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Abstract

The aim of this study was to investigate the effect of adding whole-body vibration (WBV; frequency = 35 to 40 Hz; amplitude = 4 mm) to squat training on the T-cell proliferative response of elderly patients with osteoarthritis (OA) of the knee. This study was a randomized controlled trial in which the selected variables were assessed before and after 12 weeks of training. Twenty-six subjects (72 ± 5 years of age) were divided into three groups: 1) squat training with WBV (WBV, N = 8); 2) squat training without WBV (N = 10), and 3) a control group (N = 8). Women who were ≥60 years of age and had been diagnosed with OA in at least one knee were eligible. The intervention consisted of 12 uninterrupted weeks of squatting exercise training performed 3 times/week. Peripheral blood mononuclear cells were obtained from peripheral blood collected before and after training. The proliferation of TCD4$^+$ and TCD8$^+$ cells was evaluated by flow cytometry measuring the carboxyfluorescein succinimidyl ester fluorescence decay before and after the intervention (Δ). The proliferative response of TCD4$^+$ cells (P = 0.02, effect size = 1.0) showed a significant decrease (23%) in the WBV group compared to the control group, while there was no difference between groups regarding the proliferative response of TCD8$^+$ cells (P = 0.12, effect size = 2.23). The data suggest that the addition of WBV to squat exercise training might modulate T-cell-mediated immunity, minimizing or slowing disease progression in elderly patients with OA of the knee.

Key words: Vibratory platform; Lymphocyte; Proliferation; Elderly; Osteoarthritis

Introduction

Osteoarthritis (OA) is a chronic, progressive and degenerative osteoarticular disease characterized by arthralgia, stiffness and limitations of articular function. OA most commonly occurs in the knee (1). The etiology of OA involves biomechanical, biochemical and genetic factors that contribute to the imbalance between the synthesis and destruction of articular cartilage (2). The degradation of the articular cartilage initiates an inflammatory process in which cytokine production acts as both a causative agent and a consequence of the disease (2).

Although OA has been considered to be a local form of joint inflammation by many researchers, there is now evidence indicating the involvement of a systemic inflammatory response exhibited by T cells and the presence of inflammatory markers in peripheral blood, including inflammatory cytokines and antibodies (3). Therefore, osteoarthritis has been associated with an exacerbation of the inflammatory response and inflammation,

which seems to be an important factor contributing to the change in the function of chondrocytes. This favors the development of an imbalance between anabolic and catabolic activities involved in the remodeling of the extracellular matrix of cartilage (4). Moreover, the presence of activated T cells and cytokine transcripts in chronic joint lesions of patients with OA suggests that T cells contribute to chronic inflammation in a large proportion of these patients (5).

Recent studies have documented that the cellular components of the OA synovium include activated T and B cells as well as monocytes and macrophages, which are thought to be immunocompetent (5). The literature reports an increase in the proliferative response of mononuclear cells under inflammatory conditions, such as OA (6). As reported by Sakata et al. (7), the proliferative T-cell response in OA is greater compared to controls, and the TCD4$^+$ cells, which play critical roles in producing the proinflammatory cytokines that contribute to the degradation of articular cartilage, are involved in this augmented proliferative response (7).

Although the process of articular cartilage degradation is irreversible, non-pharmacological interventions have been indicated to relieve the signs and symptoms of the disease and to delay its progression. Whole-body vibration (WBV) training has been recommended as an efficient and alternative method for improving muscle strength and proprioception in volunteers with knee OA (8,9).

During this training modality, the individual stands on a platform that generates vertical sinusoidal vibrations. These mechanical stimuli are transmitted to the body to stimulate the primary endings of the muscle spindles, which in turn activate α-motor neurons, resulting in muscle contractions comparable to the tonic vibration reflex (10). Recently, our group demonstrated improvement in the functionality and the self-perception of the disease status in OA patients after a 12-week training program in which WBV was combined with squatting exercise (11), as well as improvements in the inflammatory marker profiles of elderly patients with knee OA, with reduction in the plasma concentration of soluble tumor necrosis factor receptor 1 (sTNFR1) and sTNFR2 (12). However, to the best of our knowledge, no study has investigated the effect of this modality of intervention on the proliferative response of T cells in elderly patients with knee OA.

