THE LEVEL OF IMPROVEMENT IN THE FUNCTION OF BONE MARROW DERIVED ENDOTHELIAL PROGENITOR CELLS IS DEPENDENT ON THE VOLUME OF AEROBIC EXERCISE TRAINING

EXERCISE AND SPORTS SCIENCES

ORIGINAL ARTICLE

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ABSTRACT

Introduction: Skeletal muscle angiogenesis induced by aerobic exercise training (ET) is crucial in the improvement of the aerobic capacity. The endothelial progenitor cells (EPC) derived from bone marrow have been described for promoting both the vascular repair and angiogenesis. Although the role of the ET on the parameters of the EPC has been investigated, the effect of different volumes of ET on the EPC function in bone marrow, skeletal muscle metabolic alterations and capillarization are unknown. Objective: We hypothesized that ET improves the EPC function in bone marrow, accompanied by increase of skeletal muscle oxidative capacity and angiogenesis dependents of the increase of volume of ET. Methods: Twenty-one Wistar rats were divided into 3 groups: sedentary control (SC), trained protocol 1 (T1) and trained protocol 2 (T2). T1: swimming training consisted of 60 min, 1x/day/10 weeks, with 5% body weight load. T2 the same as T1 until 8th week, in the 9th week the rats trained 2x/day and in the 10th week 3x/day. Results: ET promoted resting bradycardia, increase of exercise tolerance, peak oxygen uptake and citrate synthase enzyme activity in the T1 group, being these adaptive responses exacerbate in the P2 group, indicating that the aerobic condition was improved in this group. ET improved the EPC function of the bone marrow in T1, and the response was exacerbated in T2 group. In parallel, increase in the number of capillaries dependent of ET volume was also observed. Conclusion: These findings suggest that the bone marrow as the main reservoir of EPC is influenced by different ET volume, possibly being responsible for the improvement of aerobic performance observed by higher endogenous EPC mobilization, active participants in the process of angiogenesis induced by ET.

Keywords: exercise, capillaries, bone marrow, endothelial progenitor cells.

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INTRODUCTION

Aerobic exercise training (ET) leads to several adaptation responses in the cardiovascular and skeletal muscular systems. In the skeletal muscle, improvement in physical performance is associated with high mitochondrial density, oxidative capacity, improvement in vascular function as well as angiogenesis. Muscular angiogenesis is indication of an important adaptation to ET, contributing in an important way to improvement in aerobic capacity represented by increase in the transport, conductance and extraction of muscular oxygen[1-4]. Many factors have been mentioned as important to the formation process of new blood vessels, where the contribution of circulating cells has recently become appreciated[5].

The discovery of the bone-marrow derived endothelial progenitor cells (EPC) in the peripheral circulation broaden the view of the angiogenic process, since an accumulation of evidence has shown that the EPC migrate to the circulation participating both in the repair process and in the formation of new blood vessels[5-9]. Asahara *et al.*[6,7] were the first ones to isolate and characterize a population of CD34+ circulating stem-cells, which could be differentiated into cells with endothelial cell phenotype, indicating hence, that the peripheral blood is a reservoir of EPC derived from the bone marrow and which would present repairing properties. Moreover, studies have shown that the EPC not only incorporate within the functional blood vessels originating new vessels, but could also release a multitude of growth factors, providing paracrine signals to the adjacent endothelial cells, which facilitate angiogenesis and indicate additional meaning to which the EPC contribute to the neovascularization and reendothelization process[10]. More recently, other studies have shown that they increase in number in response to the tissue ischemia[11] and participate in the development of collateral vessels after *ex vivo* expansion and when transplanted[12].

It is known that the EPC number and function are regulated not only by many growth factors and cytokines in response to the muscle damage and cardiovascular risk factors, but also to alterations in life style, such as practice of aerobic exercise[7-9].

Recent studies evidenced aerobic ET as responsible for promoting higher release and improving the bone-marrow derived EPC functional properties both in humans and animals, which contributes to the improvement of the endothelial function, angiogenesis and consequently, maintenance of cardiovascular homeostasis[13-19].

