remove blood and fix the muscle tissues, respectively. The left calf muscles were then removed and stored until *ex vivo* MR imaging.

All experimental procedures were approved by the institutional Animal Care and Use Committee. Animals were individually housed in a temperature controlled room on a 12:12 h light: dark cycle under the care of our Laboratory Animal Resources Department. Regular monitoring was conducted by animal care and research technicians for signs of distress, pain, injury, or disease.

HMB Administration

Standard rodent chow and water were provided *ad libitum*. The HMB group was administered HMB orally using custom made chow by Research Diets, Inc. (USA) with HMB provided by Metabolic Technologies Inc. (USA). Food consumption and body weight were measured weekly. Based on previous experience, daily food consumption typically ranges from 15-25 g/day for a 250 g rat (or 60-100 g feed/kg body weight). With these values as a reference, the HMB dosage was calculated as approximately 1% (w/w) CaHMB to obtain ~0.50 g HMB/kg body weight/day (Baxter et al., 2005). We chose a 6 g HMB intervention in accordance with prior human studies that used this dosage as the upper recommended limit (Gallagher et al., 2000; Nissen et al., 1996). We then calculated a human-to-rodent conversion to provide an appropriate and safe dosage for each animal. On the premise that a rodent's metabolism is at least 6 times greater than humans, the conversion that was calculated to achieve the target daily dose was as follows: (6 g/75 kg body weight) $\times$ 6 = 0.48 g HMB/day (Baxter et al., 2005).

Resistance training (RT)

RT was implemented using a ladder climbing apparatus as described by Lee et al. (2004). Briefly, the device was 1 m in length at a 75° incline with 1 cm of space between adjacent ladder rails. We utilized two ladders to allow for a greater number of animals to be trained simultaneously. The order of training groups was rotated to minimize any variability introduced by each trainer's methods. Loading was accomplished by attaching heavy (e.g., 100 g, 150 g, 200 g, or 500 g) and/or light loads (e.g. 10 g, 15 g, 20 g, or 40 g) to the base of the rat's tail with foam tape and a clip. The RT protocol was adapted from Lee et al. (2004), who demonstrated that ladder climbing produced a 23.3% increase in muscle mass following 8 weeks of training. Daily training consisted of 8 total repetitions beginning with one repetition at 70% of maximum weight determined from pre-test

(MAX), two repetitions at 80% MAX, two repetitions at 90% MAX, one repetition at MAX, one repetition at MAX plus 20 g, and one repetition at MAX plus 40 g. Two-minute rest intervals were provided between repetitions. A successful repetition required each rat to climb the full length of the ladder from bottom to top with gentle encouragement but without overt assistance.

Tests of functionality

Whole body strength

The whole body strength test was to assess the maximum weight that the animal could carry during one successful ladder climbing repetition. The protocol consisted of multiple trials in which loading was progressively increased until a successful repetition could not be performed. The first repetition began with a load equivalent to 50% of each rat's body weight, with 20 g being added after each successful trial. In the event of an unsuccessful attempt, loads were reduced as necessary in smaller increments and the trial repeated. For the post-training evaluation, a load calculated as the pre-test maximum weight (MAX) plus 20 g was used for the first trial, with an additional 20 g of weight added to every subsequent repetition until rats failed to successfully complete a full repetition. A 2-3 min recovery period was provided between trials. The maximum load carried without assistance in the pre- and post-tests were used for later analysis.

Grip strength

In contrast to whole body strength, this test evaluated regional strength localized to the forelimbs. In this procedure, the rat was positioned in front of a force gauge (DFS-101 Force gauge, AMETEK TCI, CA) so that they could grasp the tension sensitive steel bar of the device with their forelimbs. After visual observation of gripping, the researcher gently pulled back on the rat's tail until it released its hold on the bar. Grip strength was measured by the device as force produced in grams. Three trials were conducted with the greatest force value recorded as maximum grip strength.

Inclined plane test

The inclined plane test (Murphy et al., 1995) assessed sensorimotor function by determining the rats' ability to maintain their body position for 5 s on an inclined plane while the angle of the surface was changed from 20° to 60° at 2° intervals. A 5-min recovery period was provided between trials. The inability to remain in position for 5 s after three attempts was defined as failure, and the angle prior to failure was recorded.

Magnetic resonance diffusion tensor imaging (MR DTI)

Ex vivo MR analyses were performed on the gastrocnemius and soleus muscles using magnetic resonance diffusion tensor imaging (MR DTI). This DTI technique has been previously used to image several tissue types including nerves (van der Jagt et al., 2012), cardiac muscle (Healy et al., 2011), and skeletal muscle (Masad and Grant, 2011). DTI provides information on skeletal muscle architecture by tracking the random diffusion of water molecules as it interacts with the internal structures that restrict its movement. A major advantage of DTI is that the diffusion of molecules can probe tissue structure with a high degree of sensitivity not typically provided by usual imaging procedures, thereby making it a useful technique for studying biological tissue architecture (Heemskerk et al., 2005). Moreover, this technique allowed us to evaluate the average

dimensions of individual myofibers contained within whole muscle in contrast to the invasive biopsy that only represents a small portion of muscle. DTI yields a principle, second, and third eigenvalue denoted λ_1 , λ_2 , and λ_3 , respectively. λ_1 has been proposed to represent diffusive transport along the long axis of the myofiber while λ_2 and λ_3 constitute transport perpendicular to the long axis (i.e. cross-sections) (Heemskerk et al., 2005). Fractional anisotropy (FA) can also be calculated, which refers to the degree by which diffusion occurs restricted or unrestricted, and is inversely related to myofiber cross-sectional area. Therefore, the four parameters calculated for each region of interest (i.e. gastrocnemius and soleus) were λ_1 , λ_2 , λ_3 , and FA.