Because activated TCD4$^+$ cells may play critical roles in cartilage degradation in OA (7), it is believed that the addition of WBV to the squat exercises could reduce the proliferative response of TCD4$^+$ cells since this training modality improves functionality and the self-perception of the disease status in OA patients (11), and also improves their inflammatory marker profiles (12). Therefore, the aim of the present study was to investigate the effect of adding WBV to squat training on the T-cell proliferative response of elderly subjects with knee OA.

Material and Methods

This was a randomized controlled study in which the variables were assessed 48 h before and after a 12-week training program. For allocation of the participants to groups, a 1:1 ratio of randomization was applied. To minimize the chance of bias, we used opaque envelopes that were sealed and serially numbered. The envelopes were opened sequentially after the participant's name and further details were written on each envelope, and they were kept in a locked and secure place. The allocation sequence was concealed from the researcher who enrolled and assessed the participants. Only one researcher involved in the randomization was aware of the group assignments. The study was approved by the Internal Review Board of the Universidade Federal dos Vales do Jequitinhonha e Mucuri.

The sample for this study consisted of elderly volunteers from the community, who lived in the city of Diamantina, MG, Brazil, and were sedentary according to the International Physical Activity Questionnaire (IPAQ) (13). The volunteers were recruited through the physiotherapy clinic of the Universidade Federal dos Vales do Jequitinhonha e Mucuri and through medical referrals. To participate in the study, volunteers were required to meet the following inclusion criteria: age ≥60 years and a diagnosis of OA in at least one knee according to clinical and radiographic criteria of the American College of Rheumatology (14). The severity of OA was classified radiographically according to the Kellgren and Lawrence scale (15) (grades 0-4, with 0 being normal and 4 representing severe OA). Grade 2 (definite osteophytes and the possible narrowing of the joint space) was set as a cutoff to classify OA of the knee in our sample population. The Kellgren and Lawrence criteria were absence of any recent knee injury, no requirement for a walking aid, self-report of not having been submitted to any rehabilitation procedure in the previous three months, no regular (twice per week or more) use of aspirin or other nonsteroidal anti-inflammatory medications, and no use of corticosteroids or other medications known to affect immune function (15,16). Volunteers were excluded if they had any orthopedic, neurological, respiratory, or acute cardiac diseases that would interfere with the study or if they had any cognitive deficit demonstrated by the Mini-Mental Status Examination (17). A radiological evaluation was also performed to verify the OA diagnosis (18).

Of the 125 elderly individuals screened for eligibility, 26 satisfied the inclusion and exclusion criteria and were randomly divided into three groups: 1) the vibration group (WBV) in which the participants performed squatting exercises in addition to whole-body vibration (N = 8), 2) the exercise group (EXE) in which the participants performed squatting exercises without vibration (N = 10), and 3) the control group (CON; N = 8) in which the participants did not receive training and were instructed not to change their lifestyle during the study or engage in any new type

of physical activity. To ensure the retention of patients in the control group, weekly phone calls were made to each group member to confirm her routine activities.

Clinical and demographic data were collected from the participants in both groups (WBV and EXE) using an evaluation chart. All of the study patients (WBV, EXE, CON) underwent a clinical evaluation and blood sample collection 48 h prior to the initiation of the 12-week intervention program for the platform and squat groups. The blood samples were always collected at 8:00 am, followed by clinical testing. At the end of the training period, the volunteers in each of the three groups were reassessed. Peripheral blood was collected from the cubital vein at the beginning of the study and at the end of 12 continuous weeks of intervention to assess the proliferation of T lymphocytes. The intervention program consisted of performing squat exercise training with WBV or without WBV 3 times/week on alternate days.

Warm-up

Prior to each training session, both groups (WBV and EXE) completed a warm-up on a stationary cycle (Stone Fitness, 2001) at 70% of the predicted maximum heart rate based on each subject's age; the heart rate was monitored using a Polar heart rate monitor (model F4) for 10 min. Immediately afterwards, the participants in the WBV group were positioned with their feet 28 cm apart (14 cm to the right and 14 cm to the left of the center of the vibration stimulus), ready to begin the squat exercises on the vibratory platform, while the participants in the EXE group performed the same procedure without vibration.

Squat exercises

The structured program of squat exercises on the platform and squat groups was conducted 3 times/week, on alternate days, for 12 weeks. The squat exercise was performed starting at approximately 10° of knee flexion and continuing until 60° of knee flexion was reached. For each volunteer, a knee angle of 60° was measured before the series of exercises, and a barrier was imposed on the buttocks to limit the degree of knee flexion.

