全身律動訓練對於中年小鼠的身體組成，運動表現以及其生化反應的影響

Effect of whole-body vibration training on body composition, exercise performance and biochemical responses in middle-aged mice.

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簡述內容：

老化是生理狀態隨時間而變老的過程，不論人類或動物，身體機能會漸漸衰老，過程中可能包括心血管功能下降、肌肉和骨質的流失、身體脂肪儲存增加、認知衰退等，而久坐不動的生活方式會加速身體功能的惡化，最終可能導致行動不便或失能產生，但人體的協調與平衡機制可以由後天的表現而變化改善，因此運動成為維持身體機能最大的功臣之一，而持續規律運動不單純是改善身體的變化，對後續的連帶效應與預防老化也帶來巨大的效益。

對於老年人保持運動習慣是不可避免的，實驗採用全身垂直律動四週測試年邁老鼠不論在運動表現、體能表現、身體功能性都有明顯的進步，因此運用全身律動訓練於人體上無論於運動保養、無時間運動、懶得運動、無法自主運動者都可以達到最佳的應用價值，對於老年人的健身規劃上也帶來不可或缺的輔助效益。

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Effect of whole-body vibration training on body composition, exercise performance and biochemical responses in middle-aged mice

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ABSTRACT

Aims. Whole-body vibration (WBV) is a well-known light-resistance exercise by automatic adaptations to rapid and repeated oscillations from a vibrating platform, which is also a simple and convenient exercise for older adults. However, the potential benefits of WBV on aging-associated changes in body composition, exercise performance, and fatigue are currently unclear. The objective of the study is to investigate the beneficial effects of WBV training on body composition, exercise performance, and physical fatigue-related and biochemical responses in middle-aged mice.

Methods. In total, 24 male C57BL/6 mice aged 15 months old were randomly divided into 3 groups (n = 8 per group): sedentary control (SC), relatively low-frequency WBV (5.6 Hz, 2 mm, 0.13 g) (LV), and relatively high-frequency WBV (13 Hz, 2 mm, 0.68 g) (HV). Mice in the LV and HV groups were placed inside a vibration platform and vibrated at different frequencies and fixed amplitude (2 mm) for 15 min, 5 days/week for 4 weeks. Exercise performance, core temperature and anti-fatigue function were evaluated by forelimb grip strength and levels of serum lactate, ammonia, glucose, and creatine kinase (CK) after a 15-min swimming exercise, as were changes in body composition and biochemical variables at the end of the experiment.

Results. Relative muscle and brown adipose tissue weight (%) was significantly higher for the HV than SC mice, but relative liver weight (%) was lower. On trend analysis, WBV increased grip strength, aerobic endurance and core temperature in mice. As well, serum lactate, ammonia and CK levels were dose-dependently decreased with vibration frequency

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; BAT, brown adipose tissue; BUN, blood urine nitrogen; CK, creatine kinase; EFP, epididymal fat pad; HV, relatively high-frequency vibration; LV, relatively low-frequency vibration; LDH, lactate dehydrogenase; SC, sedentary control; TC, total cholesterol; TG, triacylglycerol; TP, total protein; WBV, whole-body vibration.

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after the swimming test. Fasting serum levels of albumin and total protein were increased and serum levels of alkaline phosphatase and creatinine decreased dose-dependently with vibration frequency. Moreover, WBV training improved the age-related abnormal morphology of skeletal muscle, liver and kidney tissues. Therefore, it could improve exercise performance and ameliorate fatigue and prevent senescence-associated biochemical and pathological alterations in middle-aged mice.

Conclusions. WBV training may be an effective intervention for health promotion in the aging population. The detailed molecular mechanism of how WBV training regulates anti-aging activity warrants further functional studies.

1. Introduction

The number of older people (aged 65 years or over) in nearly all countries of the world is increasing exponentially. According to the report of World Population Ageing [1], the older population has increased from 202 million in 1950 to 841 million in 2013 and is projected to increase to more than 2 billion in 2050. Efforts to improve health in older adults with increasing life expectancy are needed.

Aging is a natural and an irreversible process that can greatly affect the individual's health, independence and quality of life [2]. This process is associated with numerous physiological alterations, including reduced cardiovascular function, loss of muscle mass and bone, increased storage of body fat, and variable degrees of cognitive decline, all of which contribute to decreased functional capabilities and may ultimately lead to frailty [3–6]. Age-related functional deterioration can be accelerated by a sedentary lifestyle, which causes reduced mobility and independent living [7]. For instance, older people with a sedentary lifestyle are at increased risk of developing sarcopenia, which is characterized by a gradual loss of muscle mass and a decrease in muscle strength and power [8]. The condition is associated with frailty and less ability to recuperate from illness or injury [9].

Although a number of the age-related decreases in function may be inevitable, these can be alleviated by exercise, such as aerobic or resistance training [3,10]. Aerobic training can limit the age-related changes in cardiac function by improving cardiorespiratory fitness, which may lead to reduced risk of mortality and cardiovascular diseases [11,12]. As well, resistance training can prevent the loss of muscle mass, strength, and functionality, thereby offsetting age-related frailty and enhancing mobility [13,14]. Therefore, older people can safely participate in regular exercise (e.g., aerobic or strength training). However, most older adults are reluctant to exercise and are unable to tolerate aerobic or strength exercise routines on a regular basis because of cognitive and/or psychological limitations [15]. Consequently, an exercise modality that may have higher adherence rates and lower risks of side effects is needed to help older adults stay active or keep exercising.

One potential exercise modality is whole-body vibration (WBV), which has appealed to various populations, including athletes [16], postmenopausal women [17,18] and older adults because of its benefits on fitness or health [19]. WBV consists of standing on an oscillating platform that produces sinusoidal vertical vibrations. While the platform vibrates with the appropriate frequency and amplitude, it transmits the energy to the body, thus provoking reflexive muscle construction, known as the "tonic vibration reflex" [20]. WBV exercise has been found to enhance physical performance in athletes and young adults by improving muscle strength and power [16,21]. Furthermore, in recent studies, the enhanced muscular effect was found similar to that of conventional resistance exercise [22–24]. WBV has therapeutic potential to improve bone mineral density [18] and flexibility [25] and reduce pain perception [26]. Much attention has focused on the vibration-specific benefits in older adults, who are particularly frail due to aging. For instance, WBV training can increase bone strength in postmenopausal women [17,27], but no similar beneficial effect was noted in young women [28]. Likewise, WBV training may improve balance function and partly counteract aged-induced muscle frailty by enhancing muscle strength in older adults. Enhanced muscle power or balance in older adults may diminish the risk of subsequent falls [29]. Thus, older adults may benefit more from a WBV exercise modality than younger people.

Despite considerable information on muscle function with WBV training in young and older adults, the mechanisms underlying the performance enhancement effects have not been fully elucidated. Most previous studies have demonstrated that improvements in muscle strength and power after WBV training may be attributed, at least in part, to increased neuromuscular activation [16]. However, evidence of the physiological and biochemical indices of the effect of WBV training is insufficient. Moreover, whether exercise performance can be enhanced by reducing muscle fatigue following long-term WBV training is unknown. Notably, there is paucity of data on whether WBV has a concomitant effect on other organs or tissues, because this technique is transmitted to the whole body. To address these issues, we recently reported in a mouse model of diet-induced obesity that WBV training delayed the fatigue response, enhanced grip strength, mitigated fat accumulation and reduced serum total cholesterol and triacylglycerol levels [30]. More importantly, the feasibility of applying WBV training as an exercise modality in a mouse model of aging needs examining because these WBV-specific benefits may be prominent in middle-aged mice.

Thus, we aimed to investigate the beneficial effects of a 4-week WBV training regimen on body composition, exercise performance and physical fatigue in middle-aged mice. Grip strength and swim-to-exhaustion time were indicators of exercise capacity. Changes in histopathology of various organs/tissues with WBV training were examined to examine any changes in age-related decline in morphologic features.

Furthermore, serum biochemical and fatigue-related variables were examined to determine whether 4-week WBV training had health-promoting and/or anti-fatigue effects in middle-aged mice.

2. Materials and Methods

2.1. Animals

Male C57BL/6 middle-aged mice (15 months old, n = 24) were purchased from BioLASCO (Yi-Lan, Taiwan) and housed in the animal facility at National Taiwan Sport University (NTSU) at 22 °C, 50% to 60% relative humidity, and a 12-h light–dark cycle (light on 7:00 AM). Distilled water and standard laboratory chow diet (No. 5001; PMI Nutrition International, Brentwood, MO, USA) were available *ad libitum*. Before experiments, mice were acclimatized for 1 week to the environment and diet.

2.2. Ethics Statement

All animal experimental protocols complied with rules of Institutional Animal Care and Use Committee (IACUC) in National Taiwan Sports University (NTSU). The protocol was approved by IACUC of NTSU (Permit Number: IACUC-10205).

2.3. Study Design and Training Protocols

After 1-week acclimatization, 24 middle-aged mice were randomly divided into 3 groups (n = 8 each) for treatment: sedentary control (SC) (n = 8), relatively low-frequency vibration (LV) group (n = 8) and relatively high-frequency vibration (HV) (n = 8) for 4 weeks. In LV and HV groups, mice were exposed to vertical WBV on a vibration platform (Body Green, Qigong Master, BW760, Taiwan). The frequencies provided by the vibration platform were 5.6 Hz (LV group; peak acceleration, 0.13 *g*) and 13 Hz (HV group; peak acceleration, 0.68 *g*). A low-intensity vibration platform is considered to produce gravitational force < 1 *g* regardless of frequency. The peak-to-peak amplitude of the vibration was 2 mm in HV and LV groups. The accelerations, frequencies and amplitude of WBV were based on our previous work finding that exercise performance and fatigue, prevention of fat accumulation, and alteration in obesity-associated biochemical processes in obese mice may be improved by WBV training [30]. The WBV training was conducted under continuous supervision for 15 min/day, 5 days/week for 4 weeks. Each training session was regularly conducted from 8:00 to 9:00 am. Food intake and water consumption were recorded daily, and all animals were weighed weekly.

2.4. Forelimb Grip Strength

A grip strength meter (Model-RX-5, Aikoh Engineering, Nagoya, Japan) was used to measure grip power of the forelimb as we previously described [31,32].

2.5. Exhaustive Swimming Exercise

After the 4-week WBV-training intervention, mice were subjected to a swim-to-exhaustion test, with weight equivalent to 5% of their body weight attached to their tails to analyze endurance time as we previously described [31,32]. The swimming time before the exhaustion of each mouse was measured to evaluate exercise endurance capacity. Exhaustion was determined by loss of coordinated movements and failure to return to the surface within 7 s.

2.6. Core Body Temperature Measurement

The core body temperature of mice was taken by use of a digital thermometer with a rectal probe (DE-3003 K-TYPE, Deree, New Taipei City, Taiwan) before and after the 4-week WBV training. Pre- and post-training core temperature was measured in an environmental room set at 22 °C.

2.7. Biomarkers of Muscle Fatigue

The swimming exercise performance test was carried out after the 4-week WBV training to determine the effect of WBV training on muscle fatigue-related biomarkers, including serum lactate, ammonia, and glucose levels and creatine kinase (CK) activity. After a 15-min swimming test, blood samples were immediately collected from mice and prepared for analysis.

2.8. Blood Biochemical Analysis

Serum samples were analyzed for the following parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), albumin, CK, total protein (TP), blood urine nitrogen (BUN), creatinine, uric acid, total cholesterol (TC), triacylglycerol (TG) and glucose by use of an autoanalyzer (Hitachi 7080, Hitachi, Tokyo).