In this study, DTI was performed as we have previously described (Wilson et al., 2012). Data for the gastrocnemius and soleus muscles in 7-noncollinear gradient directions were acquired using a widebore 11.75-T vertical magnet with a Bruker Avance console and Micro2.5 gradients. Spin echo DTI scans were performed using a 15-mm birdcage coil with b values of 0, 500, and 1,000 s/mm² at an in-plane resolution of 50 × 50 μm^2 and a slice thickness of 500 μm . The DTI acquisition parameters included the following: TE = 20.5 ms, TR = 2.75 s, Δ = 12.7 ms, and δ = 2.1 ms. A high resolution (40 μm^3) 3D gradient-recalled echo image was also obtained (TE/TR = 10/150 ms) for anatomical and volumetric measurements. Imaging time was approximately one hour for each muscle sample. After acquisition, the images were processed in the widest region of each muscle with MedINRIA software to calculate λ_1 , λ_2 , λ_3 , and FA.

Immunohistochemistry

To quantitate satellite cell and myonuclei numbers, tissue sections 6- μm thick were made using a cryostat (Microm HM 525 Cryostat, USA). Three percent neutral-buffered formalin was used to fix the sections at room temperature for 45 min. Then, 5% goat serum was used to block the sections in PBS at room temperature for 30 min. Sections were incubated at 37°C with Pax7 antibody (Verdijk et al., 2007) (MO15020, 1:200 in 1% goat serum; Neuromics) for 1 h, and then incubated in biotinylated goat anti-mouse secondary Ab (BA-9200, 1:200 in 1% goat serum; Vector Labs) at room temperature for 30 min. Pax7 cells were presented by DAB substrate (Elite Pk-6102; Vector Labs) after the application of Vectastain ABC reagent at room temperature for 1 h. Sections were rinsed with PBS followed by 5 min in deionized (DI) water. Mayer's hematoxylin was used to counterstain nuclei for 5 min followed by rinses in warm, running tap water and DI water. As a result of staining, myonuclei and satellite cells were identified as blue and brown, respectively. High-resolution (48-bit TIFF) images were captured at 40× magnification. The same analyst counted the number of fibers, Pax7-stained cells, and myonuclei while being blinded to group assignment. Myonuclei per fiber, satellite cells per fiber, and relative satellite cell number (i.e. satellite cells/satellite cells + myonuclei) were then calculated.

RNA isolation and RT-PCR

Total RNA was extracted from pre-weighed frozen muscle samples using TRI reagent. Reverse transcription was performed to synthesize cDNA as we have previously described (Kim et al., 2005; 2007). The primer sequences for PCR were: *IGF-I* (fwd) 5'-GCATTGTGGATGAGTGTTC-3', (rev) 5'-GGC TCCTCCTACATTCTGTA-3'; *MGF* (fwd) 5'-GCATTGTGGAT GAGTGTTC-3', (rev) 5'-CTTTCTGTGTGTCGATAGG-3'; *myogenin* (fwd) 5'-ACTACCCACCGTCCATTCAC-3', (rev) 5'-

Fig. 1. Percent change in lean and fat mass from pre- to post-resistance training (RT) in the non-HMB and HMB groups. *Significant percent change within group ($p < 0.05$).

TCGGGGCACTCACTGTCTCT-3'; *MyoD* (fwd) 5'-GCGCCG CTGCCTTCTACG-3', (rev) 5'-GCCGCACTCTTCCCTGGTCT-3'; *myostatin* (fwd) 5'-CAAACAGCCTGAATCCAACCTAG-3', (rev) 5'-CCGTGAGGGGGTAGCGACAG-3'; and *ActRIIB* (fwd) 5'-ACACCGGGCATGAAGCAGAAAAC-3', (rev) 5'-CCACG-ACACCACGGACATCCTC-3'. For each PCR reaction, 18S was coamplified with each target cDNA (mRNA) resulting in a ratio of target mRNA/18S. PCR reactions were generated in 25 μ l of mixture consisting of 1 μ l of 50 mM MgCl₂, 2.5 μ l of 10x PCR buffer, 0.5 μ l of 10 mM dNTP, 0.15 μ l of DNA polymerase, a specific amount of primer, a specific amount of 18S rRNA primer/competimer mix, and nuclease-free water. The denaturing step was conducted for 3 min at 96°C, followed by a specific number of cycles for 1 min at 96°C, 45 s at a specific annealing temperature, 45 s at 72°C, and ending with 3 min at 72°C. Amplified products were separated on 2% agarose gel mixed with ethidium bromide and analyzed by densitometry.

Statistical analysis

All data are presented as mean $\pm$ standard error (SE). Total food intake was compared by *t*-test. *In vivo* data (pre- and post-RT) including whole body strength, grip strength, sensorimotor coordination, and body composition were analyzed using 2×2 (group $\times$ time) repeated measures ANOVA. All *in vitro* data including myofiber dimensions, transcript expression, satellite cell number, total myonuclei, and relative satellite cell number were analyzed by one way ANOVA. Fisher's Least Significance Difference (LSD) tests were used to localize main or interaction effects. *P*-values < 0.05 were considered significant.

RESULTS

Food consumption

No group differences were observed for total food intake (HMB $1,503 \pm 198.7$ g; non-HMB $1,510.7 \pm 96.8$ g; $p > 0.05$).

Body composition

Body composition was analyzed before and after training using DXA, which determined whole body lean and fat tissue compartments. Body weight was also evaluated at the same time-points. There was no significant group $\times$ time interaction or main group effect for body weight, lean mass, or fat mass. However, there was a significant main time effect for body

Fig. 2. Myofiber dimensions of the gastrocnemius (A) and soleus (B) as determined by magnetic resonance diffusion tensor imaging (MR DTI). λ_1 , λ_2 , and λ_3 represent length, long cross-sectional area, and short cross-sectional area of the myofiber, respectively. Fractional anisotropy (FA) indicates the degree to which diffusion occurred restricted or unrestricted and is inversely proportional to myofiber size. GAS, gastrocnemius; SOL, soleus. Values are mean $\pm$ SE. ^Significantly less than control within MR DTI parameter ($p < 0.05$). *Significantly greater than control within MR DTI parameter ($p < 0.05$).

weight that was being driven by pre- to post-training decreases in both the HMB (pre 444.17 ± 28.05 g, post 414.83 ± 25.65 g) and non-HMB (pre 448.67 ± 34.2 g, post 405.83 ± 31.9 g) groups ($p < 0.05$). There was also a significant main time effect for lean mass (+20%, pre vs. post, $p < 0.05$). Post hoc analysis indicated that the time effect was being driven by pre- to post-training lean mass increases in both the HMB (+18%) and non-HMB (+23%) groups ($p < 0.05$) (Fig. 1). Likewise, there was a significant main time effect for fat mass (-19%, pre vs. post, $p < 0.05$) that was being driven primarily by pre- to post-training reductions in the HMB group only (-22%, $p < 0.05$) (Fig. 1).