The parameters of the vibration in the platform group were based on the principles of training load progression: the frequency ranged from 35 to 40 Hz, the amplitude was 4 mm and the acceleration ranged from 2.78 to 3.26 g. The choice of vibratory frequencies and the amplitude were set so that we obtained an acceleration range between 2 and 5 g, which was suggested to be sufficient for achieving physiological effects by Delecluse et al. (19). Prior to data collection, the acceleration values of the platform were measured using the Mega accelerometer (b) (ZPP1-3D-BC, Acceleration Measuring Kit, Kuopio, Finland).

The volume load of squat training for the squat and platform groups was increased systematically during the 12-week intervention by increasing the time and number of sets (6 sets x 20 s to 8 sets x 40 s) and reducing the resting

time (40 to 25 s) (11). For patients in the platform group, the vibration acceleration was also increased by varying the vibratory frequency (35-40 Hz).

Whole-body vibration

For the volunteers in the WBV group, a commercially available vibration platform was used (FitVibe, GymnaUniphy NV, Belgium) that produces vertical synchronous vibration, causing vibration in both legs while the platform moves predominately in the vertical direction. This results in simultaneous and symmetrical movement of both sides of the body during exposure (20).

The overload used in the present study was only body weight. However, to ensure control and progressive training, a gradual increase in volume (increase in exercise time of sets and decrease in rest intervals between sets) and intensity (acceleration was increased by varying the vibration frequency from 35 to 40 Hz) was used during the 12 weeks of intervention.

Lymphocyte proliferative response

To evaluate the effect of training on the T-cell proliferative response, a blood sample (approximately 10 mL) was collected into heparinized tubes from each volunteer 48 h before training and 48 h after the last training session. Blood collection was performed between 7:30 and 8:30 am after a 12-h fast using the following stringent blood-draw criteria: no reported infection or symptoms of infection for 7 days, adequate sleep (6-9 h), no exercise or alcohol use for 24 h, no topical corticosteroid use or aspirin for 48 h, no systemic antihistamines or corticosteroid use for 1 week, and no immunizations during the previous 3 weeks.

Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation using Ficoll-Hypaque (Sigma, USA), as described by Bicalho et al. (21). Next, 1×10^7 cells/mL were suspended in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (PBS/BSA, 0.1%) and labeled with carboxyfluorescein succinimidyl ester (CFSE, 10 μM), as described by Lyons et al. (22). The cells were washed and resuspended in RPMI supplemented with 2 mM L-glutamine, antibiotic/antimycotic solution (100 IU/mL penicillin G, 100 μg/mL streptomycin and 2.5 μg/mL fungizone) and 10% fetal calf serum. The cells were stimulated with the mitogen phytohemagglutinin (PHA, 1 μg/mL) for 5 days at 37°C and 5% CO_2. After the culture period, the cells were collected, washed in PBS and incubated with monoclonal antibodies that were specific for human CD4 and CD8 and conjugated with phycoerythrin (PE) and Cy-Chrome (CY), as described by Openshaw et al. (23).

Labeled cells were then analyzed by flow cytometry (FACScan, Becton Dickinson, USA) using the region of small lymphocytes and blast cells (R1) according to the profile of size and granularity [forward scatter (FSC) x side scatter (SSC)]. The fluorescence of CFSE was measured on the FL1 channel, CD4-PE on the FL2 channel and CD8-

CY on the FL3 channel. The compensation and gain settings were determined for each experiment based on unstained cells and cells labeled with only one fluorescent dye. Thirty-thousand events in the R1 region were acquired for later analysis using the Cell Quest software (Becton Dickinson).

The proliferative index was then calculated from the data in the histograms using the following formula (24), considering that two cells with a given CFSE intensity emerged from the mitotic division of a single cell possessing a CFSE intensity immediately above: proliferative index = $(100 - Y) / Y$, where Y (%) = $X0 + X1/2 + X2/4 + X3/8 + X4/16 + X5/32 + X6/64 + X7/128$; $X0$ represents the percentage of T cells that did not divide (located in M1), and $X1-7$ represents the peak of gradual division (located in M2 to M7).

Statistical analysis

The SPSS statistical software, version 18.0, was used for the statistical analysis. P values ≤0.05 were considered to be statistically significant. We performed the analysis between groups by variation (Δ) within each group, where post-value - pre-value = delta value (Δ). The Shapiro-Wilk test was used to evaluate the normalcy of the data [delta value (Δ)]. Because the data were normally distributed for the dependent variables, parametric tests were used for the statistical analysis. The differences between groups were tested by ANOVA. The Tukey *post hoc* test was used to verify the differences between the conditions.