2.9. Histology

After collection of blood samples, mice were killed immediately by cervical dislocation. The heart, liver, lungs, kidneys, muscle, epididymal fat pad (EFP), and brown adipose tissue (BAT) were excised and weighed. The relative organ and/or tissue weights were calculated by using the animals' fasting body weights. Excised heart, liver, lungs, kidneys and muscles were fixed in 10% formalin solution, then embedded in paraffin, cut by use of a microtome at 4-μm thick and stained with hematoxylin and eosin. Histological analysis involved a light microscope equipped with a CCD camera (BX-51; Olympus, Tokyo, Japan) and representative samples were photographed.

2.10. Statistical Analysis

Data are expressed as mean ± SEM. Statistical differences were analyzed by one-way ANOVA and trend analysis involved the Cochran–Armitage test to investigate the dependence of the response on vibration frequency (i.e., trend of response-level efficacy with vibration frequency) with SAS 9.0 (SAS Inst., Cary, NC, USA). Duncan's *post-hoc* analysis was used for further comparisons within testing conditions. Relationships between clinical variables and physical performance were calculated by

Pearson's-correlation coefficients using the CORR procedure of SAS. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of WBV Training on Food and Water Intake, Body and Tissue Weight, and Relative Tissue Weight in Middle-Aged Mice

Middle-aged mice with or without a 4-week WBV training regimen showed no differences in food and water consumption or body weight (Table 1) or absolute and relative weights of EFP, heart, kidney and lung. On trend analysis, WBV training significantly decreased the absolute and relative weight of liver ($P < 0.0001$) and dose-dependently increased the absolute and relative weight of BAT with increasing vibration frequency ($P < 0.0001$) (Table 1). Furthermore, only HV training significantly increased relative muscle weight ($P = 0.0003$, Table 1). Therefore, the WBV-training regimen could be incorporated into the daily routine of mice and had no adverse effects on daily food and water consumption or body weight. Gross examination of the vital organs of all mice revealed no overt abnormalities (data not shown).

3.2. Effect of WBV Training on Forelimb Grip Strength in Middle-Aged Mice

To examine the effect of WBV training on muscle strength of mice, we used a forelimb grip strength test after 4-week WBV training. The mean force exerted was 124.3 ± 5.4, 135.3 ± 5.5, and 140.9 ± 2.2 g for the SC, LV, and HV groups, respectively (Fig. 1). Trend analysis revealed a dose-dependent increase in grip strength of mice with increasing vibration frequency ($P = 0.0009$), especially with HV ($P = 0.0193$). Our WBV regimen with relatively high frequency may be effective for improving the muscle strength of middle-aged mice.

3.3. Effect of WBV Training on Endurance Time in Middle-Aged Mice with an Exhaustive Swimming Exercise

After a 4-week WBV training, all middle-aged mice underwent a swimming endurance test to assess exercise endurance capacity. The mean endurance time of swimming to exhaustion was 5.82 ± 0.75, 9.82 ± 2.68 and 11.00 ± 1.40 min for the SC, LV, and HV groups, respectively, with no significant differences between groups but a positive dose–response trend with increasing vibration frequency ($P = 0.0003$; Fig. 2). Hence, WBV training showed a significant trend toward an increase in endurance time, with no significant difference between all groups.

3.4. Effect of WBV Training on Fatigue-Related Biochemical Parameters in Middle-Aged Mice After an Exhaustive Swimming Exercise

Mean lactate and NH_3 and CK levels were significantly lower in HV trained mice than controls (Fig. 3). In addition, except for glucose level, lactate and NH_3, levels and CK activity were decreased with increasing vibration frequency of WBV (trend analysis; $P < 0.0001$, $P < 0.0001$, $P = 0.0056$, respectively). Glucose level was increased but not significantly by vibration frequency. The glucose level was higher for the LV than SC

Table 1 – Effect of whole-body vibration (WBV) training on food and water intake, body and tissue weight, and relative tissue weight in middle-aged mice.

Characteristics	SC	LV	HV	Trend analysis *P* value #
Initial BW (g)	33.6 ± 0.7 [a]	33.5 ± 0.7 [a]	33.8 ± 0.4 [a]	0.4487
Final BW (g)	32.1 ± 0.7 [a]	31.5 ± 0.4 [a]	31.1 ± 0.3 [a]	0.1869
Food intake (g · day^{-1})	3.56 ± 0.10 [a]	3.77 ± 0.08 [a]	3.59 ± 0.07 [a]	0.3790
Water intake (ml · day^{-1})	4.99 ± 0.18 [a]	5.47 ± 0.15 [a]	5.43 ± 0.18 [a]	0.0644
Muscle (g) *	0.32 ± 0.01 [a]	0.33 ± 0.01 [a]	0.34 ± 0.00 [a]	0.1087
BAT (g)	0.083 ± 0.005 [a]	0.103 ± 0.003 [b]	0.109 ± 0.003 [b]	**<0.0001**
Liver (g)	1.45 ± 0.04 [b]	1.33 ± 0.02 [a]	1.31 ± 0.01 [a]	**<0.0001**
Kidney (g)	0.41 ± 0.01 [a]	0.41 ± 0.01 [a]	0.41 ± 0.01 [a]	0.8189
EFP (g)	0.86 ± 0.08 [a]	0.88 ± 0.04 [a]	0.79 ± 0.05 [a]	0.4518
Heart (g)	0.16 ± 0.01 [a]	0.16 ± 0.01 [a]	0.16 ± 0.01 [a]	0.4462
Lung (g)	0.41 ± 0.02 [a]	0.43 ± 0.01 [a]	0.41 ± 0.02 [a]	0.9215
Relative muscle weight (%)	1.00 ± 0.02 [a]	1.02 ± 0.02 [a]	1.09 ± 0.01 [b]	**0.0003**
Relative BAT weight (%)	0.261 ± 0.012 [a]	0.328 ± 0.010 [b]	0.345 ± 0.011 [b]	**<0.0001**
Relative liver weight (%)	4.54 ± 0.10 [b]	4.26 ± 0.05 [a]	4.24 ± 0.03 [a]	**<0.0001**
Relative kidney weight (%)	1.32 ± 0.04 [a]	1.32 ± 0.02 [a]	1.29 ± 0.02 [a]	0.8667
Relative EFP weight (%)	2.69 ± 0.22 [a]	2.78 ± 0.12 [a]	2.51 ± 0.15 [a]	0.5019
Relative heart weight (%)	0.50 ± 0.02 [a]	0.49 ± 0.02 [a]	0.52 ± 0.02 [a]	0.3684
Relative lung weight (%)	1.30 ± 0.04[a]	1.33 ± 0.03[a]	1.30 ± 0.07[a]	0.9459

Data are mean ± SEM (n = 8). Relative tissue weight (%) = tissue weight (g)/final body weight (g) × 100%. Abbreviations: BW, body weight; BAT, brown adipose tissue; EFP, epididymal fat pad; SC, sedentary control; LV, relatively low-frequency WBV (5.6 Hz, 0.13 g); HV, relatively high-frequency WBV (13 Hz, 0.68 g).

[a,b] Means with different superscripts in each row are significantly different (one-way ANOVA followed by a Duncan's post-hoc test, $P < 0.05$).

* Sum of gastrocnemius and soleus muscles.

\# Trend analysis with Cochran–Armitage test.

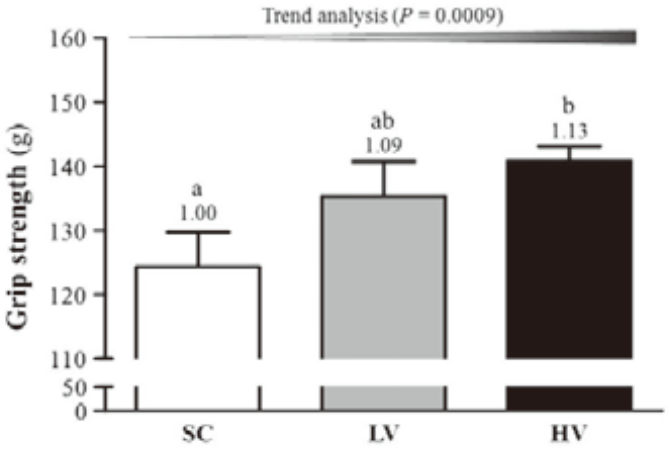

Fig. 1 – Effect of 4-week WBV training on forelimb grip strength in middle-aged mice. Data are mean ± SEM (n = 8 mice per group). Different letters indicate significant difference (one-way ANOVA followed by a Duncan's *post hoc* test, P < 0.05). Trend analysis with Cochran–Armitage test. SC, sedentary control; LV, relatively low-frequency WBV (5.6 Hz, 0.13 g); HV, relatively high-frequency WBV (13 Hz, 0.68 g).

and HV groups (P = 0.0192). Conversely, lactate level was decreased dose-dependently with vibration frequency among the 3 groups but was significant only for the HV group (P < 0.0001 vs. controls). NH_3 level and CK activity did not differ between the LV and HV groups.

3.5. *Effect of WBV Training on Core Body Temperature in Middle-Aged Mice*

Core body temperature did not differ among the groups before WBV training (Table 2). However, with 4-week WBV training, core body temperature was higher for LV and HV than SC mice. Moreover, we found a trend of increasing core body temperature with increasing vibration frequency (P < 0.0001).

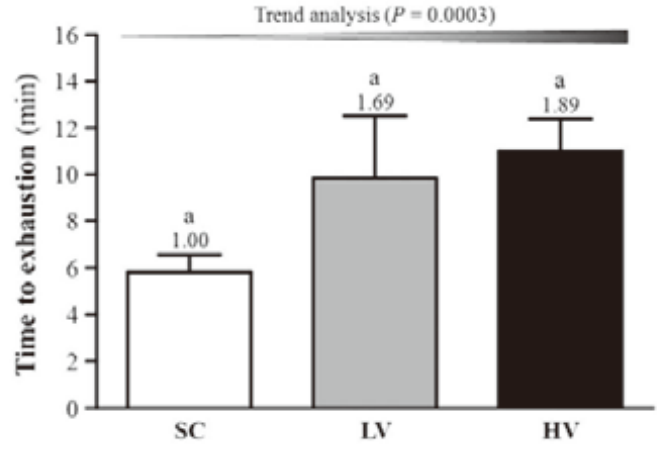

Fig. 2 – Effect of 4-week WBV training on endurance time in middle-aged mice by exhaustive swimming exercise. Data are mean ± SEM (n = 8 mice per group). Different letters indicate significant difference (one-way ANOVA followed by a Duncan's *post hoc* test, P < 0.05). Trend analysis with Cochran–Armitage test. SC, sedentary control; LV, relatively low-frequency WBV (5.6 Hz, 0.13 g); HV, relatively high-frequency WBV (13 Hz, 0.68 g).

3.6. *Effect of WBV Training on General Blood Biochemistry in Middle-Aged Mice*

In response to WBV training, blood ALB and TP levels were higher for LV and HV than SC mice (both P < 0.05; Table 3), with no differences in blood ALB and TP levels between the LV and HV groups but with a dose–response increase in blood ALB and TP levels with increasing vibration frequency (trend analysis, P = 0.0012 and P = 0.0407, respectively). In contrast, we found a dose–response decrease in ALP level and creatinine activity (trend analysis P < 0.0001 and P = 0.0020, respectively) with WBV training. ALP level and creatinine activity were significantly lower, by 15.0% (P = 0.0248) and 10.3% (P = 0.0095), respectively, for HV than SC mice. ALP level and creatinine activity were lower but not significantly for HV than LV mice.

3.7. *Correlation Between Biochemical Variables and Physical Activities*

To test whether the blood biochemical parameters are directly related to the physical performance during the test, Pearson correlation coefficients were calculated. There were several meaningful correlations (Table 4). First of all, the lactate level was significantly correlated with ammonia, CK, and glucose but not grip strength or exhaustive swimming time (Table 4). The correlation between ammonia and CK was the strongest (r = 0.7923, P < 0.001) and glucose also showed high correlation with grip strength and exhaustive swimming time (r = 0.7103, r = 0.6324; both P < 0.001, respectively). Exhaustive swimming time was correlated with grip strength (r = 0.7247, P < 0.001).