Strength and sensorimotor coordination

To provide verification of training adaptations consistent with myofiber hypertrophy (e.g. enhanced muscle performance), three tests of functionality were administered including whole body strength, grip (limb) strength, and sensorimotor coordina-

Table 1. Functionality pre- and post-resistance training (RT) in HMB and non-HMB groups

	HMB		Non-HMB	
	Pre-RT	Post-RT	Pre-RT	Post-RT
Whole body strength (g/g of BW)	0.59 ± 0.06	2.28 ± 0.15*	0.63 ± 0.08	2.13 ± 0.10*
Grip strength (g/g of BW)	4.74 ± 0.38	5.38 ± 0.22*	4.55 ± 0.35	5.45 ± 0.29*
Sensorimotor coordination (°)	43.67 ± 0.59	43.66 ± 1.82	43.44 ± 0.53	43.00 ± 2.24

Values are mean ± SE. BW, body weight.

*Significantly greater than Pre-RT within group ($p < 0.05$)

tion. Results for the functionality tests are presented in Table 1. There was no significant group × time interaction or main group effect for whole body strength; however, there was a significant main time effect ($p < 0.05$) because whole body strength increased after 10 wks of RT (+262%). Likewise, there was no significant group × time interaction or main group effect for grip strength; however, there was a significant main time effect ($p < 0.05$) as reflected by increased limb strength after training (+17%). Post hoc tests revealed that the time effect observed for grip strength was being driven by RT-induced gains in both the HMB (+14%) and non-HMB groups (+20%) ($p < 0.05$). With respect to sensorimotor coordination, ANOVA revealed no significant interaction or main effects.

DTI analysis of myofiber size

The gastrocnemius and soleus muscles of the left hindlimb were the regions of interest for DTI analysis. For each region of interest, three eigenvalues (λ) and fractional anisotropy (FA) were calculated. λ_1 , λ_2 , and λ_3 represented length, long cross-sectional area, and short cross-sectional area of the myofiber while FA indicated the degree to which diffusion occurred restricted or unrestricted (i.e. uniformity of diffusion), which is inversely proportional to myofiber size. Both the HMB and non-HMB groups showed significantly greater λ_2 (HMB +9%, non-HMB +9%) and λ_3 (HMB +13%, non-HMB +12%) in the gastrocnemius compared to control ($p < 0.05$) (Fig. 2A). For eigenvalues calculated from the soleus, both groups demonstrated significantly greater λ_1 (HMB +9%, non-HMB +8%), λ_2 (HMB +18%, non-HMB +17%), and λ_3 (HMB +22%, non-HMB +20%) compared to control ($p < 0.05$) (Fig. 2B). Regarding FA, both groups showed decreased FA in the gastrocnemius (HMB -13%, non-HMB -10%) and soleus (HMB -30%, non-HMB -25%) relative to control ($p < 0.05$) (Figs. 2A and 2B). There were no significant differences in any DTI parameter between the HMB and non-HMB groups. There were also no group differences in wet weight of the gastrocnemius (control 2285.00 ± 77.73 mg, HMB 2250.00 ± 99.73 mg, and non-HMB 2211.67 ± 87.69 mg) and soleus (control 172.50 ± 7.5 mg, HMB 170.00 ± 9.66 mg, and non-HMB 163.33 ± 12.30 mg) ($p > 0.05$). When muscle wet weight was normalized to body weight, there were still no significant changes observed in gastrocnemius (control 3.22 ± 0.15 mg·g of BW^{-1} , HMB 2.83 ± 0.04 mg·g of BW^{-1} , and non-HMB 2.95 ± 0.20 mg·g of BW^{-1}) and soleus muscles (control 0.38 ± 0.03 mg·g of BW^{-1} , HMB 0.41 ± 0.03 mg·g of BW^{-1} , and non-HMB 0.41 ± 0.08 mg·g of BW^{-1}) ($p > 0.05$).

Fig. 3. Immunohistochemical determination of satellite cells per fiber (A), myonuclei per fiber (B), and relative satellite cell number [C; (satellite cells / satellite cells + myonuclei)]. (D) Transverse sections of mounted muscle were cut 6 μ m thick from the midbelly of the gastrocnemius and soleus muscles and stained with antibodies against Pax7. Pax7-stained satellite cells were identified as any nuclei localized to the myofiber membrane and stained brown or with a brown rim (arrows). Sections were counterstained with hematoxylin to identify myonuclei, which are stained blue (arrow heads). Images were captured by light microscopy at 40 $\times$ magnification and analyzed by a single investigator blinded to the sample. Values are mean $\pm$ SE. *Significantly greater than control within muscle ($p < 0.05$).

Fig. 4. mRNA expression of mitogenic and myogenic genes normalized to 18S in the gastrocnemius and soleus muscles. (A) *IGF-I*, (B) *MGF*, (C) *myostatin*, (D) *activin type IIB receptor (ActRIIB)*, (E) *myogenin*, (F) *MyoD*. Values are mean $\pm$ SE. *Significantly greater than control within muscle ($p < 0.05$).

