Original Article

Blood flow restriction during the resting periods of high-intensity resistance training does not alter performance but decreases MIR-1 and MIR-133A levels in human skeletal muscle

Ferenc Torma [a], Peter Bakonyi [a], Zsolt Regdon [b], Zoltan Gombos [a], Matyas Jokai [a], Gergely Babszki [a], Marcell Fridvalszki [a], Laszló Virág [b], Hisashi Naito [c], Syed.Rehan Iftikhar Bukhari [d], Zsolt Radak [a,e,*]

[a] Research Center of Molecular Exercise Science, University of Physical Education, Budapest, Hungary
[b] Department of Medical Chemistry, Faculty of Medicine, University of Debrecen, Hungary
[c] Department of Exercise Physiology, Graduate School of Health and Sports Science & Medicine, Juntendo University, Japan
[d] Doctoral School of Clinical Medicine, Semmelweis University, Budapest, Hungary
[e] Faculty of Sport Sciences, Waseda University, Japan

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ABSTRACT

Blood flow restriction (BFR) during exercise bouts has been used to induce hypertrophy of skeletal muscle, even with low loads. However, the effects of BFR during the rest periods between sets are not known. We have tested the hypothesis that BFR during rest periods between sets of high-intensity resistance training would enhance performance. Twenty-two young adult male university students were recruited for the current study, with $n = 11$ assigned to BFR and $n = 11$ to a control group. The results revealed that four weeks training at 70% of 1 RM, five sets and 10 repetitions, three times a week with and without BFR, resulted in similar progress in maximal strength and in the number of maximal repetitions. The miR-1 and miR-133a decreased significantly in the vastus lateralis muscle of BFR group compared to the group without BFR, while no significant differences in the levels of miR133b, miR206, miR486, and miR499 were found between groups. In conclusion, it seems that BFR restrictions during rest periods of high-intensity resistance training, do not provide benefit for enhanced performance after a four-week training program. However, BFR-induced downregulation of miR-1 and miR-133a might cause different adaptive responses of skeletal muscle to high intensity resistance training.

Introduction

Skeletal muscle is the most abundant tissue in the human body, accounting for about 60% of the total protein content and 40% of body mass. Although it plays an important metabolic role by picking up circulating carbohydrate, or regulating immune function via myokine production,[1] its contracting ability is the most unique among organs. Indeed, muscle mass and function strongly limit human physical performance. Resistance training is one of the most widely used methods to improve performance or reduce the age-associated decline in muscle function.[2] Although most studies suggest that resistance training with loads around 70% of one repetition maximum (1 RM) is most appropriate to increase muscle hypertrophy,[3] it is clear that aerobic endurance training can also cause increased protein synthesis in skeletal muscle and

lead to hypertrophy.[4] Indeed, wide range approaches have been reported to increase muscle mass and function, which are dependent on endogenous components such as genetic, epigenetic, and exogenous factors like nutrition, intensity, duration, or the nature of the resting periods.[5]

Blood flow restriction training (BFR) is used to increase muscle performance, especially to enhance exercise-induced neural adaptation,[6] and muscle mass,[7] but it is also applied to improve endurance capacity.[8] Blood flow is restricted by cuff pressure, which is maintained during exercise and rest periods between sets. BFR during resistance training with low intensity appears to have similar effects as high intensity resistance training.[9] However, low intensity training cannot recruit fast twitch fibers, which activation is important in all dynamic sports. Therefore, it could be useful for those who have limitations, to engage in lifting high loads. BFR affects central hemodynamic parameters.[10] It has

been reported that BRF with maintained occlusion pressure during rest periods, could lead to increased brachial systolic blood pressure.[11] We have reported that occlusion during rest periods of high intensity resistance training (70% of 1RM) resulted in decreases in the levels of microRNA-206 and increases in PAX7 content in the vastus lateralis muscle of the BFR leg after acute bouts of training.[12] However, the effects of BFR during rest periods of chronic high intensity resistance training are not known.

In those protocols when the occlusion cuff is inflated and deflated, skeletal muscle is subjected to ischemia/reperfusion periods, which leads to increase generation of reactive oxygen species (ROS).[13] Oxidative challenge is controlled by complex antioxidant and oxidative damage repair mechanisms, and microRNA-s (miRs), which are a unique subset of noncoding RNA and are also involved in the regulation of oxidative stress.[14] In addition, it has been shown that miRs are modulated by acute BFR[12] and they also play an important role in muscle hypertrophy[14], on the other hand, it is not known, whether the adaptation to chronic resistance training with BFR would involve the modulation of miRs.