To check the size of the differences between the pre- and post-training periods and between groups, we analyzed the magnitude of the effects (25,26).

Sample size

The sample size necessary to detect a difference between groups of PBMCs with a proliferation index of 6.06 and a standard deviation value of 1.87 (8) was calculated considering a significance level of 0.05 and statistical power of 0.8. We calculated that at least 2 participants would be required in each group.

For the dependent variable (lymphocyte proliferative response), the statistical power of the study was calculated from the results of the magnitude of the effect of the deltas obtained from comparison of the results before and after training (26,27). For comparison between groups, the magnitude of the effect was high for both TCD4^{+} (Cohen's f =
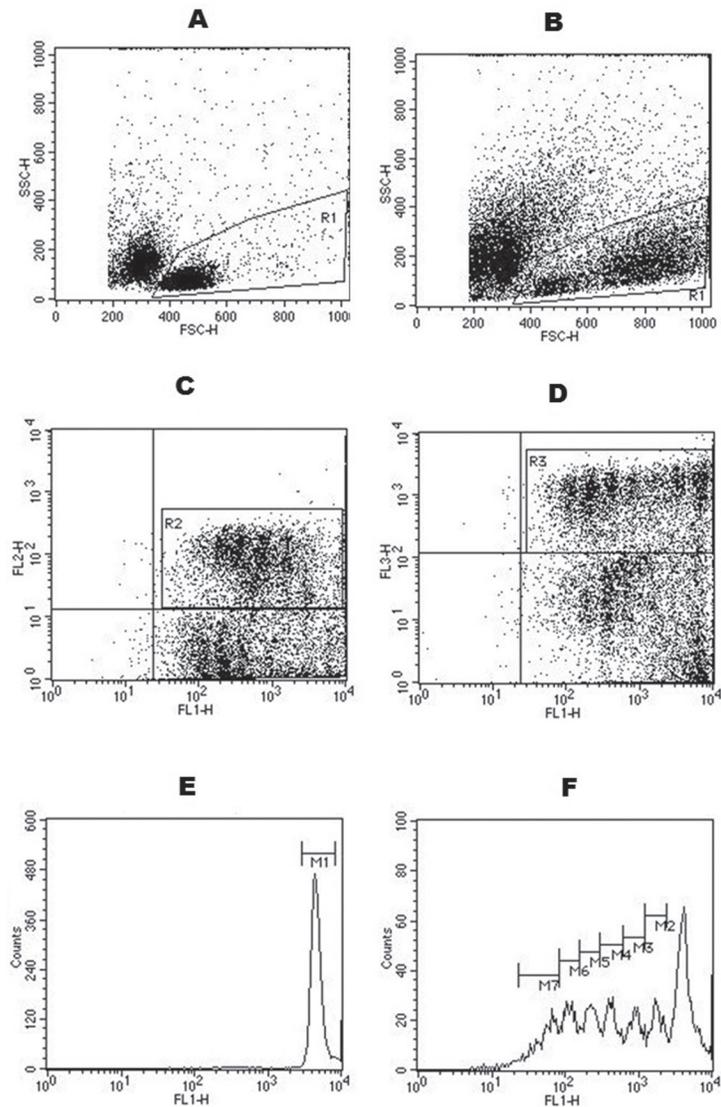

Figure 1. Analysis of cell proliferation using carboxyfluorescein succinimidyl ester (CFSE) in flow cytometry. *A*, Control for unstimulated cultures; *B*, phytohemagglutinin (PHA)-stimulated cultures; *C*, CFSE x CD4; *D*, CFSE x CD8; *E*, CFSE-stained cells derived from unstimulated cultures; *F*, CFSE-stained cells in cultures stimulated with PHA. SSC and FSC = side and forward scatter, respectively.

1.00) and TCD8^{+} cells (Cohen's f = 2.23), indicating that the study had a statistical power above 85% for these variables, and the minimum sample size to obtain a statistical power of 80% is 5 individuals per group (27).