Satellite cell and myonuclei number

Satellite cells and myonuclei were quantitated using immunohistochemical methods. Satellite cells per fiber, myonuclei per fiber, and relative satellite cell number (i.e. satellite cells/satellite cells + myonuclei) were analyzed to evaluate the extent to which HMB influenced the satellite cell pool in old female rats subjected to RT. Satellite cell number was significantly greater ($p < 0.05$) in the soleus but not the gastrocnemius of both the HMB (+108%) and non-HMB (+100%) groups compared to control; however, the two groups were not different from each other (Fig. 3A). Likewise, myonuclei number was significantly greater ($p < 0.05$) in the soleus but not the gastrocnemius of both the HMB (+32%) and non-HMB (+32%) groups compared to control (Fig. 3B). These increases in total myonuclei were not different between the two groups. Relative satellite cell number was also significantly greater ($p < 0.05$) in the soleus but not the gastrocnemius of the HMB (+52%) and non-HMB (+47%) groups compared to control, with no differences between the two groups (Fig. 3C).

mRNA expression of mitogenic and myogenic regulators

Mitogenic mRNA expression

Insulin-like growth factor I (*IGF-I*) and mechanogrowth factor (*MGF*) mRNA levels were determined to represent markers positively regulating cell cycle activity. We also evaluated mRNA expression of myostatin and its membrane bound receptor activin type IIB (*ActRIIB*) to serve as markers negatively regulating cell cycle activity. There was a significant group effect in which *IGF-I* mRNA was greater in soleus muscles of the HMB group (+33%) compared to control ($p < 0.05$) (Fig. 4A). Comparable increases in *IGF-I* mRNA expression were also observed in soleus muscles of the non-HMB group though this did not reach significance (+30%, $p = 0.056$). *MGF* mRNA expression was significantly greater in soleus muscles of both the HMB (+32%) and non-HMB (+40%) groups compared to control ($p < 0.05$) (Fig. 4B). No significant group differences were found for either positive regulator in the gastrocnemius (Figs. 4A and 4B). Regarding negative cell cycle regulators (i.e. *myostatin* and *ActRIIB*), no significant group differences were observed in either the gastrocnemius or soleus muscles (Figs. 4C and 4D).

Myogenic mRNA expression

Transcript levels of the myogenic regulatory factors *MyoD* and *myogenin* were analyzed as indices of regenerative capacity. *Myogenin* mRNA was significantly greater in soleus muscles of both the HMB (+69%) and non-HMB (+70%) groups compared to control ($p < 0.05$) (Fig. 4E). However, no significant group differences in *myogenin* mRNA were found for the gastrocnemius (Fig. 4E). There were also no significant group differences in *MyoD* mRNA expression in either the gastrocnemius or soleus muscles (Fig. 4F).

DISCUSSION

The primary purpose of this investigation was to test the hypothesis that HMB provision to aged female rodents would enhance the satellite cell myogenic program, thereby facilitating RT-induced myofiber hypertrophy. The rationale for this approach stemmed from previous work documenting: 1) a blunted hypertrophic response to RT in old females (Kosek et al., 2006), which may be at least partly attributed to reduced satellite cell number and an impaired regenerative capacity; and 2) the ability of HMB to effectively stimulate the myogenic program (Kornasio et al., 2009; Moore et al., 2005). Our major findings from the present study was that HMB administration did not further augment myofiber size, muscle satellite cell number, regenerative markers, or *in vivo* lean tissue and function. In fact, both the HMB and non-HMB groups showed comparable changes in each of these variables. This suggests that the RT stimulus itself likely accounted for the favorable adaptive responses seen in both experimental groups. Consequently, our data did not support the predicted outcome whereby HMB provision promotes regenerative processes and enhances RT-induced myofiber growth.

HMB did not enhance RT-mediated increases in myofiber size or lean mass

Animals provided with HMB had significantly greater gastrocnemius and soleus myofiber size following RT compared to control as indexed by the principal eigenvalues and FA. Importantly however, the percentage increase from control displayed by the HMB group was similar to that obtained from animals assigned to RT only (non-HMB group). This agrees with our findings on DXA-determined whole body lean tissue as both experimental groups exhibited comparable gains that were not significantly different from each other. Moreover, both experimental groups increased whole body strength and grip strength to a similar degree relative to control. Collectively, these findings point to the absence of a demonstrable additive effect of HMB that results in greater hypertrophy, lean tissue, or functional performance than what would have been achieved by RT alone.

To our knowledge, few studies, if any at all, have examined the combined effects of HMB and RT at the myofiber level in humans or animals. However, several investigations have evaluated its impact on whole body lean mass in humans. For instance, Nissen et al. (1996) reported that HMB supplementation during a high frequency, short-term RT program (6 d/wk for 7 wks) significantly increased lean body mass by approximately 2 kg compared to the placebo condition. Likewise, Gallagher et al. (2000) found that subjects supplemented with HMB during 8 wks of RT also improved lean body mass by roughly 2 kg compared to placebo. However, despite some support for the efficacy of HMB in conditions of increased loading, positive adaptations have not always been evident. Similar improvements in

lean tissue between RT vs. HMB + RT groups have been reported in studies of comparable HMB dosage and RT frequency/duration (Jowko et al., 2001; Kreider et al., 1999; Ransone et al., 2003; Vukovich et al., 2001). Thus, the collective body of work addressing the ergogenic effect of HMB on loaded skeletal muscle appears equivocal at best.

In accordance with previous reports acknowledging no additive HMB effect during RT, we also did not observe greater lean mass improvements in the HMB group vs. the non-HMB group. It is possible that our relatively intense RT stimulus itself may have overridden the HMB effect on lean mass and myofiber size. In other words, the potential benefits conferred by HMB provision may have been subordinate to the signals and ensuing adaptive responses induced by the particular RT program employed in the current work. For instance, HMB provision without concurrent RT has been reported to evoke a 1.2% gain in lean mass (Baier et al., 2009). In the present study, we found a significant main time effect for lean mass (pre- vs. post-RT +20%) that was being driven by comparable RT-induced increases in both experimental groups (HMB +18%, non-HMB +23%). These values constitute considerably greater percentage changes compared to what others have previously reported for HMB alone. Consequently, we may not have been able to discern or detect any statistically significant differences resulting from the potential additive effect of HMB intake because it was disguised by the pronounced adaptations to our RT stimulus, although this is merely speculation.