Therefore, in the present investigation, we were interested in the effects of high-intensity resistance training (70% of 1 RM) with BFR during rest periods on the gain of maximal and submaximal strength and on the levels of skeletal muscle generated miRs.

Materials and methods

Participants

Twenty-two young men volunteered for the study (see Table 1 for characteristics). The participants were randomly allocated into two equal groups: blood flow restricted (BFR) and control (C). All volunteers were informed about the purpose of the study and detailed written information was provided about the study procedures. Before each participant was enrolled a medical checkup was conducted and a medical questionnaire was filled out to rule out any cardiological, neurological, metabolic, or musculoskeletal abnormalities. Participants were non-smokers, but active university students. During the four weeks of BFR training and the one week prior to and after the program, participants were asked to avoid additional resistance training and consumption of any dietary supplements, but they were encouraged to continue their normal dietary patterns. All procedures were approved by the Ethical Committee of the University of Physical Education (ET-KEB/No8/2017), and the research was conducted according to the standards of the Declaration of Helsinki.

Initial testing and conditioning

All participants were familiar with the squat exercise, but for additional safety and for performance measurement purposes the exercise was performed in a squatting frame where the applied resistance travelled in a fixed vertical path. Before the training session, participants were subjected to a 3RM test to set the training resistance. Three -repetition-maximum was tested according to the National Strength and Conditioning Association (USA) guidelines. Briefly, before the test, a 10 min warm-up session was performed on a cycle ergometer. During the testing phase, participants performed ten repetitions with a resistance corresponding to half of their body weight. This was followed by one set of 4–6 reps with full body weight. In the final block, volunteers had four trials to reach the 3RM that was defined as 90–93% of the 1RM.[15] The

load was gradually increased and a minimum of two and a maximum of 5 min rest periods were permitted between trials. A repetition was accepted as successful if no external help was needed to move the weight or if the upper thigh had reached a position parallel to the ground during each squat.

Four days before the first training session and three days after the last muscle biopsy, 3RM was measured. After two days of recovery, a power endurance test was applied in which an all-out squat effort with 70% of 1RM was measured. These tests were performed before, and after the four week training sessions (Fig. 1).

Exercise training and blood flow restriction

The training period lasted four weeks with three training sessions per week. This exercise protocol has been shown to increase the performance of BFR training[16] and miRs are modulated even after a single bout of exercise.[17,18] Prior to the resistance exercise, 20 min of cycling on an ergometer was applied as a warm-up. During the exercise sessions, participants performed five sets of squats with ten repetitions at 70% of 1RM with a warm-up session with 30% and 50% of the 1RM. Sixty seconds of BFR was applied between sets during the 2 min rest periods, including all 30%, 50% and 70% sessions. The occlusion cuff (11-cm-wide tourniquet #8; Mizuho, Tokyo, Japan) was put on bilaterally at the upper part of the thigh until the cuff reached the gluteal fold. The cuff pressure was set to 220–230 mmHg.[19] During the rest periods participants stayed in a semi-horizontal position on a bench with a 45° inclination, with outstretched lower limbs. Both the occlusion and the control groups received the same 70% 1RM load, but vascular occlusion was not applied to the control group.

During the exercise sessions, individual performance was monitored
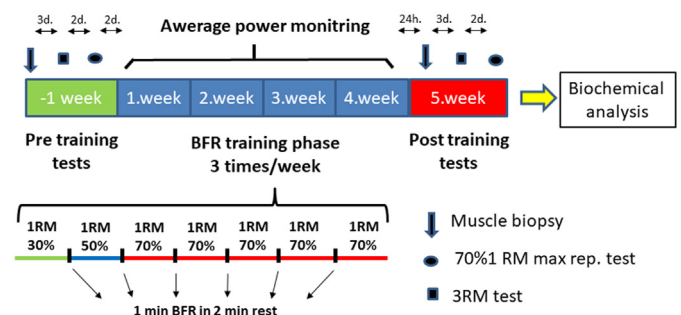
Study layout

Fig. 1. The layout of the study.
The protocol of the study is summarized in this figure. One week prior to the first BFR exercise, muscle biopsy samples were taken (black arrow). Three days following the biopsy participants conducted a 3RM test (black rectangle), two days afterwards a 70% 1RM test (black circle) was performed. After two days of recovery subjects started the BRF protocol (details in the text). Twenty four hours after the last BFR exercise session muscle biopsy samples were taken, followed by functional tests with similar timing to the pre-workout period. Abbreviations: d. = day, h. = hours.