Results

To analyze cell proliferation using CSFE in flow cytometry, the R1 region was defined by dot plots in graphs of FSC x SSC and comprised lymphocytes and blast cells to

control for unstimulated (Figure 1A) and PHA-stimulated cultures (Figure 1B). The events in R1 were then analyzed for fluorescence from dot plots of FL1 x FL2 (CFSE x CD4) (Figure 1C) and FL1 x FL3 (CFSE x CD8, Figure 1D), from which regions R2 and R3 were defined, which included the CFSE$^+$CD4$^+$ cells and the CFSE$^+$CD8$^+$ cells, respectively. The events in R1, R2 and R3 were sequentially analyzed for CFSE fluorescence intensity using histograms (Figure 1E and F). The M1 region was defined as a region of CFSE-stained cells derived from unstimulated cultures, which represents the peak of quiescent cells (Figure 1E). The M2 to M7 regions were defined according to the peaks of different CFSE intensities in cultures stimulated with PHA (Figure 1F).

There was no significant difference between groups with respect to mean age and anthropometric data. No significant differences were found in the data collected prior to training between groups, thus confirming the baseline homogeneity of the groups (Table 1).

The variation (Δ) in the proliferative response of TCD4$^+$ cells (P = 0.02, effect size = 1.00) was significantly reduced in the WBV group compared to the CON group (Figure 2A), while there was no difference between groups regarding the variation

(Δ) in the proliferative response of TCD8$^+$ cells (P = 0.12, effect size = 2.23; Figure 2B).

Discussion

In this study, we investigated the effect of adding WBV to squat training on the T-cell proliferative response in elderly subjects with knee OA, since OA is a chronic inflammatory disease and, as we have previously demonstrated (11), WBV improves the clinical condition of OA patients. The findings

Table 1. Sample characterization and baseline variables of the whole-body vibration group (WBV), exercise group (EXE) and control group (CON).

Characteristics	WBV (N = 8)	EXE (N = 10)	CON (N = 8)
Age (years)	75 ± 7	71 ± 4	72 ± 6
Body mass (kg)	72.75 ± 11.41	74.24 ± 9.95	63.71 ± 10.15
Height (m)	1.58 ± 0.05	1.57 ± 0.08	1.55 ± 0.08
Index of TCD4$^+$ cells proliferation	3.20 ± 0.96	2.46 ± 1.22	1.42 ± 1.00
Index of TCD8$^+$ cells proliferation	2.40 ± 1.28	2.79 ± 2.31	1.95 ± 1.42
Grade 2 (%)	20	20	37.5
Grade 3 (%)	30	30	37.5
Grade 4 (%)	50	50	25

Grades 2, 3, and 4 = severity of knee osteoarthritis in percent. Data are reported as means ± SD. There were no statistically significant differences among groups for any parameter (ANOVA).
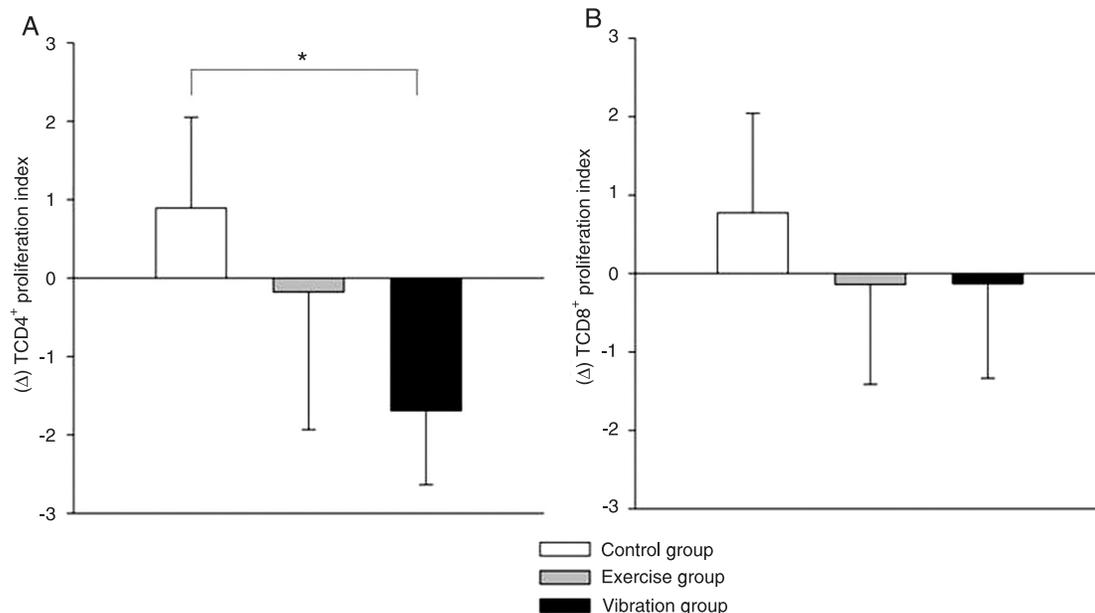