3.8. *Effect of WBV Training on Histopathology in Middle-Aged Mice*

To investigate an effect on organs after 4-week WBV training, we used histopathology. In SC mice, the liver, muscle, heart, kidney and lung tissues differed histopathologically as compared with younger counterparts, which suggests that these changes were related to natural aging. In liver, middle-aged SC mice showed mild histopathological changes, including the distribution of fat droplets within hepatocytes (Fig. 4A). Middle-aged SC mice showed skeletal muscle bundles with vacuole-like spaces and fragmented myofibers caused by myofiber atrophy due to aging-related changes (Fig. 4B). Middle-aged heart sections showed vacuolization of cardiomyocytes (Fig. 4C). Middle-aged kidney sections showed numerous renal tubules containing hyaline casts (Fig. 4D). Moreover, middle-aged lung sections appeared to have slight emphysema (Fig. 4E). These histopathological characteristics were ameliorated or not seen in middle-aged LV and HV mice after 4-week WBV training. However, LV and HV mice showed similar histopathological changes in all sections. Nevertheless, our WBV-training regimen reversed a variety of aging-related histopathological changes to some degree. These changes seemed to be ameliorated with increasing vibration frequency especially in the skeletal muscle sections.

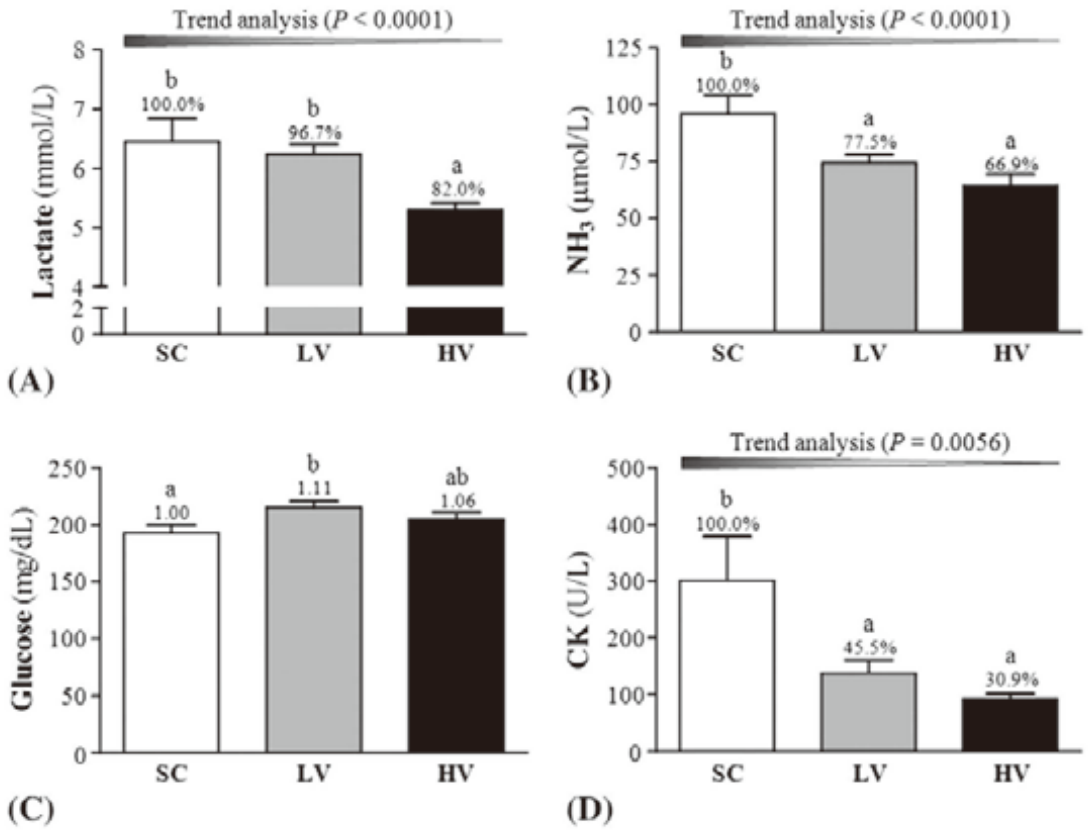

Fig. 3 – Effect of 4-week WBV training on fatigue-related biochemical parameters in middle-aged mice by exhaustive swimming exercise. Serum lactate (A), ammonia (B), and glucose (C) levels, and creatinine kinase (CK) activity (D). Data are mean ± SEM (n = 8 mice per group). Different letters indicate significant difference (one-way ANOVA followed by a Duncan's *post hoc* test, $P < 0.05$). Trend analysis with Cochran–Armitage test. SC, sedentary control; LV, relatively low-frequency WBV (5.6 Hz, 0.13 g); HV, relatively high-frequency WBV (13 Hz, 0.68 g).

4. Discussion

WBV training can be beneficial in counteracting age-induced decreases in muscle function. Clinical studies show enhanced muscle strength and contraction velocity with the training in older adults [33] and increased lower body strength in postmenopausal women [27]. However, we lack data on post-training blood biochemical parameters. Muscle fatigue, as indicated by blood biomarkers, may affect exercise performance. Furthermore, in animal models, most WBV studies have focused on bone health rather than exercise performance and fatigue response. On trend analysis, WBV increased grip strength, aerobic endurance and core temperature. As well, serum lactate, ammonia and CK levels were dose-dependently decreased with WBV training frequency after the swimming test. Fasting serum levels of ALB and total protein were increased and serum levels of ALP and CK decreased dose-dependently with WBV. Moreover, WBV training ameliorated the age-related abnormal morphology of skeletal muscle, liver and kidney tissues. Therefore, the training could improve exercise performance and alleviate fatigue and prevent senescence-associated biochemical and pathological alterations in middle-aged mice. WBV training may be an effective intervention for health promotion in the aging population.

High frequency and magnitude of WBV (i.e., 13 Hz and 0.68 g) may improve exercise performance. WBV at low frequency and magnitude (5.6 Hz and 0.13 g) seemed to be of intermediate efficacy. WBV training dose-dependently

Table 2 – Changes in core body temperature pre- and post-WBV training in middle-aged mice.

Core body temperature (°C)	SC	LV	HV	Trend analysis[#] P value
Pre-training	37.3 ± 0.1[a]	37.3 ± 0.1[a]	37.5 ± 0.1[a]	0.0845
Post-training	37.3 ± 0.1[a]	38.1 ± 0.0[b]	38.3 ± 0.1[b]	**<0.0001**

Data are mean ± SEM (n = 8). SC, sedentary control; LV, relatively low-frequency WBV (5.6 Hz, 0.13 g); HV, relatively high-frequency WBV (13 Hz, 0.68 g).

[a,b] Means with different superscripts in each row are significantly different (one-way ANOVA followed by Duncan's post-hoc test, $P < 0.05$).

[#] Trend analysis with Cochran–Armitage test.

Table 3 – Effect of WBV training on biochemical parameters in middle-aged mice.

Variables	SC	LV	HV	Trend analysis *P* Value #
AST (U/L)	95 ± 23[a]	57 ± 1[a]	60 ± 2[a]	0.4254
ALT (U/L)	42 ± 5[a]	37 ± 1[a]	37 ± 2[a]	0.9316
ALP (U/L)	290 ± 11[b]	273 ± 16[ab]	247 ± 5[a]	**<0.0001**
LDH (U/L)	375 ± 52[a]	325 ± 15[a]	367 ± 13[a]	0.3482
Albumin (g/dL)	3.6 ± 0.2[a]	3.8 ± 0.0[b]	3.8 ± 0.0[b]	**0.0012**
CK (U/L)	318 ± 146[a]	71 ± 4[a]	76 ± 6[a]	0.5512
TP (g/dL)	5.8 ± 0.0[a]	5.9 ± 0.0[b]	5.9 ± 0.1[b]	**0.0407**
BUN (mg/dL)	29.5 ± 0.7[a]	28.4 ± 0.5[a]	27.6 ± 0.7[a]	0.1452
Creatinine (mg/dL)	0.29 ± 0.01[b]	0.27 ± 0.01[ab]	0.26 ± 0.01[a]	**0.0020**
Uric acid (mg/dL)	1.8 ± 0.1[a]	1.7 ± 0.1[a]	1.7 ± 0.1[a]	0.6712
TC (mg/dL)	105 ± 2[a]	104 ± 2[a]	102 ± 2[a]	0.5198
TG (mg/dL)	51 ± 4[a]	50 ± 5[a]	40 ± 4[a]	0.1225
Glucose (mg/dL)	155 ± 6[a]	153 ± 5[a]	156 ± 5[a]	0.6593

Data are mean ± SEM (n = 8). SC, sedentary control; LV, relatively low-frequency WBV (5.6 Hz, 0.13 g); HV, relatively high-frequency WBV (13 Hz, 0.68 g). Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; CK, creatine kinase; TP, total protein; BUN, blood urine nitrogen; CREA, creatinine; TC, total cholesterol; TG, triacylglycerol.
[a,b] Means with different superscripts in each row are significantly different (one-way ANOVA followed by Duncan's post-hoc test, $P < 0.05$).
Trend analysis with Cochran–Armitage test.

increased forelimb grip strength of middle-aged mice. This observation is in agreement with previous studies showing the beneficial effect of WBV on improving muscle strength in older adults [33] and mice [34]. However, the mechanisms by which WBV influences muscle strength are still not well understood. Muscle strength and lean mass are highly interrelated [35]. Therefore, the WBV effect with HV on relative muscle mass might be responsible for the observed increases in muscle strength in middle-aged mice. We assumed that the increased muscle strength in middle-aged mice following HV training might be associated with the suppressed loss of muscle mass. WBV-exposed middle-aged mice showed higher relative muscle weight than sedentary controls, with no change in body weight and significantly only at high-frequency vibration and magnitude (13 Hz and 6.8 g). Therefore, HV WBV may be more effective than low frequency in ameliorating aged-induced muscle loss and increasing grip strength. This finding is consistent with a previous study showing that lean mass in untrained females was slightly increased after 24 weeks of WBV, although body weight was not altered [24]. HV might induce muscle hypertrophy. The muscle hypertrophic effect of WBV at high frequency has been reported previously [34]. Further clinical studies are warranted to investigate whether WBV training improves muscle strength and preserves muscle mass of older adults to counteract the effects of aging-associated sarcopenia.

Table 4 – Correlation between biochemical variables and physical activities.

Variables	Lactate	Ammonia	CK	Glucose	GS	ST
Lactate	1					
Ammonia	0.7192**	1				
CK	0.6316**	0.7923**	1			
Glucose	0.1843*	0.1279	0.0952	1		
GS	0.0796	0.0516	0.0703	0.7103**	1	
ST	0.0528	0.0188	0.0162	0.6324**	0.7247**	1

Pearson's correlation analysis of averaged values for each variable (n = 24). GS: grip strength; ST: exhaustive swimming time.
* Significant at $P < 0.05$.
** Significant at $P < 0.001$.

The swim-to-exhaustion time was higher but not significantly with WBV at LV and HV than in sedentary mice. However, we found a significant dose–response relationship by vibration frequency with swim-to-exhaustion time. This finding may be explained in part by the individual variability in muscle endurance of animals. The animals we used were middle-aged; therefore, aging *per se* may affect the outcomes of the swimming exercise task. For example, the responsiveness of skeletal tissue to mechanical loads is blunted by aging [36].