Table 1
Age, weight, height and 70% of 1 RM of Sample characteristics.

Sample Group	Sample Size	Age (Years)	Weight (kg)	Height [cm]	Work Load (kg)
Control mean ± SD	11	23.9 ± 1.7	77 ± 8.9	182.3 ± 7.8	82.2 ± 18.6
BFR mean ± SD	11	24.1 ± 6.1	81.3 ± 6	184.5 ± 7.2	86.7 ± 12.9

Abbreviation: SD = standard deviation.

by measuring vertical power output with a linear encoder (MuscleLab Power, Ergotest, Norway). Average power was defined as the average power output during the 70%RM sets.

Muscle microbiopsy

The first muscle micro biopsy for investigating initial conditions was taken 72 h before the first physical performance test; the second sample, representing the post-training condition, was taken 24 h after the last exercise session. Subjects arrived at the laboratory in a fasted state and had been instructed to avoid coffee and other beverage consumption except for water. Muscle micro biopsies were taken with a semiautomatic needle (EASY-RAM 14Gauge $\times$ 100 mm (Length) using local analgesia (20 mg/mL lidocaine-hydrochloride; EGIS, Budapest, Hungary) from the right vastus lateralis muscle at the site located approximately two-thirds of the distance below the anterior superior iliac spine and the upper margin of the patella. Muscle samples were quickly rinsed with PBS pH 7.4 and frozen with liquid nitrogen. Samples were kept at $-80\,^\circ$C until RNA extraction.

MicroRNA

Muscle RNA content was extracted by using Trizol reagents (TRI Reagent®: TR 118, MRC Inc., Cincinnati, OH, USA) from 5 to 10 mg muscle tissue sample. RNA purity was assessed by absorbance at 260 nm and 280 nm. Extracted RNA with greater than a 1.8 ratio were utilized for subsequent procedures and analysis. Total RNA (10 ng) was reverse transcribed by the MicroRNA Reverse Transcription Kit (TaqMan™ Advanced miRNA cDNA Synthesis Kit: A28007, Applied Biosystems, Foster City, CA, USA). Each target miRNA was quantified according to the manufacturer's protocol with minor modifications. TaqMan Micro-RNA Assays were used as follows: miR-486, miR-499, miR-206, miR1, miR-133a, and miR-233b. All materials were supplied by Applied Biosystems (TaqMan™ Advanced miRNA Assay: A25576, Applied Biosystems). qRT-PCR was performed using TaqMan Fast Universal PCR Master Mix (Cat#: 4366072, Applied Biosystems) on a 7500 Fast Real-Time PCR System according to the manufacturer's protocol. All samples were run in triplicate and normalized as described earlier.[20] Briefly, miR-191 was used as a normalizer in each sample and the target miR expression levels were calculated by the $\Delta\Delta$CT method.

Statistics

For statistical analysis, the Statistica 13 program (TIBCO 13.4.0.14) was used. Normality was tested with the Shaphiro-Wilk Test. All presented variables followed a normal distribution. To determine statistical differences between the pre and post-training state Student's paired *t*-test (two-tailed) was used. Group differences were tested with Student's independent *t*-test. The measured and calculated data of the squat exercise were evaluated by repeated measures ANOVA with Greenhouse-Geisser correction and Tukey's HSD post hoc analysis. To test if there was any connection between measured variables, the Pearson correlation coefficient was calculated.

Results

Both the BFR and the control group significantly improved during the exercise treatment (Greenhouse–Geisser adjusted $p < 0.05$). Exercise time had a strong effect on the average power output (Exercise time effect: $p = 5.0E\text{-}14$, partial $\eta^2 = 0.585$). In contrast, results revealed neither significant differences between control and BFR groups nor significant group effects (Group effect: $p = 0.89$, partial $\eta^2 = 0.001$; Exercise time-*Group effect: $p = 0.37$, partial $\eta^2 = 0.054$) on the average power throughout the 12 training sessions of the four-week exercise program (Fig. 2).