Although the ET effect on the EPC parameters has been evidenced, studies with alteration in the ET volume on the EPC and the capillarization have not been found yet. Therefore, the aim of this study is to verify: 1) if the increase of the ET volume induces remarkable improvement in the EPC function of the bone marrow of female rats; and 2) if the increase of the ET volume increases

even more the capillarization of the soleus muscle. The authors raise the hypothesis that ET improves the EPC function of the bone marrow, followed by greater capillarization and muscular oxidative capacity dependent on the ET volume increase, which is relevant to the more active skeletal musculature being it necessary so that better physical performance is reached.

MATERIAL AND METHODS

Experimental animals

In order to make this study possible, 21 female Wistar rats were used. The animals were obtained at the Central Animal Facility of the Institute of Biomedical Sciences of the University of São Paulo (ICB-USP). The animals weighed between 180 and 200 g at the beginning of the protocol.

The animals used in this study were kept in plastic cages and were grouped in three or four per box and separated by experimental group. Room temperature in the premises was kept between 22º-24ºC and light was controlled in a 12-hour inverted light-dark cycle. Water and food were offered *ad libitum*.

All procedures were according to the Ethical Principles in Animal Experimentation adopted by the Brazilian College of Animal Experimentation, being this research Project approved by the Ethics Committee of the Physical Education and Sports School of the University of São Paulo.

Animal identification

The animals were randomly divided in three groups with seven animals each, according to the experimental protocol:

- Wistar female rats sedentary control (SC);
- Wistar female rats trained with protocol 1 (P1); and
- Wistar female rats trained with protocol 2 (P2).

Protocols of aerobic physical training

Protocol 1 (P1) – the animals were trained in swimming during 10 weeks, 60- min sessions, once a day, five times/week, with gradual increase of work load (weight on the tail in percentage of body weight). Increasing adaptation was performed until the animals reached 5% of their body weight[20].

Protocol 2 (P2) – in this training protocol, the animals trained until the eight week with the same previous protocol (P1). On the ninth week, the animals trained twice a week, 60-min sessions with a four-hour interval between sessions. On the 10th week, they trained three times a day, 60-min sessions with a four-hour interval between sessions. The rats were identified and weekly weighed for correction of training overload due to their body weight increase. This training protocol with increase of exercise volume has been used with the purpose to mimic a high-performance exercise training[21].

The used protocols were characterized as low and moderate intensity and long duration trainings, being efficient in promoting cardiovascular adaptations and increase of muscular oxidative capacity[20,21].

Pre and post the ET period, the animals were submitted to hemodynamic analyses, exercise tolerance test and oxygen uptake peak. After 24 hours from the last training session, the animals were killed by anesthesia with an intraperitoneal injection of sodium pentobarbital (80 mg/kg). The necessary samples were collected and accordingly stored for histological, biochemical and cellular analyses.

Direct measurement of blood pressure and heart rate

After the ET period, the animals were cannulated and kept in individual cages, where they remained for at least 24 hours before the experiment of blood pressure and heart rate record was started. The cannula on the animal was connected to a polyethylene tube (PE 100) which was connected to an electromagnetic transducer (P23 Db; Gould-Statham) which, on its turn, was connected to an amplifier (General Purpose Amplifier-Stemtech, Inc.). The analog signal of the blood pressure was converted to digital (Stemtech, Inc.), recorded in real time in a micro-computer with CODAS System and analyzed through the program compatible with *Windows*, with sampling frequency of 1,000 Hz per channel. Beat-by-beat values of the systolic blood pressure (SBP), diastolic blood pressure (DBP) mean blood pressure (MBP) and heart rate (HR) were obtained from this program.

Evaluation of the tolerance to maximal exercise

The evaluation of the maximal exercise protocol was performed with the animals from the three groups being placed on the treadmill. The exercise test started immediately after the animal was placed on the treadmill. Initial velocity was of 6 m/min (no inclination), which constitutes a scale protocol for with velocity increase of 3 m/min at every 3 min, until maximum velocity supported by the animals was reached. The criterion for determination of the animal exhaustion and test interruption was the moment at which the rat was not able to run due to the velocity increase on the treadmill. This evaluation was conducted after the training period to compare the performance response of the animals among the groups. The total duration time of the exercise test (min) was compared for each rat.