This phenomenon of increased time to exhaustion might be linked to changes in fatigue-related biomarkers in the trained middle-aged mice. Reduced exercise performance may be attributed to exercise-induced fatigue, which can be further characterized by the accumulation of metabolites, such as lactate and ammonia, activation of CK [37], and depletion of blood glucose [38]. In the present study, we found significant dose-dependent inhibitory effects of WBV training on the above indices, except blood glucose level, in middle-aged mice after a 15-min swimming task. The observed changes for the LV and HV groups suggested a delayed fatigue-response potential, which might explain the trend toward an increase in time to exhaustion in a swimming test as compared with the SC group. This is the first study to investigate the effect of chronic WBV training on the muscle fatigue response to exhaustive exercise in middle-aged mice. Thus, 4-week WBV training, regardless of high frequency or low frequency, could slightly improve the endurance capability of middle-aged mice by delaying the onset of fatigue. However, how WBV training results in prolonging fatigue remains to be further elucidated. WBV may have an effect on neuromuscular function. In one study, young and old mice exposed to WBV with low-intensity vibration (30 Hz with 0.3 g peak acceleration) showed enhanced neuromuscular dynamics and strength behavior, as evidenced by increased

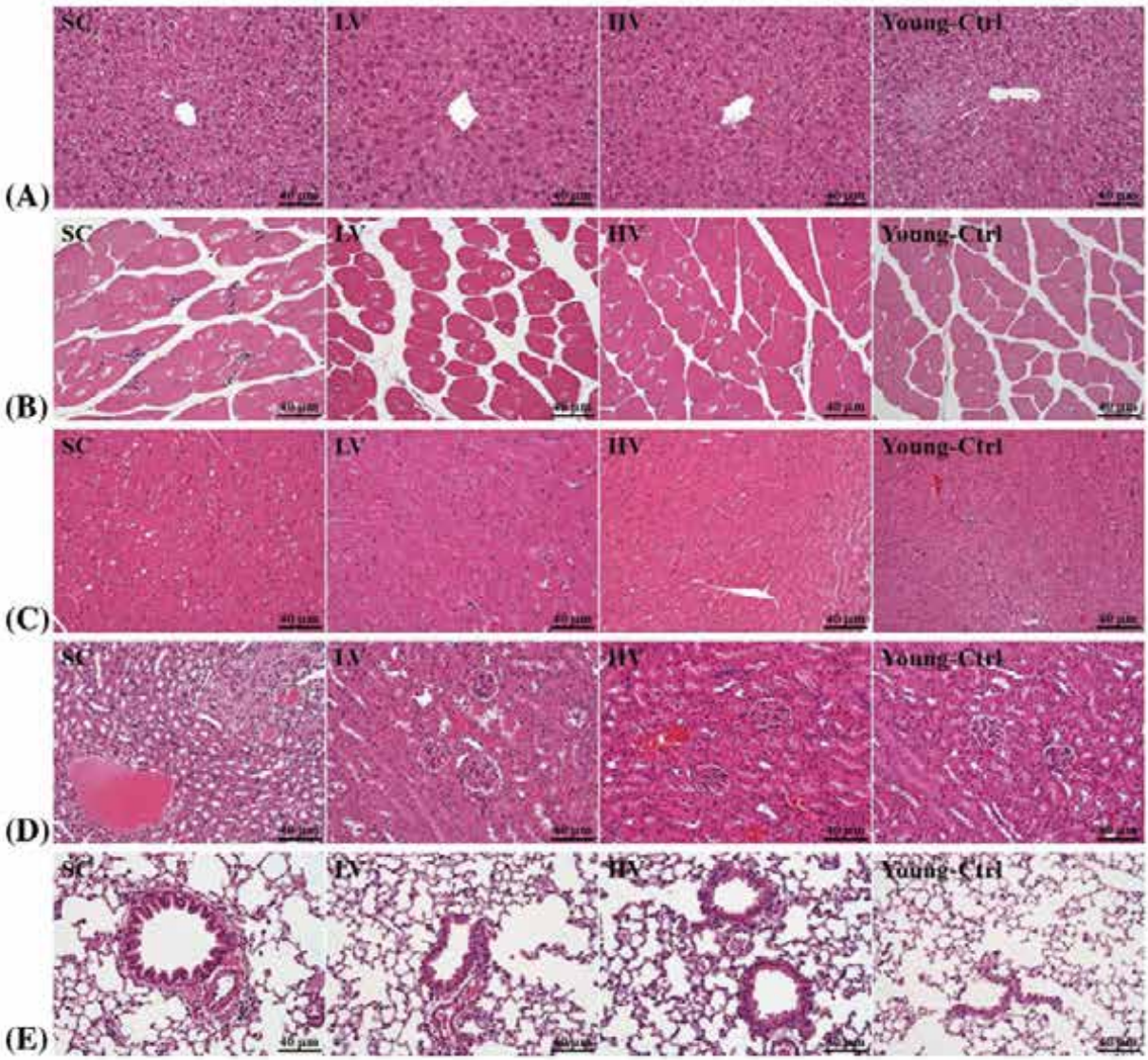

Fig. 4 – Effect of WBV training on body tissues. Representative hematoxylin- and eosin-stained photomicrographs of liver (A), skeletal muscle (B), heart (C), kidney (D) and lung (E) sections from middle-aged mice groups and young control (Young-Ctrl) mice without WBV training. SC, sedentary control; LV, relatively low-frequency WBV (5.6 Hz, 0.13 g); HV, relatively high-frequency WBV (13 Hz, 0.68 g). Original magnification 200× for all photomicrographs. Scale bar, 40 μm.

isometric force production and grip strength [34]. Thus, the fatiguing effects of intensive synaptic muscle stimulation may be attenuated by WBV at low-intensity vibration, which in turn augments fatigue resistance of muscle [34].

Our WBV-trained middle-aged mice showed significantly increased BAT mass and relative BAT weight accompanied by a small but significant increase in core body temperature. WBV training may protect against aged-related impairment of BAT thermogenic ability. BAT plays an important role in thermogenesis, which is responsible for regulating body temperature and energy balance [39,40]. In addition, BAT thermogenesis is involved in preventing obesity [41]. However, aging can affect this thermoregulation and lead to diminished cold tolerance and propensity to obesity [42]. Previous studies demonstrated that old animals showed reduced ability to regulate the body temperature because of age-induced decrease in capacity of BAT thermogenesis [43]. Increased BAT mass and relative BAT weight with WBV training in middle-aged mice might reflect improved BAT thermogenic ability to result in increased core body temperature. Moreover, the increased BAT mass and weight could limit weight gain and fat accumulation [44], so WBV training might have an anti-obesity effect. We reported the anti-obesity effect of WBV in our previous study (with the same training protocol as used in this study) focusing on the obese mouse model [30]. Thus, the WBV used in the present study would have had the same effect. However, we did not demonstrate a significant preventive effect of WBV on weight gain, which suggests that the animal model with different experimental conditions may explain such diverse results. Nevertheless, we showed the beneficial effects of WBV on activated BAT thermogenesis of middle-aged mice with concomitant increase in core body temperature.

As a fourth component, we sought to investigate the effect of 4-week WBV training on liver, skeletal muscle, heart, kidney and lung of middle-aged mice in that the vibration stimulation was for the whole body. These tissues were selected because they contribute to whole-body physiology and can be perturbed by aging and influenced by WBV exposure. We examined the concomitant effects of WBV on

various tissue pathologies with aging. Light microscopy of these sections from sedentary middle-aged mice showed overt morphological alterations in these tissues as compared with young controls. This finding suggests perturbations in tissue pathologies in response to natural aging, which may lead to loss of normal physiological/biochemical functions. These age-induced pathologic changes were ameliorated or reversed by WBV training, which demonstrates the anti-aging potential of WBV to maintain the functions of various organs at cellular levels (such as liver and kidney). However, the involved mechanism responsible for these pathological changes remains a matter for speculation.

Nevertheless, the histopathological findings can be further supported by results from parallel studies showing significant changes in blood biochemical parameters such as albumin, total protein, ALP and CK. Blood biochemistry is used to assess the functions of internal organs. For example, blood albumin, total protein and ALP can serve as prognostic indicators of liver damage because of their correlation with extent of hepatic dysfunction [45,46], whereas creatinine can be used as a biomarker of renal dysfunction [47]. Our finding of increased blood albumin and total protein levels and reduced ALP activity due to WBV training represents the ameliorating effects of WBV on metabolic functions of the liver with respect to aging. Liver plays an important role in biosynthesis of albumin [48] and our observations of increased blood albumin and total protein could be attributed to improved metabolic functions of liver in middle-aged mice receiving a 4-week WBV training regimen. Elevated creatinine in blood seems to evidence renal impairment. Creatinine level is gradually increased with age, which implies loss of normal renal function with age [47]. Thus, the reduced creatinine level we observed supports that renal function of middle-aged mice may be attenuated by WBV training. Thus, altered histopathlogic and biochemical conditions in response to WBV training may have distinct biological advantages to aging animals. Despite the use of WBV in this population eliciting health-enhancing gains [20], its risks of other organ systems do not appear to have been described [49]. Therefore, the lack of harmful effects found in tissue histopathology and blood biochemistry could suggest that WBV training at low intensity, regardless of frequency, may be safe and well tolerated by aging animals.

Although a number of variables were significantly altered dose-dependently by 4-week WBV training as demonstrated by our trend analysis findings, we were unable to detect the significant differences between the LV and HV conditions for most variables. For example, WBV with HV condition achieved significantly increased grip strength and relative muscle weight and decreased levels of lactate (i.e., post-exercise) and CK, as demonstrated by post-hoc analyses. Consideration should be given to WBV protocols (i.e., frequency, amplitude and duration) that varied greatly among studies, which likely affected study outcomes, further limiting the generalizability of the reported results. Thus, WBV protocols should be compared in the present and published studies. Because of the lack of evidence of optimal WBV protocols, we chose the vibration protocols corresponding to the results of our previous study [30] and suggest that low intensity (i.e., acceleration level < 1 g), regardless of frequency, may be used during chronic WBV to achieve significant gains in grip strength, prevention of muscle loss, fatigue-resistance, and histopathological and biological alterations in aging animals. Previous studies in various populations have also shown that WBV training with low intensity may have greater advantages than high intensity, although these studies focused on bone health [18,27,28].

5. Conclusions

In summary, we report the novel findings that 4 weeks of WBV training of low intensity in middle-aged mice altered 1) exercise performance, as shown by significantly increased forelimb grip strength and modest improvements in the swim-to-exhaustion time in a swimming task; 2) fatigue-resistance, as illustrated by decreased post-exercise lactate and ammonia levels and CK activity; 3) body composition, as evidenced by increased relative muscle weight as well as BAT and relative BAT weight; 4) age-related pathologies in 5 tissues, as evidenced by mild pathological changes particularly in muscle, liver and kidney; and 5) biochemical parameters, as evidenced by increased albumin and total protein levels and decreased alkaline phosphatase activity and creatinine level. In addition, increased BAT and relative BAT weight may be responsible for the increased core body temperature in WBV-trained middle-aged mice. Most changes in these variables were dose-dependent. To our knowledge this is the first study of low-intensity WBV training to incorporate functional and clinically relevant biochemical and physiological aging animal data with histopathological data. The improvements in exercise performance and fatigue-resistance are likely due to the pleiotropic effects of WBV training at multiple levels, including physiology and functionality. For example, WBV training might modulate pathological tissue susceptibilities to aging in aged animals, and the nature of such modulations implies that the effects of WBV might occur in multiple organ systems that as a whole could contribute to sustained increases in exercise performance and fatigue-resistance. These results may be of potential clinical value but warrant the investigation of the underlying mechanisms of the action of WBV training and its potential clinical application as an ergogenic aid to fitness for older adults.

Author Contributions

Ching-I Lin, Yen-Shuo Chiu and Chi-Chang Huang analyzed the data, interpreted the results, prepared figures, and wrote the manuscript. Nai-Wen Kan and Chi-Chang Huang designed the experiments. Wen-Chyuan Chen, Nai-Wen Kan and Li Wei contributed reagents, materials and analysis platforms. Wen-Ching Huang and Wen-Chyuan Chen carried out the laboratory experiments.

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Disclosure Summary

The authors have no conflicts of interests.