When we assessed 90% 1 RM and the number of maximal repetitions

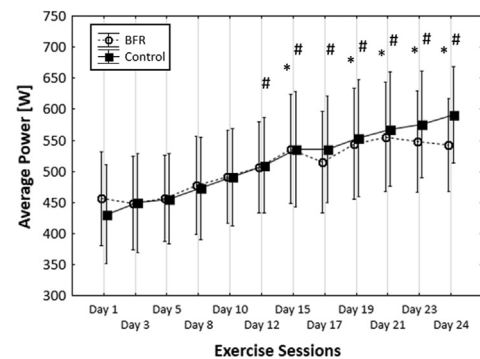## Changes at the average power during the training period

Fig. 2. Changes at the average power during the training period.
The average performance of the subjects was measured in every training session during the four week training period. Significant differences were not detected between the groups. However, the average performance significantly increased after 12 training sessions. Mean $\pm$ SD, marks: * $p < 0.05$ vs. initial value in BFR group, # $p < 0.05$ vs. initial value in C group.

of 70% of 1RM, no significant differences were found between groups (Fig. 3). In both the control and BFR groups the 90% 1RM increased 1.15 and 1.20 fold respectively, and maximal repetition with 70% of 1RM increased 1.64 fold in the control group and 1.54 fold in the BFR group.

From the micro-biopsy samples, we measured myomir content of miR1, miR133a, miR133b, miR206, miR486, and miR499. Despite the similar results for the functional tests (90% of 1 MR, and max repetition of 70% of 1RM), both miR1 (fold difference: 0.73, $p = 0.0197$) and miR133a (fold difference = 0.75; $p = 0.0423$) levels decreased significantly only in the muscle samples of the BFR group (Fig. 4). Remarkably, a significant positive correlation was found between the relative expression level of miR-133a and miR-1 indicating a probable common physiological control.

No significant differences in the levels of miR133b, miR206, miR486, and miR499 were found between groups and initial values (Fig. 5).

Discussion

We have tested whether BFR during rest periods of high-intensity resistance training would benefit maximal strength in young male subjects. Despite the encouraging results of our previous acute study with the same exercise protocol,[12] the present study shows that four weeks of training with and without BFR results in a similar development of strength in young untrained subjects. The reason behind this discrepancy is not known, but emphasize the difference between acute and chronic exercise-induced adaptation. On the other hand, it has been shown that BFR in high intensity-exercise training did not increase the performance.[21] On the other hand, in this case, the BFR during the rest periods of resistance training resulted in alteration of miR-1 and miR-133a. It is generally believed that microRNAs interact with mRNAs in the cytoplasm, and in most cases, they block the transition of the genetic code to proteins. However, recent information suggests that microRNA can modulate transcriptional control and even translocate to mitochondria.[22] One of the modulated microRNA in the present study, the miR1, has been shown to stimulate the myogenic program of mitochondrial genome-encoded transcripts, and represses it in the cytoplasm.[23] Therefore, downregulation of miR1 by BFR restriction during rest periods might have the opposite effect, i.e. downregulation of the mitochondrial DNA-associated myogenic program and stimulation of nuclear DNA driven myogenic translation. In our previous study using the same experimental protocol, we found induction of PAX7 protein, suggesting activation of satellite cells. This is in accordance with the observations