When considering that no differences in myofiber size or lean tissue were observed between the HMB and non-HMB groups, the nature of the RT stimulus itself merits further discussion given that it likely accounted for the adaptive responses which occurred (i.e. main time effect). We attempted to replicate human RT using a ladder climbing device that animals were required to ascend with weights attached to their tails. Intensity was prescribed as a percentage of pre-test maximum weight carried through one complete repetition up the ladder. Training loads often reached two times body weight, which animals carried a distance of one meter through a near vertical plane for multiple repetitions resulting in a high level of total work. This training stimulus effectively improved myofiber size, lean tissue, and strength to a similar degree in both experimental groups. While our original intent was to provide HMB as a means to promote regenerative events and facilitate myofiber growth during increased skeletal muscle loading, our findings suggest that HMB intake does not appear necessary when using RT of sufficient intensity in aged animals. Indeed, gastrocnemius and soleus myofiber cross-sectional area obtained from DTI analysis (i.e. λ_2 and λ_3) increased significantly in both the HMB and non-HMB groups with no differences between the two. The increase in soleus myofiber size is particularly noteworthy because we have previously reported that type I myofibers, which predominate in slow muscles such as the soleus, did not hypertrophy in older men and women following chronic RT (Kosek et al., 2006). In fact, only young men increased type I myofiber size following RT (+18%) (Kosek et al., 2006), which is similar to the training-induced increase in soleus myofiber size observed in the current work (λ_2 +17~18%; λ_3 +20~22%).

HMB did not enhance RT-mediated increase in satellite cell number

HMB has previously been shown to stimulate proliferation and differentiation of muscle cell cultures *in vitro* (Kornasio et al., 2009). It has also been reported to enhance satellite cell mitotic activity and myofiber cross-sectional area in non-mammalian

species (Moore et al., 2005). Because of these direct promotive effects on myogenesis, HMB has been suggested to be a useful therapy against muscle wasting conditions (Zanchi et al., 2011). The rationale for the current study was to expand on our previous work in which old females displayed a diminished hypertrophic response to RT compared to other age and gender groups, possibly due to an impaired or slowed regenerative response to mechanical load (Kosek et al., 2006). We intended to determine whether HMB provision would regulate components of the myogenic program in a manner which would facilitate RT-induced myofiber growth in old female rodents. Both the non-HMB and HMB groups significantly increased satellite cell number (+100~108%), myonuclei (+32%), and relative satellite cell number (+47~52%) to a similar degree in the soleus compared to control. This suggests that HMB did not provide an additional stimulus to expand the satellite cell pool or enhance myonuclear addition. Consequently, the responses seen in these immunohistological measures most likely occurred as a result of the RT stimulus itself.

It is not entirely clear why we did not observe a clear additive effect of HMB on the satellite cell population. As alluded to in the preceding section addressing myofiber size and lean tissue, the possibility exists that the adaptive responses to RT masked the potential benefits of HMB supplementation. In other words, the RT stimulus could have induced changes in satellite cell number and myonuclei that exceeded those associated with HMB alone, therefore, it may have been difficult to detect any additive HMB effect. Indeed, long-term RT without supplementation has been shown to expand the satellite cell pool and promote myonuclear addition (Petrella et al., 2006). The percent increases in satellite cell and myonuclear content in the present study corresponded well to our previous work in which satellite cell and myonuclei number increased by 117% and 26%, respectively, in humans that demonstrated robust RT-induced hypertrophy (Petrella et al., 2008). The extensive expansion of the satellite cell population in response to RT has been suggested to represent a reservoir that has accumulated in anticipation of future mechanical stimuli (Petrella et al., 2008). It may also reflect an improved ability to maintain myofiber integrity or support growth with advancing age. Collectively, our immunohistological data are consistent with our assessments of myofiber cross-sectional area and lean mass because comparable increases in each of these variables were demonstrated by both experimental groups, lending further support to the likelihood of adaptations arising predominantly from RT. Importantly, it also suggests that sufficiently intense RT without HMB can generate robust expansion of the satellite cell pool in old muscle, particularly females.

HMB and RT regulation of mitogenic and myogenic genes

We found that *IGF-I* mRNA increased significantly in soleus muscles of the HMB group only compared to control (+33%, $p < 0.05$). However, we also observed a strong trend for increased *IGF-I* mRNA in the non-HMB group (+30%, $p = 0.056$). Because of this, we could surmise that *IGF-I* mRNA increased similarly in both experimental groups primarily from the RT stimulus itself, which agrees with numerous investigations demonstrating the induction of muscle *IGF-I* by high tension loading (Bamman et al., 2001; Kim et al., 2005; Petrella et al., 2006; Sun et al., 2006). For *MGF* mRNA levels, significant increases were observed in soleus muscles of both the HMB (+32%) and non-HMB (+40%) groups compared to control ($p < 0.05$). This is not surprising given that *MGF* is considered the load sensitive counterpart of *IGF-I* that contains potency in stimulating the

activation, proliferation, and incorporation of satellite cells (Kandalla et al., 2011). Conversely, we did not find any group differences in *myostatin* or *ActRIIB* mRNA expression. Taken together, the upregulated mRNA expression of positive mitogenic/myogenic regulators in both the HMB and non-HMB groups along with no change in negative regulators suggests that RT induces gene transcription of factors which favor satellite cell activity. Such an explanation would also at least partly account for the robust expansion of satellite cell number observed in the current study.

The myogenic regulatory factors *Myf5*, *MyoD*, *Mrf4*, and *myogenin* constitute other major components governing satellite cell activity. The expression patterns of these proteins provide some of the signals that direct satellite cell progeny to proliferate, differentiate into myocytes, or return to quiescence (Le Grand and Rudnicki, 2007; Yablonka-Reuveni et al., 2008). In the current work, *MyoD* and *myogenin* mRNA levels were determined to serve as surrogate markers of regenerative capacity. We did not detect any group differences in *MyoD* mRNA expression; however, *myogenin* levels increased significantly in the soleus of both the HMB (+69%) and non-HMB (+70%) groups compared to control. The similar increases in *myogenin* mRNA displayed by both experimental groups points to a predominant RT effect, a pattern that also applies to our results for myofiber size, immunohistological parameters, and mitogenic transcripts. The lack of change in *MyoD* mRNA does not fully agree with our previous work that noted an induction of this myogenic regulatory factor in old muscle following chronic resistance loading (Kosek et al., 2006). Because HMB alone has also been reported to increase *MyoD* mRNA and protein expression in muscle cell culture (Kornasio et al., 2009), it would have been reasonable to at least expect comparable elevations in both the HMB and non-HMB groups (reflecting a predominant influence of RT), if not a greater response when combined with HMB. Regardless, our data do not appear to support this assertion.