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低強度垂直律動會促進C2C12肌小管內成肌細胞的形成

Low-magnitude vertical vibration enhances myotube formation in C2C12 myoblasts.

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簡述內容：

隨著年齡增長，肌肉生成膠原蛋白的速度逐漸減緩，加上能讓皮膚迅速回彈的彈力蛋白減少，甚至發生斷裂，就好比為什麼嬰幼兒的皮膚可以如此的Q彈光澤，而高齡長者的皮膚呈現卻是充滿皺紋與鬆弛；皮膚狀態在25歲左右開始自然衰老，30歲之後身體肌肉衰竭的速度也會大於生長速度，人們的肌肉開始以每年0.5%到1%的速度減少，這也是為什麼過30歲後外表老化的速度變得更明顯的原因。

本實驗採用全身垂直律動研究，分別以不同頻率進行測試(0Hz、5Hz、8Hz、10Hz)，證實即使以低強度的垂直律動，亦可明顯促進肌肉細胞活化與膠原蛋白增生，有效減緩老化速度，因此若以全身垂直律動輔助作為每日的運動保養，除了證實可以延緩身體老化的速度，亦可輔助對於肌肉萎縮的相關治療帶來正向的健康效益。

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Low-magnitude vertical vibration enhances myotube formation in C2C12 myoblasts

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Wang CZ, Wang GJ, Ho ML, Wang YH, Yeh ML, Chen CH. Low-magnitude vertical vibration enhances myotube formation in C2C12 myoblasts. *J Appl Physiol* 109: 840–848, 2010. First published July 15, 2010; doi:10.1152/japplphysiol.00115.2010.—Whole body vibration training is widely used in rehabilitation and sports activities to improve muscle strength, balance, and flexibility. However, the molecular mechanisms of vertical vibration (VV) training and their effect on the myogenesis of myoblasts remain undefined. This study was undertaken to address the hypothesis that VV can enhance the expression of ECM proteins and myogenic regulatory factors (MRFs) in myoblasts and, in turn, increase myotube formation. Using real-time PCR, Western blot analysis, and immunofluorescence studies, we examined the effect of VV treatment with frequencies of 5, 8, or 10 Hz on the expression of ECM proteins and MRFs as well as myotube formation in C2C12 myoblasts. We showed that VV stimulation is safe and effective at stimulating myogenesis in C2C12 myoblasts. The levels of expression of the ECM proteins type I collagen and decorin were the highest after VV treatment at frequencies of 8 and 10 Hz. Expression of the MRFs MyoD and myogenin increased after VV stimulation in a time- and dose-dependent manner. The total number of myotubes formed, as well as the length and the average area of myotubes, were substantially increased following VV treatment at frequencies of 8 to 10 Hz. In conclusion, VV treatment at frequencies of 8 to 10 Hz can stimulate the expression of ECM proteins and MRFs in myoblasts and, in turn, increase myotube formation.

whole body vibration; myoblast; myogenesis; myotubes

WHOLE BODY VIBRATION, A MECHANICAL load, is widely used in rehabilitation and sports training to improve muscle strength, balance, and flexibility (8, 17). It has been demonstrated to enhance performance in both untrained (38) and athletic (13) young adults, healthy elderly people (17), and frail institutionalized patients (3). Recent studies of muscle regeneration have been focused on myogenic regulatory factors (MRFs) and the ECM microenvironment (35); however, the effect of vertical vibration (VV), a type of mechanical load, on these factors remains to be elucidated.

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Four MRFs (MyoD, Myf-5, myogenin, and MRF4) have been shown to induce myogenic conversion of nonmuscle cell lines, including fibroblasts, chondrocytes, and neurons (41). In addition, these MRFs are upregulated in satellite cells, the stem cells of adult skeletal muscles, after reactivation from a mitotically quiescent state following stress induced by exercise or trauma (28). Each MRF has been shown to play a specific role in myogenesis (1, 28, 41). Of interest, induction of MyoD expression is a key step in the commitment of somite cells to the myogenic lineage and has become a marker of activated and proliferating satellite cells (20). Myogenin, additionally, plays a critical role in the myogenesis of myoblasts and is a marker of terminal myoblast differentiation (33, 48). Previous gene knockout studies have shown that the myogenin gene product is required for myofiber formation during muscle development (7). Specifically, in differentiating C2C12 cells, myogenin promotes the fusion of myotubes and the formation of myofibers (33). A unique temporal expression pattern also exists for these MRFs, with early MyoD activation leading to subsequent myogenin expression (48). Thus, the expression of both MyoD and myogenin indicates a complete progression of myoblast proliferation, differentiation, and fusion into myotubes, which then leads to muscle regeneration and growth.

In addition to MRFs, it has been shown that the ECM is required to ensure myoblast migration, proliferation, and differentiation (19). Similarly, the ECM has been shown to be capable of adapting to changes in the external environment, such as mechanical loading or inactivity and disuse, specifically with collagen levels responding to altered levels of physical activity (14, 19). Central to this regulatory role for collagen in muscle differentiation is the interaction between type I collagen and proteoglycans such as decorin (45). It has been shown that the decorin core protein can influence the rate and extent of collagen fibrillogenesis (40, 55). One possible mechanism for the role of decorin has been suggested in a recent study (18), which demonstrated that decorin enhances myoblast proliferation and differentiation by suppressing myostatin, a member of the TGF-β superfamily, that has inhibitory effects on myoblast proliferation and differentiation. Accordingly, expression of decorin and collagen I results in enhanced myoblast proliferation and leads to subsequent differentiation of myotube hypertrophy. Thus, measuring these ECM components, in addition to the expression of MRFs, can provide a

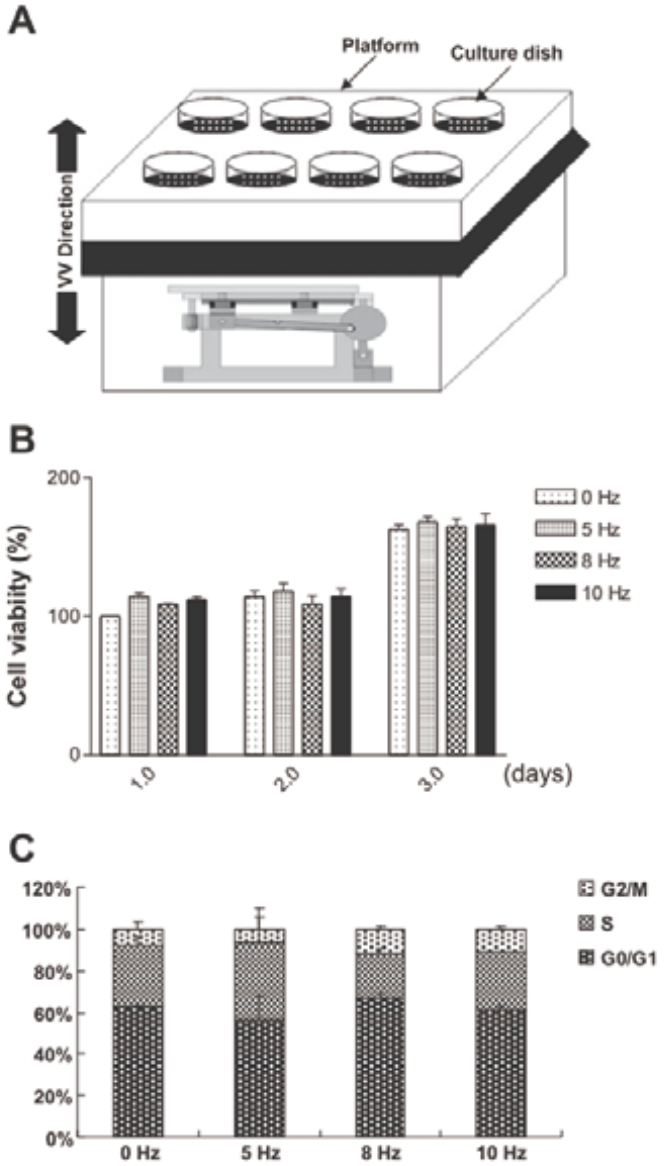

Fig. 1. *A*: schematic diagram of the whole body vertical vibration (VV) device. *B*: quantification of cell viability after VV using the methylthiazoletetetrazolium assay. *C*: cell cycle distribution of VV-treated cells was analyzed by using flow cytometry 24 h after treatment. Percentages of cells in G0/G1, S, and G2/M phase are presented. Data are means ± SD of 3 independent experiments.

good indication of the onset of myogenesis. Previous studies have demonstrated gene upregulation due to exercise and skeletal muscle stretching (35), but none has utilized VV as a source of stimulation.

C2C12 cells, a mouse myoblast satellite cell line (2, 47), have been used extensively as an in vitro model for studying the differentiation and regeneration of skeletal muscle (49). In this study, we investigated the effect of VV treatment, a form of mechanical stimulation, on the induction of myogenesis in C2C12 mouse myoblasts. We hypothesize that VV stimulation enhances myotube formation by inducing the gene expression of ECM components and MRFs in C2C12 myoblasts. Accordingly, we evaluated the effect of VV treatment on the expression of MyoD, an indicator of myoblast initiation, and myogenin, an indicator of terminal myoblast differentiation. In addition, the effect of VV treatment on the expression of the ECM components type I collagen and decorin was evaluated.

METHODS

Antibodies. Anti-Myo-D (C-20) and anti-myogenin (5-FD) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal sarcomeric myosin antibody (MF-20) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA). DMSO, LY294002, propidium iodide, and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO). Mouse anti-β-actin was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Cell culture and VV stimulation. The mouse myoblast satellite cell line, C2C12 (CRL-1772; American Type Culture Convention, Manassas, VA), was maintained in DMEM (GIBCO-BRL, Grand Island, NY) containing 10% FBS (GIBCO-BRL) in a humidified atmosphere of 5% CO_2 at 37°C. Cells were used before the 10th passage. To evaluate the effect of VV stimulation on the myogenesis of C2C12 myoblasts, cells were seeded at a high cell density (1×10^4 cell/cm^2). When cells grew to confluency, they were subjected to vibration stimulation, generated by a vertical platform (BodyGreen, Albany, North Shore, New Zealand), at a frequency of 5, 8, or 10 Hz with 0.4 mm amplitude for 10 min/day (Fig. 1*A*). For the phosphoinositide 3-kinase (PI3-kinase) inhibitor (LY294002) experiment, confluent C2C12 cells were pretreated with DMSO as a vehicle control or LY294002 (10 μM) for 60 min, and then, with medium containing either DMSO or LY294002, the cells were stimulated with VV for 3 days and cultured for an additional 3 days (total of 6 days) to examine myotube formation by immunofluorescence.

Methylthiazoletetetrazolium assay. The methylthiazoletetetrazolium (MTT) assay is based on the cleavage of yellow tetrazolium salt (thiazol blue tetrazolium bromide) into insoluble purple formazan by metabolically active cells and can be used to evaluate the viability of VV-treated cells. Briefly, cells were seeded in 24-well plates, washed with PBS, and then 200 μl of MTT solution (0.5 mg/ml in PBS at pH 7.2) was added to the wells. After an incubation period of 4 h, the MTT solution was removed, the cells were lysed, and the formazan crystals were dissolved by adding 200 μl of DMSO/well at 37°C for 5 min. The optical density of the solubilized formazan in each well was quantified spectrophotometrically using an ELISA reader (Tecan Sunrise; TECAN Deutschland, Crailsheim, Germany) at a wavelength of 570 nm.

Quantitative real-time PCR. Total RNA was isolated from cells using Trizol reagent (GIBCO-BRL). Reverse transcription of the RNA into cDNA was performed using oligo(dT) primers, and the Moloney murine leukemia virus reverse transcriptase. Quantitative real-time PCR was performed in the iQ5 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) using the iQ SYBR green supermix (Bio-Rad). Reactions were performed in a 25-μl mixture containing cDNA, specific primers for each gene and the iQ SYBR green supermix. Primer sequences used are shown in Table 1.