Figure 2. Variation of the proliferation index (Δ) of *A*, TCD4$^+$ and *B*, TCD8$^+$ cells before and after exercise. Δ is reported for the vibration group (N = 8), exercise group (N = 10) and control group (N = 8). Data are reported as means ± SD. *P ≤ 0.05 compared to control (ANOVA).

of the present investigation demonstrated that the addition of WBV to squat exercise training reduces the proliferative response of TCD4^{+} cells but not the number of TCD8^{+} cells in elderly subjects with knee OA. As we did not find any difference in the proliferative response of TCD4^{+} cells between the squat exercise and control groups, we conclude that the addition of WBV to squat exercises training is necessary to provide an additional overload to squat exercises.

Although OA has been considered to be primarily a disease of biomechanical alterations (28), accumulating evidence supports the involvement of the inflammatory response in the pathogenesis of the disease, with TCD4^{+} cells playing an important role in this process (5). Because chondrocytes respond to cytokines and chemokines present in the synovial fluid and joint tissues, activated TCD4^{+} cells infiltrate the synovial membrane of patients with OA (5,29) and secrete inflammatory cytokines, such as IFN-α and IL-2 (30,31), which directly or indirectly contribute to the ongoing inflammation and destruction of joint cartilage and decrease the anabolic activity of the joint. Considering this, we hypothesized that the addition of WBV to squat exercise training could minimize or slow disease progression in elderly patients with OA of the knee because activated TCD4^{+} cells may play critical roles in inflammation and cartilage degradation in OA and seem to be directly involved in the immune response to autologous chondrocyte antigens (7). In this context, our group has previously reported that WBV training improves balance, gait performance and self-reported pain and reduces the serum levels of the soluble forms of the TNF-α receptors sTNFR1 and sTNFR2 (12), suggesting that this modality of training can improve the inflammatory control of OA, thus allowing clinical improvement. The reduced proliferative response of TCD4^{+} cells in OA patients following WBV training reported in the present study further supports our hypothesis.

WBV training did not modulate the proliferative response of TCD8^{+} cells in OA patients. The role of TCD8^{+} cells in the pathogenesis of OA is less well understood than that of TCD4^{+} cells, although TCD8^{+} cells have been observed in the synovial membranes of OA patients (31), and some investigators have suggested that these cells may exert cytotoxic activities against chondrocytes (8), thus contributing to the destruction of joint cartilage. However, Kummer et al. (32) reported that up to 75% of the granzyme-positive synovial lymphocytes are natural killer cells, and only less

than 5% are CD3^{+}CD8^{+} cytotoxic cells, suggesting that natural killer (NK), and not TCD8^{+} cells, may have some role in cytotoxic cartilage destruction in OA. Although WBV training did not modulate the *in vitro* TCD8^{+} cell proliferative response, it is possible that the lower TCD4^{+} cell response may also attenuate the cytotoxic response of TCD8^{+} cells, considering the role that TCD4^{+} cells play in the development of the primary TCD8^{+} cell response (33). This possibility should be experimentally investigated.

To the best of our knowledge, this is the first randomized study to investigate the effect of the addition of WVB to squat exercise training on the proliferative response of T cells in elderly subjects with OA of the knee. We cannot say whether the effect of WBV on the proliferative response of T lymphocytes from patients with OA reported here results from a direct modulation of T-cell function by vibration or if this modulation is secondary to an improvement in neuromuscular properties. Better neuromuscular control could result in greater joint stabilization, so that the shear stress on the cartilage would be smaller, resulting in reduced production of proinflammatory cytokines and reduced stimulation of T lymphocytes, a possibility that, however, has not yet been properly investigated.

The present study has several limitations, and the results must be interpreted within the context of its design. It is important to note that specific frequencies and amplitudes were used; therefore, the results cannot be extrapolated to other parameters of vibration. Furthermore, it should be emphasized that PBMCs, rather than local inflammatory cells, were used. Moreover, PHA is a general mitogen that stimulates the proliferation of a wide variety of lymphocyte clones, which could mask the responses of those clones specifically involved in the immune response to OA.

Since the addition of WBV to squat exercise training decreases the proliferative response of TCD4^{+} cells in elderly subjects with OA of the knee, it is believed that this modality of training might modulate T-cell-mediated immunity in this population, minimizing or slowing the disease progression in elderly patients.

Acknowledgments

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