Interestingly, the myofibers of the gastrocnemius hypertrophied without noticeable changes in genes related to growth/regeneration, the satellite cell pool, or myonuclear incorporation, suggesting the involvement of an alternative pathway. Although we did not evaluate protein phosphorylation, it is certainly possible that the Akt/mTOR/p70S6k pathway downstream to *IGF-I* could have been activated in both the HMB and non-HMB groups as a result of RT. Upregulation of this signaling pathway by mechanical loading is a well-established mechanism leading to increased protein synthesis and subsequent muscle growth (Bodine et al., 2001; Nakai et al., 2010).

CONCLUSION

This study examined whether HMB provision to aged female rodents would enhance the satellite cell myogenic program and facilitate RT-induced myofiber growth. Animals provided with HMB did not exhibit greater gastrocnemius and soleus myofiber size, satellite cell number, myonuclear addition, or *in vivo* lean mass and functionality following RT. In fact, the HMB group demonstrated similar gains in these parameters compared to their counterparts that performed RT only, suggesting that RT of sufficient intensity can independently serve as an effective countermeasure to promote myofiber hypertrophy in slow and fast muscle of older females. It is particularly noteworthy that the soleus myofibers hypertrophied as a result of the RT program employed in the current work as we have previously reported that type I myofiber cross-sectional area in old females

does not respond to RT. Taken together, our data do not appear to provide evidence of an additive effect of HMB during intense RT in aged female animals; however, it does substantiate the therapeutic value of sufficiently intense RT for older females. Follow-up mechanistic work may be warranted to evaluate the efficacy of HMB in conjunction with moderate rather than high intensity RT.

ACKNOWLEDGMENTS

This work was partially supported by The Florida State University and Metabolic Technologies, Inc. MR data were collected at the FAMU-FSU College of Engineering and supported by the US National Science Foundation (IOS-0718499) and a User Collaborative Group Project grant through the National High Magnetic Field Laboratory (NSF DMR-0654118) awarded to Dr. Grant. The authors acknowledge Dr. Sukho Lee for his collaborative effort on the ladder climbing apparatus and Drs. Neema Bakhshalian, Chris Boehm, Michael Zourdos, Jacob Wilson, and Paul Henning for their technical assistance on this study.