Table 1. *Primer sequences for real-time PCR*

Gene	Primer sequence	Gene ID number
MyoD	5′-GCTTCTATCGCCGCCACTCC-3′	NM_010866
	5′-CGCACATGCTCATCCTCACG-3′	
Myogenin	5′-GCATGCAAGGTGTGTAAGAG-3′	NM_031189
	5′-GCGCAGGATCTCCACTTTAG-3′	
Decorin	5′-ACAGCATCACCGTTATGGAGAATG-3′	NM_007833
	5′-TCACAGCCGAGTAGGAAGCC-3′	
Collagen I	5′-TCAGAGGCGAAGGCAACAGTC-3′	NM_007742
	5′-GCAGGCGGGAGGTCTTGG-3′	
β-*actin*	5′-CCAACCGTGAAAAGATGACC-3′	NM_007393
	5′-ACCAGAGGCATACAGGGACA-3′	

Specific PCR products were detected by measuring the fluorescence of SYBR Green, a double-stranded DNA-binding dye (30). After the real-time PCR reaction, a dissociation (melting) curve was generated to check the specificity of the PCR reaction. The relative mRNA expression level was calculated from the threshold cycle value of each PCR product and normalized to that of β-actin by using the comparative threshold cycle method (25). All real-time PCR experiments were performed in triplicate and repeated at least three times.

Western blot analysis. Cells were washed twice with ice-cold PBS containing 1 mM sodium vanadate and then lysed in modified radioimmunoprecipitation assay buffer (150 mM NaCl, 1 mM EGTA, 50 mM Tris [pH 7.4], 10% glycerol, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) with protease inhibitor (Complete Protease Inhibitor Cocktail Tablets; Roche Diagnostics, Taipei, Taiwan) and 1 mM sodium vanadate. The lysates were cleared by centrifugation at 14,000 rpm for 15 min at 4°C, subjected to SDS-PAGE, immunoblotted with antibodies as indicated, and were then developed with an enhanced chemiluminescence reagent (ECL System; Amersham Pharmacia Biotech).

Cell cycle analysis. Cells were detached and flushed into a single-cell suspension with Hank's buffered solution. After centrifugation, cells were fixed with ice-cold 70% alcohol and treated with RNase (100 mg/ml in PBS) at 37°C for 1 h. Cells were then stained with propidium iodide (400 μg/ml in PBS) in the dark followed by filtration through a 41-μm pore size filter before analysis. The DNA content of individual cells was measured using flow cytometry (FACScan; Becton Dickinson, Mountain View, CA) with excitation set at 488 nm. Data were analyzed using a cell-fit software program and represented as histograms.

Immunofluorescence. Cells on coverslips were washed three times with PBS and then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After being washed three times with PBS, cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min, rinsed with PBS, and then immunostained with anti-myosin antibody (MF-20) for 1 h at room temperature. After being washed three times with PBS, the coverslips were exposed to Alexa Fluor 488-labeled secondary antibodies (Molecular Probes, Eugene, OR) for 1 h, and then the nuclei were stained with DAPI. The coverslips were mounted in anti-fade solution (Molecular Probes). Images of samples from three independent experiments were captured on a fluorescence microscope. A total of 15 representative images per sample were scored for myotube number, myotube length, myotube nuclei, and area occupied by myotubes relative to the total area using Image Pro Plus software (Media Cybernetics, Silver Spring, MD). The fusion index (%) was determined by dividing the number of nuclei within multinucleated myotubes by the total number of nuclei analyzed. In each case, staining without primary antibody was done with a side-by-side parallel specimen (as a negative control), which yielded a blank image.

Statistical analysis. Each value represents the mean ± SE of at least three independent experiments. One-way ANOVA or Student's *t*-test was used to test for statistical differences. Statistical significance was set at $P < 0.05$.

RESULTS

Effect of VV stimulation on the viability and cell cycle profile of C2C12 myoblasts. To evaluate the cell viability of C2C12 myoblasts after VV stimulation, we seeded cells at a subconfluent cell density (7×10^3 cell/cm^2) in each experimental group (0, 5, 8, and 10 Hz) to allow for cell proliferation. Under these cell culture conditions, confluent cell layers of C2C12 myoblasts were observed after 3 days of culture in each group, which indicates similar levels of cell proliferation. As shown in Fig. 1*B*, there was no significant difference in the cell viability between the VV-treated groups after 3 days. Furthermore, when cells were seeded at a higher density (1×10^4 cell/cm^2), which allows for more rapid generation of high confluency, and were subsequently stimulated with VV (5, 8, and 10 Hz) for 3 days, the viability of C2C12 myoblasts was also not different from that of control cells (0 Hz) (data not shown). To investigate whether VV changes the cell cycle profile of myoblasts, we measured the cell cycle profile of C2C12 myoblasts 24 h after vibration treatment. As shown in Fig. 1*C*, there was no significant difference in the cell cycle profile of VV-treated cells compared with control cells.

Effect of VV stimulation on type I collagen and decorin expression. Type I collagen and decorin are the major components of the ECM in muscle structure and are important for the myogenic differentiation of myoblasts (32). We investigated whether VV stimulation regulated type I collagen and decorin expression in C2C12 myoblasts. It has been reported that myogenic differentiation can be induced by a reduction in serum concentration (51). In this study, we cultured C2C12 cells in complete medium; thus, vibration-induced effects on myogenesis could be measured independently of mitogen deprivation-induced effects. The gene expression of type I collagen and decorin in C2C12 myoblasts with VV stimulation (0, 5, 8, and 10 Hz) was determined using real-time PCR for up to 3 days (Figs. 2). Overall, the expression of type I collagen and decorin significantly increased within 3 days after VV stimulation. The gene expression of type I collagen in VV-stimulated groups (5, 8, and 10 Hz) was about threefold higher than

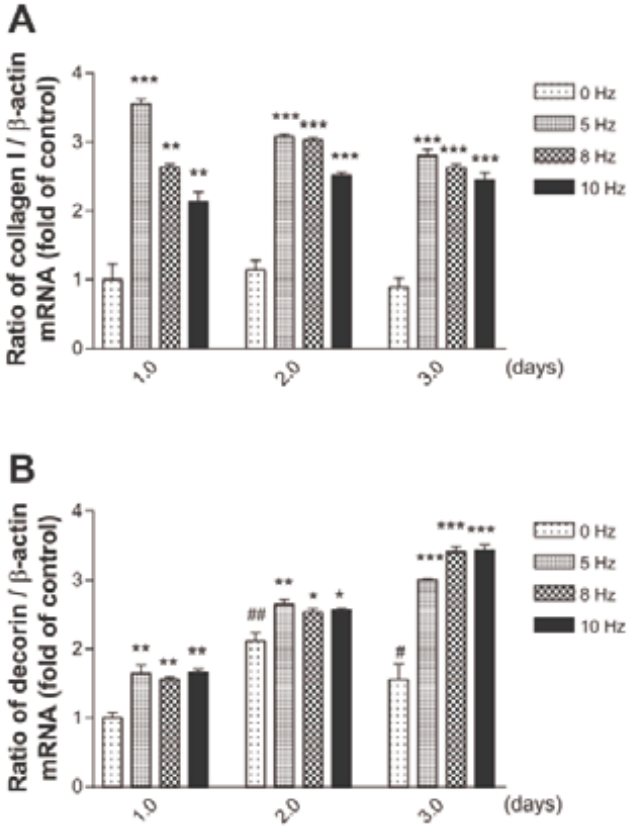

Fig. 2. Effect of VV stimulation on the gene expression of type I collagen and decorin. Cells were cultured for up to 3 days with or without VV stimulation. The mRNA expression of type I collagen (*A*) and decorin (*B*) were determined using real-time PCR. Data are means ± SD of 3 independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for VV-treated vs. control cells (0 Hz); $\#P < 0.05$, $\#\#P < 0.01$ for control cells on *days 2* and *3* vs. control cells on *day 1*.

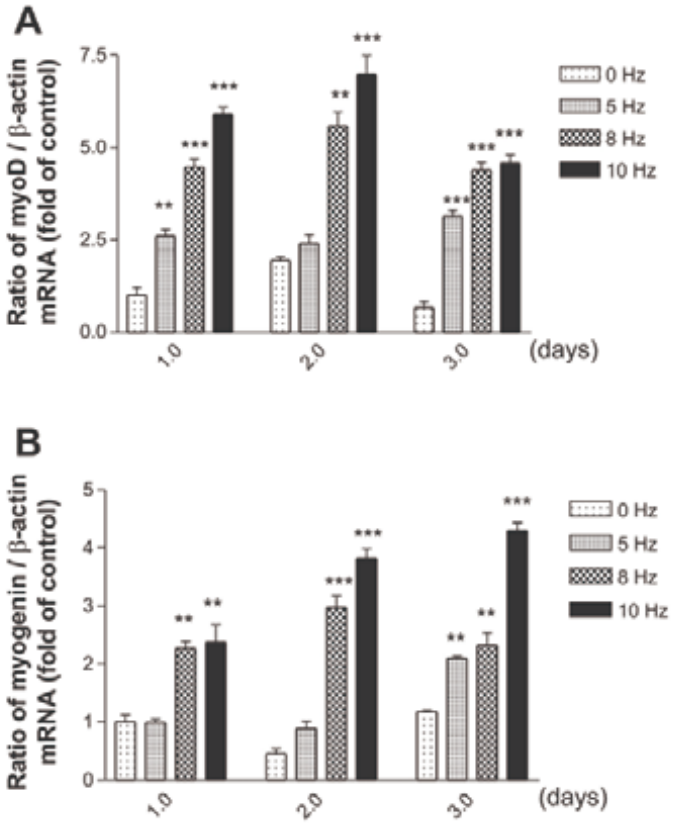

Fig. 3. Effect of VV stimulation on MyoD (*A*) and myogenin (*B*) gene expression. Cells were cultured for 3 days with or without VV stimulation. The mRNA expression of MyoD (*A*) and myogenin (*B*) were determined using real-time PCR. Data are means ± SD of 3 independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ for VV-treated vs. control cells (0 Hz).

that of the control group (0 Hz) (Fig. 2*A*). Notably, expression of type I collagen was highest in the 5 Hz VV-stimulated group, although all VV-treated groups had levels of type I collagen expression that were significantly higher than that of control cells. The gene expression of decorin was also significantly increased from *days 1* through *3* in VV-treated groups (5, 8, and 10 Hz), compared with that of the control group (Fig. 2*B*), with the highest decorin expression in the 10 Hz VV-stimulated group.

VV stimulation augments the expression of MyoD and myogenin. MRFs, including MyoD and myogenin, are the master regulators in the early and terminal differential stages of myogenesis (31, 39). To determine whether VV stimulation promoted myotube formation via regulation of MyoD or myogenin expression, we compared the expression of MyoD and myogenin in C2C12 cells with or without VV stimulation. Real-time PCR analysis showed that MyoD and myogenin gene expression increased in C2C12 cells in a dose-dependent manner with VV stimulation (Fig. 3). In the 5 Hz VV-treated group, gene expression of MyoD was significantly greater than control at *days 1* and *3* (Fig. 3*A*). In addition, MyoD gene expression in the 8 and 10 Hz VV-treated groups was higher than that of the 5 Hz VV-treated group (Fig. 3*A*). In the 5 Hz VV-treated group, gene expression of myogenin was significantly higher than that of the control group at *days 2* and *3* but was lower than those of the 8 and 10 Hz VV-treated groups (Fig. 3*B*). In cells with 8 Hz VV stimulation, myogenin gene expression was increased about twofold over that of control (0 Hz) cells at *days 1* and *3* and threefold higher than that of the control group at *day 2*. In cells that received 10 Hz VV stimulation, myogenin gene expression was about twofold higher than that of the control group at *day 1* and fourfold higher than control cells at *days 2* and *3*.