3 RMax and és 70% of the 1RM results

90% Max. Load
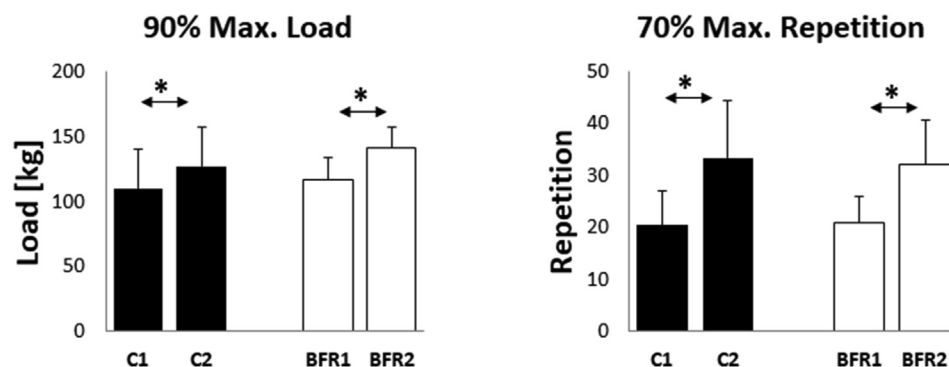

70% Max. Repetition

Fig. 3. 3 RM and 70% of the 1RM results
The three repetition maximum and the 70% 1 RM maximum were measured before and after four weeks of training. C1: result in the control group before the training period, C2: result in control group after the training period, BFR1: result in the occlusion group before the training period, BFR2: result in the occlusion group after the training period. Results are expressed as mean ± SD, $n = 9$ in each group, *$p < 0.05$ compared to initial values in BFR, $p < 0.05$ compared to initial values in control group.

miR-1, miR-133a levels and the relationship between miR-1 and miR-133a
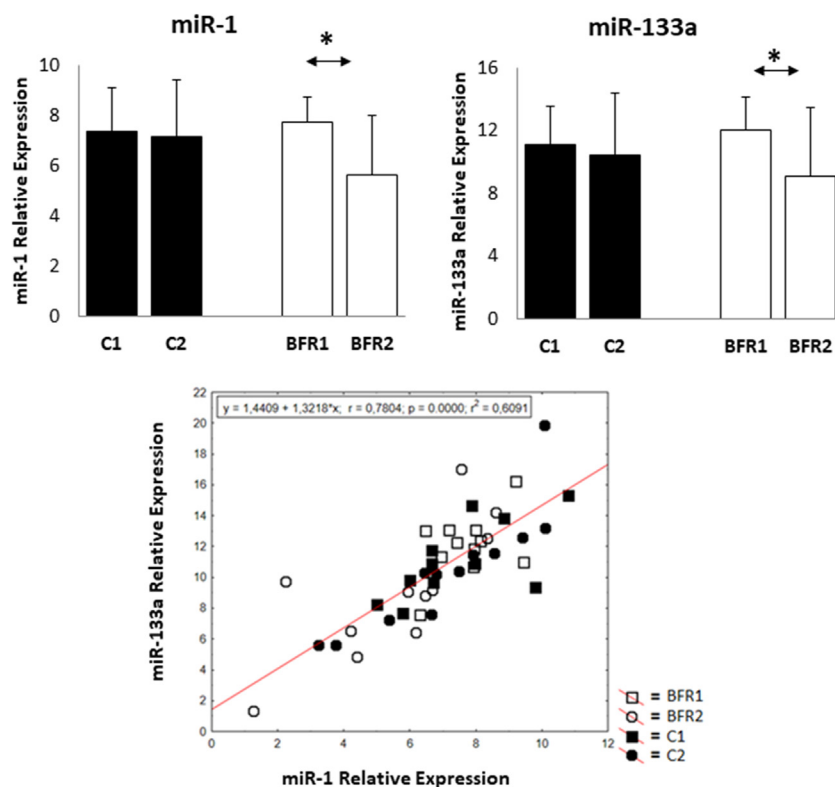
miR-1

miR-133a

Fig. 4. miR-1, miR-133a levels and the relationship between miR-1 and miR-133a
BFR decreased the levels of miR-1 (A) and miR-133a (B) compared to values of the subjects trained without BFR. A strong correlation was found between miR-1 and miR-133a. C1: result in the control group before the training period, C2: result in the control group after the training period, BFR1: result in the blood flow restriction group before the training period, BFR2: result in the blood flow restriction group after the training period. Results are expressed as mean ± SD, $n = 11$ in each group, * $p < 0.05$ compared to initial values.

that the levels of miR1decreased in the skeletal muscle of young men, compared to elderly subjects, when subjected to resistance training and acute protein anabolic loading.[24] In addition, we have shown previously that during overload-induced hypertrophy of rat skeletal muscle the level of miR-1 decreased and this was associated with increased levels of IGF1-Ea and IGF-1Eb/mechano-growth factor mRNAs.[25]