REFERENCES

- Adams, G.R. (2006). Satellite cell proliferation and skeletal muscle hypertrophy. *Appl. Physiol. Nutr. Metab.* 31, 782-790.
- Baier, S., Johannsen, D., Abumrad, N., Rathmacher, J.A., Nissen, S., and Flakoll, P. (2009). Year-long changes in protein metabolism in elderly men and women supplemented with a nutrition cocktail of beta-hydroxy-beta-methylbutyrate (HMB), L-arginine, and L-lysine. *J. Parenter. Enteral Nutr.* (JPEN) 33, 71-82.
- Bamman, M.M., Shipp, J.R., Jiang, J., Gower, B.A., Hunter, G.R., Goodman, A., McLafferty, C.L., Jr., and Urban, R.J. (2001). Mechanical load increases muscle IGF-I and androgen receptor mRNA concentrations in humans. *Am. J. Physiol. Endocrinol. Metab.* 280, E383-390.
- Bamman, M.M., Hill, V.J., Adams, G.R., Haddad, F., Wetzstein, C.J., Gower, B.A., Ahmed, A., and Hunter, G.R. (2003). Gender differences in resistance-training-induced myofiber hypertrophy among older adults. *J. Gerontol. A Biol. Sci. Med. Sci.* 58, 108-116.
- Barani, A.E., Durieux, A.C., Sabido, O., and Freyssen, D. (2003). Age-related changes in the mitotic and metabolic characteristics of muscle-derived cells. *J. Appl. Physiol.* 95, 2089-2098.
- Baxter, J.H., Carlos, J.L., Thurmond, J., Rehani, R.N., Bultman, J., and Frost, D. (2005). Dietary toxicity of calcium beta-hydroxy-beta-methyl butyrate (CaHMB). *Food Chem. Toxicol.* 43, 1731-1741.
- Bodine, S.C., Stitt, T.N., Gonzalez, M., Kline, W.O., Stover, G.L., Bauerlein, R., Zlotchenko, E., Scrimgeour, A., Lawrence, J.C., Glass, D.J., et al. (2001). Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy *in vivo*. *Nat. Cell Biol.* 3, 1014-1019.
- Day, K., Shefer, G., Shearer, A., and Yablonka-Reuveni, Z. (2010). The depletion of skeletal muscle satellite cells with age is concomitant with reduced capacity of single progenitors to produce reserve progeny. *Dev. Biol.* 340, 330-343.
- Eley, H.L., Russell, S.T., and Tisdale, M.J. (2008). Attenuation of depression of muscle protein synthesis induced by lipopolysaccharide, tumor necrosis factor, and angiotensin II by beta-hydroxy-beta-methylbutyrate. *Am. J. Physiol. Endocrinol. Metab.* 295, E1409-1416.
- Fiatarone, M.A., O'Neill, E.F., Ryan, N.D., Clements, K.M., Solares, G.R., Nelson, M.E., Roberts, S.B., Kehayias, J.J., Lipsitz, L.A., and Evans, W.J. (1994). Exercise training and nutritional supplementation for physical frailty in very elderly people. *New Eng. J. Med.* 330, 1769-1775.
- Fielding, R.A., Vellas, B., Evans, W.J., Bhasin, S., Morley, J.E., Newman, A.B., Abellan van Kan, G., Andrieu, S., Bauer, J., Breuille, D., et al. (2011). Sarcopenia: an undiagnosed condition in older adults. Current consensus definition: prevalence, etiology, and consequences. International working group on sarcopenia. *J. Am. Med. Dir. Assoc.* 12, 249-256.
- Gallagher, P.M., Carrithers, J.A., Godard, M.P., Schulze, K.E., and Trappe, S.W. (2000). Beta-hydroxy-beta-methylbutyrate ingestion, Part I: effects on strength and fat free mass. *Med. Sci. Sports Exerc.* 32, 2109-2115.
- Gallegly, J.C., Turesky, N.A., Strotman, B.A., Gurley, C.M., Peterson, C.A., and Dupont-Versteegden, E.E. (2004). Satellite cell regulation of muscle mass is altered at old age. *J. Appl. Physiol.* 97, 1082-1090.
- Hao, Y., Jackson, J.R., Wang, Y., Edens, N., Pereira, S.L., and Alway, S.E. (2011). beta-Hydroxy-beta-methylbutyrate reduces myonuclear apoptosis during recovery from hind limb suspension-induced muscle fiber atrophy in aged rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 301, R701-715.
- Healy, L.J., Jiang, Y., and Hsu, E.W. (2011). Quantitative comparison of myocardial fiber structure between mice, rabbit, and sheep using diffusion tensor cardiovascular magnetic resonance. *J. Cardiovasc. Magn. Reson.* 13, 74.
- Heemskerk, A.M., Strijkers, G.J., Vilanova, A., Drost, M.R., and Nicolay, K. (2005). Determination of mouse skeletal muscle architecture using three-dimensional diffusion tensor imaging. *Magn. Reson. Med.* 53, 1333-1340.
- Ivey, F.M., Tracy, B.L., Lemmer, J.T., NessAiver, M., Metter, E.J., Fozard, J.L., and Hurley, B.F. (2000). Effects of strength training and detraining on muscle quality: age and gender comparisons. *J. Gerontol. A Biol. Sci. Med. Sci.* 55, B152-157; discussion B158-159.
- Jowko, E., Ostaszewski, P., Jank, M., Sacharuk, J., Zieniewicz, A., Wilczak, J., and Nissen, S. (2001). Creatine and beta-hydroxy-beta-methylbutyrate (HMB) additively increase lean body mass and muscle strength during a weight-training program. *Nutrition* 17, 558-566.
- Kandalla, P.K., Goldspink, G., Butler-Browne, G., and Mouly, V. (2011). Mechano growth factor E peptide (MGF-E), derived from an isoform of IGF-1, activates human muscle progenitor cells and induces an increase in their fusion potential at different ages. *Mech. Ageing Dev.* 132, 154-162.
- Kim, J.S., Kosek, D.J., Petrella, J.K., Cross, J.M., and Bamman, M.M. (2005). Resting and load-induced levels of myogenic gene transcripts differ between older adults with demonstrable sarcopenia and young men and women. *J. Appl. Physiol.* 99, 2149-2158.
- Kim, J.S., Petrella, J.K., Cross, J.M., and Bamman, M.M. (2007). Load-mediated downregulation of myostatin mRNA is not sufficient to promote myofiber hypertrophy in humans: a cluster analysis. *J. Appl. Physiol.* 103, 1488-1495.
- Kook, S.H., Choi, K.C., Son, Y.O., Lee, K.Y., Hwang, I.H., Lee, H.J., Chang, J.S., Choi, I.H., and Lee, J.C. (2006). Satellite cells isolated from adult Hanwoo muscle can proliferate and differentiate into myoblasts and adipose-like cells. *Mol. Cells* 22, 239-245.
- Kornasio, R., Riederer, I., Butler-Browne, G., Mouly, V., Uni, Z., and Hlevy, O. (2009). Beta-hydroxy-beta-methylbutyrate (HMB) stimulates myogenic cell proliferation, differentiation and survival via the MAPK/ERK and PI3K/Akt pathways. *Biochim. Biophys. Acta* 1793, 755-763.
- Kosek, D.J., Kim, J.S., Petrella, J.K., Cross, J.M., and Bamman, M.M. (2006). Efficacy of 3 days/wk resistance training on myofiber hypertrophy and myogenic mechanisms in young vs. older adults. *J. Appl. Physiol.* 101, 531-544.
- Kreider, R.B., Ferreira, M., Wilson, M., and Almada, A.L. (1999). Effects of calcium beta-hydroxy-beta-methylbutyrate (HMB) supplementation during resistance-training on markers of catabolism, body composition and strength. *Int. J. Sports Med.* 20, 503-509.
- Le Grand, F., and Rudnicki, M.A. (2007). Skeletal muscle satellite cells and adult myogenesis. *Curr. Opin. Cell Biol.* 19, 628-633.
- Lee, S., Barton, E.R., Sweeney, H.L., and Farrar, R.P. (2004). Viral expression of insulin-like growth factor-I enhances muscle hypertrophy in resistance-trained rats. *J. Appl. Physiol.* 96, 1097-1104.
- Leiter, J.R., Peeler, J., and Anderson, J.E. (2011). Exercise-induced muscle growth is muscle-specific and age-dependent. *Muscle Nerve* 43, 828-838.
- Lepper, C., Partridge, T.A., and Fan, C.M. (2011). An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. *Development* 138, 3639-3646.
- Marsh, D.R., Criswell, D.S., Carson, J.A., and Booth, F.W. (1997). Myogenic regulatory factors during regeneration of skeletal muscle in young, adult, and old rats. *J. Appl. Physiol.* 83, 1270-1275.