We confirmed the expression of MyoD and myogenin in VV-stimulated C2C12 cells using Western blot analysis (Fig. 4*A*). Consistent with the real-time PCR results, quantitative Western blot results showed that the expression of MyoD (Fig. 4*B*) and myogenin proteins (Fig. 4*C*) was significantly increased in VV-treated groups (5, 8, and 10 Hz) in a dose-dependent manner (Fig. 4*B*).

VV stimulation enhances myotube formation. Multicellular myotubes are formed when C2C12 myoblasts differentiate and fuse with each other (51). To investigate the myogenic effect of

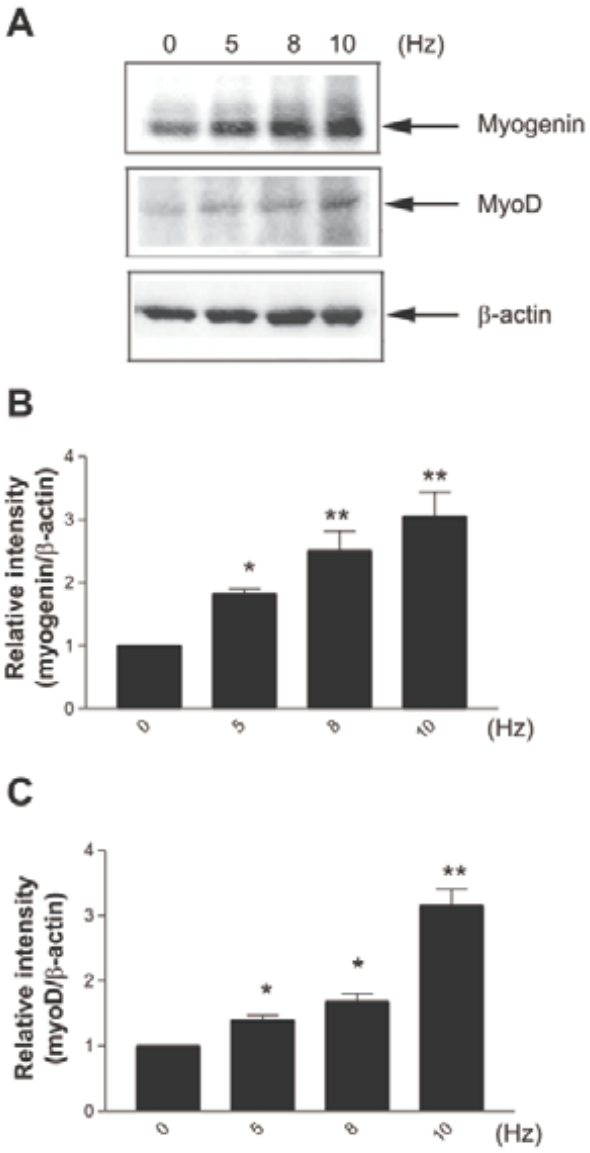

Fig. 4. Effect of VV stimulation on the protein expression of Myo-D and myogenin at *day 3*. The cells were cultured for 3 days with or without VV stimulation. *A*: protein expression of MyoD and myogenin were determined by Western blot analysis. The blots were quantified, and the ratio of myogenin vs. β-actin (*B*) and MyoD vs. β-actin (*C*) were measured and expressed as ratios compared with control cells (0 Hz) for which the ratio was defined as 1. Data are means ± SD of 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ for VV-treated vs. control cells (0 Hz).

VV stimulation in C2C12 myoblasts, confluent cells were stimulated with VV for 3 days and cultured for an additional 3 days (total of 6 days) or 6 days (total of 9 days) to examine myotube formation by immunofluorescence. Immunofluorescence of VV-stimulated C2C12 cells was performed using antibodies directed against the muscle marker sarcomeric myosin (MF-20), which binds to the myosin heavy chain of vertebrate striated muscle cells. Figs. 5*A* and 6*A* show a culture

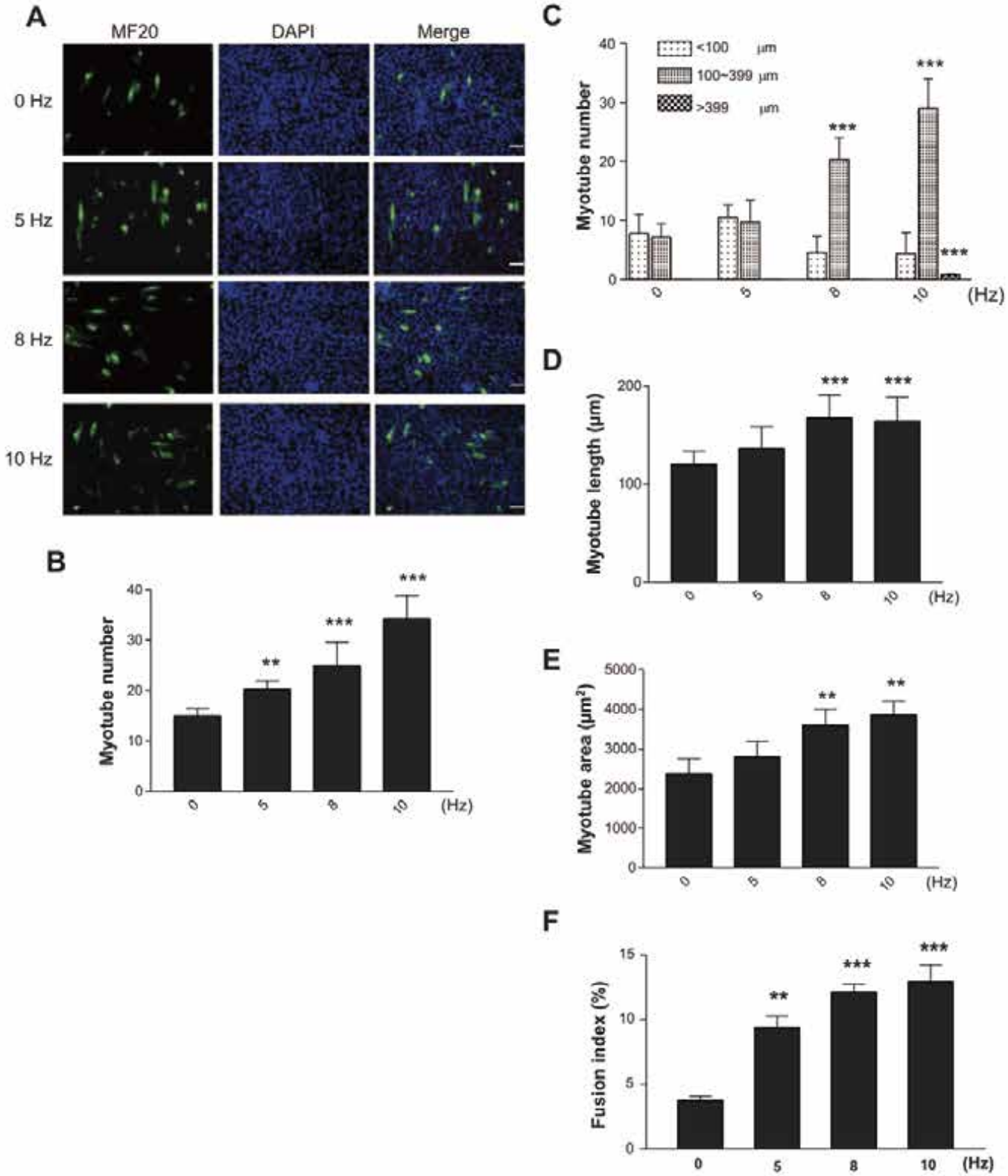

Fig. 5. Effect of VV stimulation on myotube formation at *day 6*. *A*: fluorescence images of myosin-stained C2C12 myoblasts with or without VV stimulation. Green, myosin (MF20); blue, nuclear staining with 4,6-diamidino-2-phenylindole (DAPI). Bar = 100 μm. Quantification of myotube numbers (*B*), the number of myotubes of different lengths (*C*), average myotube length (*D*), average area of individual myotubes stained with MF-20 (*E*) and % fusion index (*F*). Data are means ± SD of 3 independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ for VV-treated vs. control cells (0 Hz).

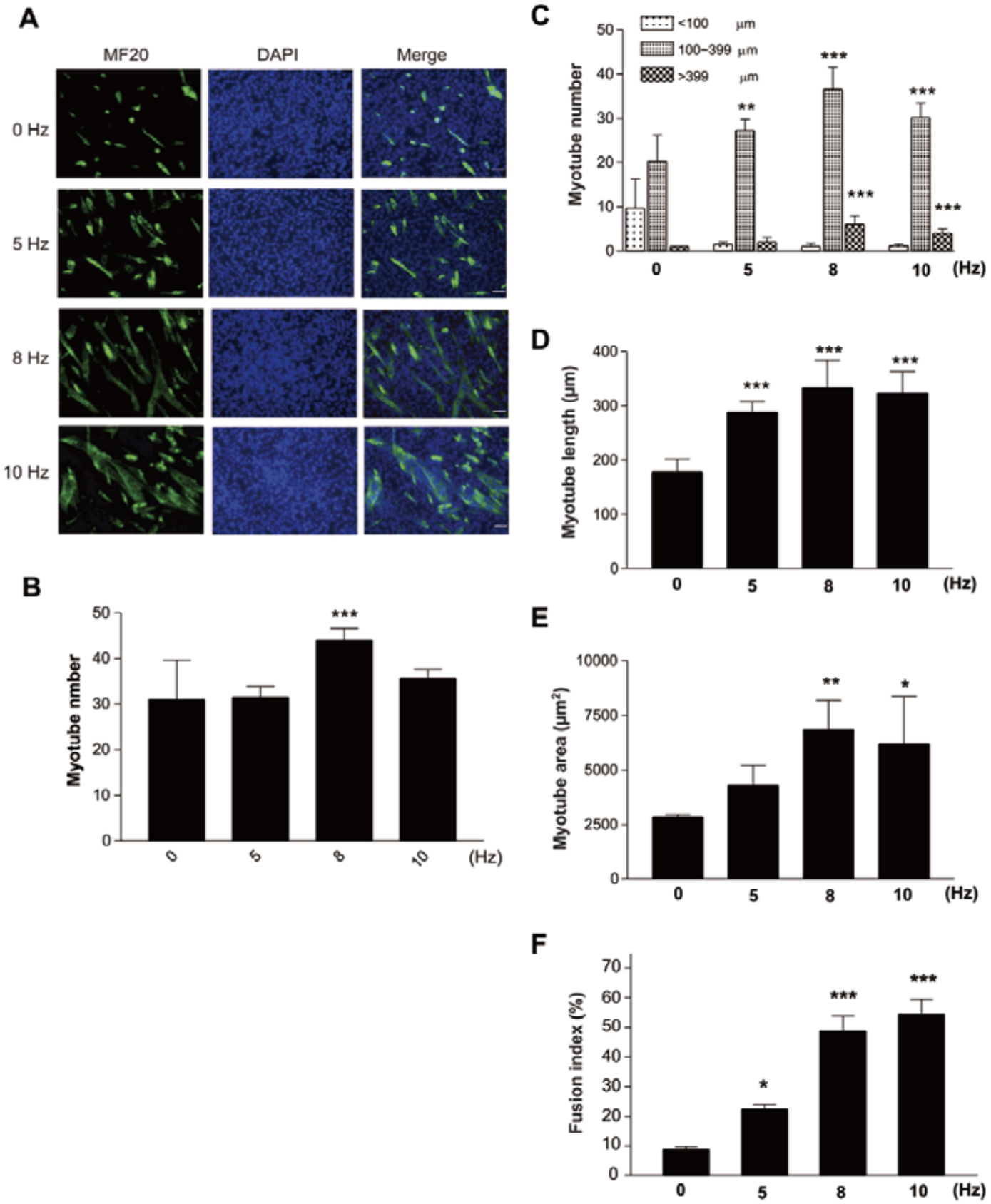

Fig. 6. Effect of VV stimulation on myotube formation at *day 9*. *A*: fluorescence images of myosin-stained C2C12 myoblasts with or without VV stimulation. Green, myosin (MF20); blue, nuclear staining with DAPI. Bar = 100 µm. Quantification of myotube numbers (*B*), number of myotubes of different lengths (*C*), average myotube length (*D*), average area of individual myotubes stained with MF-20 (*E*), and % fusion index (*F*). Data are means ± SD of 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for VV-treated vs. control cells (0 Hz).

of multicellular myotubes with antimyosin staining at *day 6* and *day 9*, respectively. Only myotubes show myosin staining, while undifferentiated myoblasts show little or no myosin staining. The nuclei of all cells are labeled with DAPI. The nuclear (cell) numbers were similar in each VV-treated group, consistent with the cell viability results in Fig. 1*B*.