The other miR which changed significantly in our study was miR133a. Liu and co-workers[26] generated transgenic mice with the deletion of miR-133a and miR-133a-2 and found that these mice developed myopathy in type II fibers and impaired mitochondrial function, among other impairments. The level of miR-133a increases as a result of

an acute bout of endurance exercise and decreases following 12 weeks of endurance training.[27] Interestingly miR-133a deficiency in mice resulted in downregulation of genes which are associated with mitochondrial biogenesis,[28] which seems to contradict the requirements of endurance training-induced adaptation. One of the targets of miR-133a is the SIRT1 protein, and we have found that in an overload-induced hypertrophy model, decreased levels of miR-133a are negatively correlated with nicotinamide phosphoribosyltransferase (NAMPT), which is important for the biosynthesis of NAD.[25] However, it is important to note that it is very difficult to identify the targets of miRs due to the fact that single miRs control hundreds of genes and there is significant overlapping in the

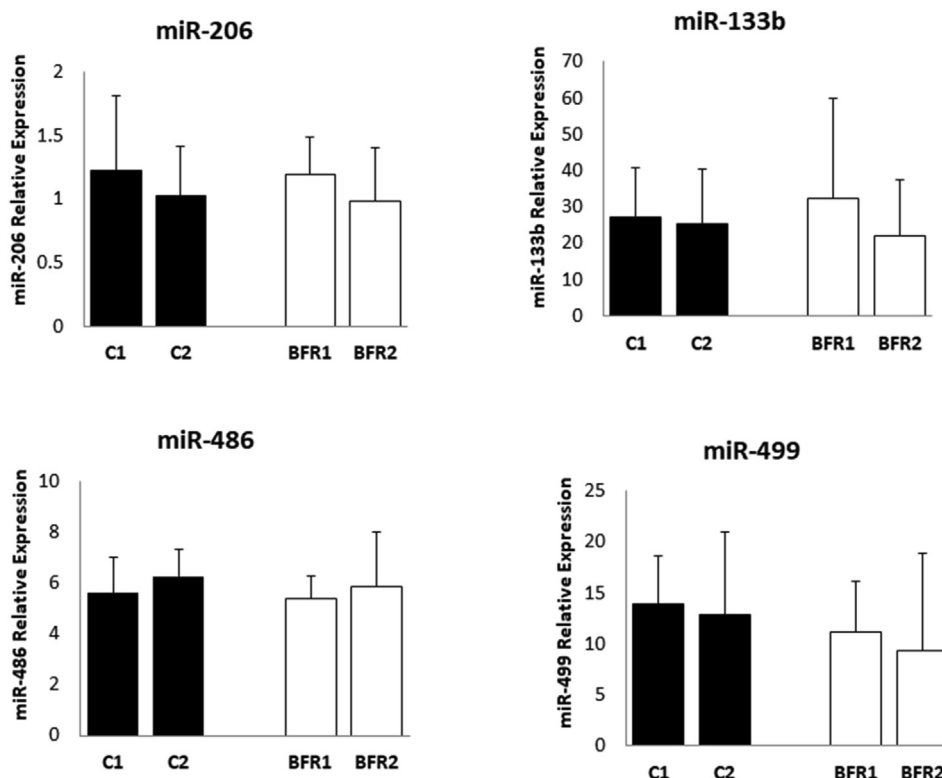## The levels of miR-206, miR-133b, miR-486, and miR-499

Fig. 5. The levels of miR-206, miR-133b, miR-486, and miR-499
BFR training did not alter the levels of miR-206, miR-133b, miR-486 and miR-499 compared to the training without BFR or the initial data.

targeted gene clusters.[29]

Despite the seemingly beneficial changes in miR1 and Mir133a levels, we could not detect significant changes at the measured performance tests. It has to emphasized that sports performance is very complex, and not easy to find biomarkers which always correlate well with the performance.[2] On the other hand, it cannot be excluded, that certain performance, such as strength endurance would change when evaluated by different types of tests. We might point out that a limitation of this study could be the small biopsy sample size.

Conclusion

It seems that BFR restrictions during the rest periods of high-intensity resistance training do not provide benefit for increased performance after a four week training program. The possible functional effects of down-regulated miR-1 and miR-133a remain to be explored.

Submission statement

We state that our paper is not submitted elsewhere.

Ethical approval statement

All procedures were approved by the Ethical Committee of the University of Physical Education (ET-KEB/No8/2017), and the research was conducted according to the standards of the Declaration of Helsinki.

Authors' contributions

FT, PB, ZR, ZG, MJ, GB, MF, SRIB, contributed in measurements and

LVHN and ZR drafted the paper.

Conflict of interest

We state no conflict of interest in our paper.

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