- Masad, I.S., and Grant, S.C. (2011). A retunable surface coil for high field 31P and 1H magnetic resonance evaluations of the living mouse leg. *Physiol. Meas.* 32, 1061-1081.
- Mauro, A. (1961). Satellite cell of skeletal muscle fibers. *J. Biophys. Biochem. Cytol.* 9, 493-495.
- Mayhew, D.L., Kim, J.S., Cross, J.M., Ferrando, A.A., and Bamman, M.M. (2009). Translational signaling responses preceding resistance training-mediated myofiber hypertrophy in young and old humans. *J. Appl. Physiol.* 107, 1655-1662.
- Moore, D.T., Ferket, P.R., and Mozdzia, P.E. (2005). The effect of early nutrition on satellite cell dynamics in the young turkey. *Poult Sci.* 84, 748-756.
- Murphy, M.P., Rick, J.T., Milgram, N.W., and Ivy, G.O. (1995). A simple and rapid test of sensorimotor function in the aged rat. *Neurobiol. Learn. Mem.* 64, 181-186.
- Nakai, N., Kawano, F., Oke, Y., Nomura, S., Ohira, T., Fujita, R., and Ohira, Y. (2010). Mechanical stretch activates signaling events for protein translation initiation and elongation in C2C12 myoblasts. *Mol. Cells* 30, 513-518.
- Nissen, S., Sharp, R., Ray, M., Rathmacher, J.A., Rice, D., Fuller, J.C., Jr., Connelly, A.S., and Abumrad, N. (1996). Effect of leucine metabolite beta-hydroxy-beta-methylbutyrate on muscle metabolism during resistance-exercise training. *J. Appl. Physiol.* 81, 2095-2104.
- Petrella, J.K., Kim, J.S., Cross, J.M., Kosek, D.J., and Bamman, M.M. (2006). Efficacy of myonuclear addition may explain differential myofiber growth among resistance-trained young and older men and women. *Am. J. Physiol. Endocrinol. Metab.* 291, E937-946.
- Petrella, J.K., Kim, J.S., Mayhew, D.L., Cross, J.M., and Bamman, M.M. (2008). Potent myofiber hypertrophy during resistance training in humans is associated with satellite cell-mediated myonuclear addition: a cluster analysis. *J. Appl. Physiol.* 104, 1736-1742.
- Pimentel, G.D., Rosa, J.C., Lira, F.S., Zanchi, N.E., Ropelle, E.R., Oyama, L.M., Oller do Nascimento, C.M., de Mello, M.T., Tufik, S., and Santos, R.V. (2011). beta-Hydroxy-beta-methylbutyrate (HMBeta) supplementation stimulates skeletal muscle hypertrophy in rats via the mTOR pathway. *Nutr. Metab. (Lond)* 8, 11.
- Ransone, J., Neighbors, K., Lefavi, R., and Chromiak, J. (2003). The effect of beta-hydroxy beta-methylbutyrate on muscular strength and body composition in collegiate football players. *J. Strength Cond. Res.* 17, 34-39.
- Sambasivan, R., Yao, R., Kissenpfennig, A., Van Wittenberghe, L., Paldi, A., Gayraud-Morel, B., Guenou, H., Malissen, B., Tajbakhsh, S., and Galy, A. (2011). Pax7-expressing satellite cells are indispensable for adult skeletal muscle regeneration. *Development* 138, 3647-3656.
- Shefer, G., Van de Mark, D.P., Richardson, J.B., and Yablonka-Reuveni, Z. (2006). Satellite-cell pool size does matter: defining the myogenic potency of aging skeletal muscle. *Dev. Biol.* 294, 50-66.
- Shefer, G., Rauner, G., Yablonka-Reuveni, Z., and Benayahu, D. (2010). Reduced satellite cell numbers and myogenic capacity in aging can be alleviated by endurance exercise. *PLoS One* 5, e13307.
- Smith, H.J., Wyke, S.M., and Tisdale, M.J. (2004). Mechanism of the attenuation of proteolysis-inducing factor stimulated protein degradation in muscle by beta-hydroxy-beta-methylbutyrate. *Cancer Res.* 64, 8731-8735.
- Smith, H.J., Mukerji, P., and Tisdale, M.J. (2005). Attenuation of proteasome-induced proteolysis in skeletal muscle by {beta}-hydroxy-{beta}-methylbutyrate in cancer-induced muscle loss. *Cancer Res.* 65, 277-283.
- Sun, D.F., Chen, Y., and Rabkin, R. (2006). Work-induced changes in skeletal muscle IGF-1 and myostatin gene expression in uremia. *Kidney Int.* 70, 453-459.
- Tracy, B.L., Ivey, F.M., Hurlbut, D., Martel, G.F., Lemmer, J.T., Siegel, E.L., Metter, E.J., Fozard, J.L., Fleg, J.L., and Hurley, B.F. (1999). Muscle quality. II. Effects of strength training in 65- to 75-yr-old men and women. *J. Appl. Physiol.* 86, 195-201.
- Van Koeveering, M., and Nissen, S. (1992). Oxidation of leucine and alpha-ketoisocaproate to beta-hydroxy-beta-methylbutyrate *in vivo*. *Am. J. Physiol.* 262, E27-31.
- van der Jagt, P.K., Dik, P., Froeling, M., Kwee, T.C., Nievelstein, R.A., Ten Haken, B., and Leemans, A. (2012). Architectural configuration and microstructural properties of the sacral plexus: A diffusion tensor MRI and fiber tractography study. *Neuroimage*.
- Verdijk, L.B., Koopman, R., Schaart, G., Meijer, K., Savelberg, H.H., and van Loon, L.J. (2007). Satellite cell content is specifically reduced in type II skeletal muscle fibers in the elderly. *Am. J. Physiol. Endocrinol. Metab.* 292, E151-157.
- Vukovich, M.D., Stubbs, N.B., and Bohlken, R.M. (2001). Body composition in 70-year-old adults responds to dietary beta-hydroxy-beta-methylbutyrate similarly to that of young adults. *J. Nutr.* 131, 2049-2052.
- Welle, S., Totterman, S., and Thornton, C. (1996). Effect of age on muscle hypertrophy induced by resistance training. *J. Gerontol. A Biol. Sci. Med. Sci.* 51, M270-275.
- Wilson, J.M., Grant, S.C., Lee, S.R., Masad, I.S., Park, Y.M., Henning, P.C., Stout, J.R., Loenneke, J.P., Arjmandi, B.H., Panton, L.B., et al. (2012). Beta-hydroxy-beta-methyl-butyrate blunts negative age-related changes in body composition, functionality and myofiber dimensions in rats. *J. Int. Soc. Sports Nutr.* 9, 18.
- Yablonka-Reuveni, Z., Day, K., Vine, A., and Shefer, G. (2008). Defining the transcriptional signature of skeletal muscle stem cells. *J. Anim. Sci.* 86, E207-216.
- Yarasheski, K.E., Pak-Loduca, J., Hasten, D.L., Obert, K.A., Brown, M.B., and Sinacore, D.R. (1999). Resistance exercise training increases mixed muscle protein synthesis rate in frail women and men ≥ 76 yr old. *Am. J. Physiol.* 277, E118-125.
- Zanchi, N.E., Gerlinger-Romero, F., Guimaraes-Ferreira, L., de Siqueira Filho, M.A., Felitti, V., Lira, F.S., Seelaender, M., and Lancha, A.H., Jr. (2011). HMB supplementation: clinical and athletic performance-related effects and mechanisms of action. *Amino Acids* 40, 1015-1025.