To assess the inductive effect of VV stimulation on myotube formation in myoblasts, the number, length, fusion index, and area of multinucleated fused myotubes were measured. In the control group (0 Hz), there was minimal myotube formation (in terms of number), and the myotubes were less organized (length and thickness) than those formed in the groups with VV stimulation (5, 8, and 10 Hz) (Figs. 5 and 6). In all VV-treated groups, the majority of the cells cultured for 9 days showed self-assembly of individual myotubes (Fig. 6), which were longer and thicker than those observed at *day 6* (Fig. 5). At *day 6* (Fig. 5*B*), total myotube numbers in the VV-treated groups of 5, 8, and 10 Hz were 1.3-, 2-, and 2.5-times greater (20 ± 1.7, 25 ± 4.6, and 34 ± 4.7, respectively) than that of the control group (0 Hz; 15 ± 1.4). At *day 9* (Fig. 6*B*), total myotube numbers in the VV-stimulated groups of 5, 8, and 10 Hz were 1-, 1.5-, and 1.2-times, greater (32 ± 2.4, 44 ± 2.6, and 36 ± 1.9, respectively) than that of the control group (0 Hz; 31 ± 8.7).

Moreover, the myotubes formed in the 8 and 10 Hz VV groups were not only longer but also thicker than those in the 5 Hz and control groups. At *day 6* (Fig. 5, *C* and *D*), average myotube lengths were 170 ± 22 and 171 ± 26 μm in the 8 and 10 Hz VV-treated groups, respectively, compared with 151 ± 25 μm in the 5 Hz VV-treated group and 135 ± 12 μm in the control (0 Hz) group. At *day 9* (Fig. 6, *C* and *D*), the myotube lengths were 288 ± 19, 333 ± 51, and 324 ± 39 μm in the 5, 8, and 10 Hz VV-treated groups, respectively, which were significantly greater than the length of 178 ± 23 μm measured in the control (0 Hz) group. We also measured the average area of individual myotubes stained with myosin antibody (MF-20). At *day 6* (Fig. 5*E*), the average areas of individual myotubes stained with MF-20 were 3,599 ± 399 and 3,862 ± 348 μm^2 in the 8 and 10 Hz VV-stimulated groups, respectively, which were significantly greater than the areas of 2,800 ± 388 μm^2 in the 5 Hz VV-treated group and 2,383 ± 370 μm^2 in the control (0 Hz) group. At *day 9* (Fig. 6*E*), the average areas of individual myotubes stained with MF-20 were 6,851 ± 1,330 and 6,177 ± 2,179 μm^2 in the 8 and 10 Hz VV-treated groups, respectively, which were significantly greater than the areas of 4,285 ± 891 μm^2 in the 5 Hz VV-treated group and 2,846 ± 110 μm^2 in the control (0 Hz) group. Furthermore, the fusion indices of all of the VV-treated groups (5, 8, and 10 Hz) were significantly higher than that of the control group (Figs. 5*F* and 6*F*). These results indicate that VV stimulation, especially in the 8 and 10 Hz VV-stimulated groups, was able to markedly enhance cell fusion and myotube formation in C2C12 myoblasts.

PI3-kinase inhibitor (LY294002) efficiently suppressed the VV-induced myotube formation. To determine whether VV stimulation promoted myotube formation via regulation of PI3-kinase signaling, we compared the myotube formation in C2C12 cells with (10 Hz) or without (0 Hz) VV treatment using the PI3-kinase inhibitor (LY294002). The results showed that LY294002 efficiently suppressed the VV-induced (10 Hz) myotube formation in C2C12 cells (Figs. 7).

DISCUSSION

Mechanical load is widely used in rehabilitation and sports activities to improve joint flexibility in humans. Many studies are designed to investigate the cellular effects of mechanical load such as magnetic stimulation (52) or flow stress (5). Mechanotransduction from a mechanical load acting on a cell can initiate a sequence of signaling events that leads to changes in transcription, translation, or cell proliferation (37). Mechanical load on skeletal muscle has been shown to increase muscle IGF-I mRNA expression (27). Muscle fiber properties and sarcomere length can also be regulated by different mechanical loads (46). It is thus clear that mechanical stimulation plays an important role in initiating optimal changes in gene expression in myocytes. Previous studies indicate that short bouts of passive stretching, a procedure commonly used to improve joint range-of-motion in humans, increased MyoD gene expression in rat muscle (35, 53). A recent study using C2C12 cells showed that the application of mechanical stretching increased the expression of MyoD and myogenin at early times (12 h) after the stretching and that this effect gradually decreased thereafter (1). The results of our study showed that the application of VV, a type of mechanical load, can promote MRF (MyoD and myogenin) expression in a time- and dose-dependent manner and enhance myogenesis in C2C12 satellite myoblasts. The molecular mechanisms of VV-induced myogenesis in C2C12 cells may include enhancement of the expression of ECM components (type I collagen and decorin) and MRFs (MyoD and myogenin) in myoblasts; these factors may, in turn, increase myotube formation, which may enhance muscle activities (42).

There are various methods of delivering VV to the body, such as via a seesaw, horizontal, or vertical platform (34). The optimal frequency for whole body training varies between studies (9). One recent study has shown that low-magnitude mechanical load with

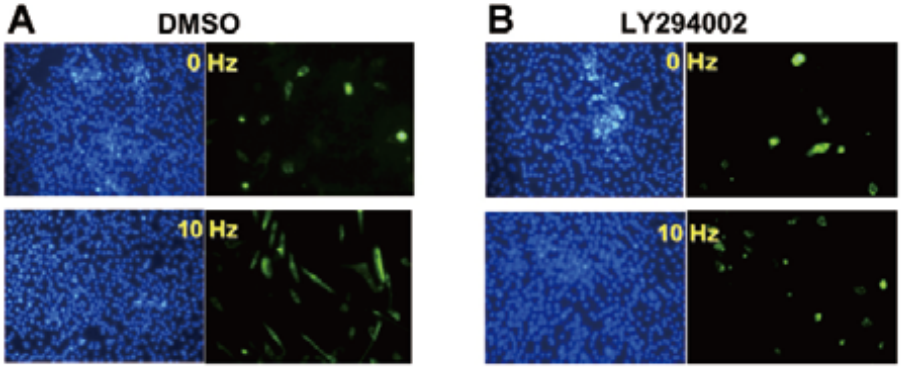

Fig. 7. Effect of the PI3-kinase inhibitor LY294002 on myotube formation in C2C12 cells with (10 Hz) or without (0 Hz) VV treatment. *A*: vehicle control (DMSO) treatment. *B*: LY294002 treatment. Green, myosin (MF20); blue, nuclear staining with DAPI.

a frequency over 30 Hz is effective in osteoporosis (15). Moreover, lower frequencies, from 4–20 Hz, offer increased muscle force, which contributes to spinal stability (4). Another study, using 8 Hz vibration in a training program, showed enhancement in muscle activity and strength (44). Accordingly, we used the VV model with frequencies from 5 to 10 Hz, which provided adequate and balanced transmission of force throughout the target cells for simulating and studying the mechanisms of whole body vibration training on enhancing muscle activity. In this study, we demonstrate that both MyoD and myogenin play important roles in the development and maintenance of striated muscle during myogenesis, with expression levels of both factors increasing in proportion to the number of days in culture and degree of VV stimulation. Furthermore, our results show that type I collagen and decorin mRNA expression is the highest after VV treatment at frequencies of 8 and 10 Hz. Moreover, the most effective frequency of VV treatment for the expression of myoD and myogenin was 10 Hz. The greatest effect of VV stimulation on myotube formation was also seen at frequencies of 8 to 10 Hz. Therefore, the optimal frequency of VV treatment for inducing myogenesis of myoblasts should be between 8 and 10 Hz.

The ECM maintains cell structure and regulates cell behavior through mechanotransduction. Type I collagen is the main structural element of skeletal muscle that enhances myoblast differentiation and myotube formation (21). Type I collagens are commonly used scaffolds for skeletal muscle tissue engineering (11). Collagen scaffolds can increase the transmitted forces of mechanical load on embedded muscle cells during myotube formation (36). In our study, type I collagen synthesis increased after VV treatment, which may increase the transmitted forces of VV on myoblast cells and subsequently enhance myogenesis. Decorin, which is associated with type I collagen, can govern the rate of collagen fibrillogenesis (43). Decorin also promotes myogenesis by enhancing the proliferation and differentiation of myoblasts (29) and accelerates muscle regeneration and repair (24). Our results support this role for decorin in myogenesis and show that decorin expression increases after VV treatment, which can subsequently promote myotube formation of the myoblasts.

MyoD and myogenin, important regulators of myogenic transcription, can activate muscular differentiation of myoblasts into myotubes (10), and regulate the proliferation of muscle satellite cells (54). MyoD and myogenin have been regarded as important regulators through their role in the adaptation of muscle cells to mechanical load (50). The induction of MyoD expression in the context of electrical and mechanical stimulation is sensitive to stimulation at high frequency (23). Consistent with these data, our study shows that VV treatment results in pronounced increases in MyoD and myogenin expression at a frequency of 10 Hz, compared with 5 or 8 Hz, which can subsequently enhance myogenesis of the myoblast.

Myotube formation plays an important role in restoring muscle function. The effects of myotube length, size, and number are related to muscle contraction during gait performance (12, 16). In this study, we demonstrate that VV stimulation positively increases the total number of myotubes that are formed as well as the length and area per myotube. Most notably, the number of myotubes (>100 μm in length) and myotube hypertrophy were increased substantially in the VV groups treated at higher frequencies (i.e., 8 to 10 Hz). Therefore, VV stimulation has positive effects on these myotube characteristics and may promote higher myogenic contractile ability. Recent studies indicate that PI3-kinase-Akt signaling pathway is a crucial regulator of skeletal muscle hypertrophy and myotube formation (6, 22, 26). Our results support this role for PI3-kinase signaling in myogenesis and show that VV-induced myotube formation was suppressed by using the PI3-kinase inhibitor (LY294002). It is tempting to speculate which members of PI3-kinase downstream signaling pathways mediate VV-regulated hypertrophy and myotube formation in myoblasts. Experiments are now underway to investigate the role of Akt in the downstream signaling pathway of PI3-kinase.

In summary, our investigation of the effects of VV on C2C12 myoblasts has shown that the procedure is both safe and effective in stimulating myogenesis. Our results demonstrate that a low frequency of VV can increase myotube formation, expression of the MRFs MyoD and myogenin, and expression of the ECM components type I collagen and decorin. While many clinical trials have already demonstrated positive results from VV stimulation in various populations, this study provides some insight into how these clinically observed increases in muscle strength and balance occur. Nevertheless, the in vitro model of C2C12 satellite myoblasts that we used may not completely reflect the true physiology of regenerating muscle. Further research using primary satellite cells or an in vivo animal model is required to determine the extent of these molecular effects over a greater range of VV frequencies and their implications for clinical use.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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