Evaluation of the oxygen uptake (VO_2)

The VO_2 peak was measured by measurement of the determination of the expired fraction of oxygen (FeO_2) during the progressive exercise test until exhaustion. In this protocol the rats were placed in a metabolic box on the treadmill, which served as a mixing chamber for the expired gas. This chamber is connected to a T-shaped tube for air samples removal (1,000 mL/min) so that the FeO_2 can be assessed in a gas analyzer. The other exit of the 'T' tube is used for inspiration of the air in continuous flow (2,500 mL/min), regulated by a vacuum pump. The front part of the metabolic box has an opening of 2 mm from the surface, which allows the room air entrance from one direction only, picked by the vacuum pump. The air flow in the metabolic box is of 3,500 mL/min.

The animal was placed in the metabolic box for a recovery period of 30 minutes for recording of the baseline status, and the test was then initiated with velocity of 3 m/min. During each stage (3 min) of performed exercise, the FeO_2 of the gas present in the air in the metabolic box was analyzed. When reaching exhaustion, the animal was kept in the metabolic box for approximately 3 min and expired fractions were recorded to verify the animal's recovery as well as functioning of the analyzers.

The VO_2 was calculated through the following mathematical formula: VO_2 = air flow x (FiO_2-FeO_2) / body weight. Where: VO_2 = $mL.kg^{-1}.min^{-1}$, air flow = 1,000 mLmin (analyzer) + 2,500 mLmin (vacuum pump) = 3,500 mLmin, FiO_2 = fraction of inspired oxygen

(room air), FeO_2 = fraction of expired oxygen (mixing box), body weight = kg.

Evaluation of the citrate synthase enzyme activity

The activity of the citrate synthase enzyme was measured in the homogenized of the soleus muscle of the animals, in a spectrophotometer as described by Srere[22]. The soleus muscle was homogenized with phosphate buffer (PBS) in the 1:10 ratio (1 g of tissue : 10 ml of PBS). The samples were centrifuged at 1,500 g, during 10 min at 4ºC. The supernatant was removed and used for measurement of the enzymatic activity.

The enzymatic kinetics was performed in a spectrophotometer and was determined by the quantification of the intensity of the color made by the reaction between the coenzyme A (CoA) and the oxaloacetic acid in the presence of the 5,5'ditio-bis 2 nitrobenzoic acid (DTNB). The reading was performed at 25ºC, during an interval of 160 seconds, in 412 nm. The enzyme activity was determined by the difference between the initial and final absorbance, divided by the difference of time of absorbance. The results were expressed in $\mu mol.mL^{-1}.mg\ protein^{-1}$. The protein was determined by the Bradford method using bovine serum albumin as standard (BSA, 1 mg/mL).

Extraction of the mononuclear cells of the bone marrow

The bone marrow cells were obtained from the animals' femur and tibia. After the lower limbs had been hygienized, an incision was made so that the coxofemoral articulation could be seen. After the ligaments were ruptured and the adjacent muscular tissue was removed, the femur was immersed in a *falcon* tube containing PBS (*phosphate-buffered saline*) and penicillin G (100 U/mL) and streptomycin (100 µg/mL). The same procedure was repeated for the tibia's removal.

Under sterile conditions, the bone marrow was extracted from the bone by washing of the cavity with culture medium *Dulbecco's Modified Eagle's Medium* (DMEM, Gibco, NY, USA), with a syringe and a needle. After successive washing and homogenization of the medium containing cells, a separation gradient was performed with Ficoll (Ficoll-Paque, GE Healthcare, Uppsala, Sweden) for separation of the portion of mononuclear cells (MNC). The material was centrifuged for 25 minutes at 1,000 g (± 23ºC) for separation of the cells. After having been separated, the cells were washed with PBS and again submitted to centrifuging for five minutes at 1,000 g, and later suspended again in small volume of PBS 1X for subsequent count in a microscope (Nikon, NY, USA). After that, they were taken to function analysis according to methodology described as follows:

Functional evaluation of the endothelial progenitor cells by formation of colony-forming unit

The EPC presents properties such as clonogenicity, which is the ability to double cells, demonstrating the power of their cells to form colonies. Basically, the clonogenic assay involves three phases: 1) the first phase consists in the separation and quantification of the MNC, as previously described; 2) the second phase involves the cellular culture of these cells, which received $5x10^6$ <NC on six-well plates containing fibronectin (BD BioCoat, CA, USA). The cells were cultivated in endothelial medium specific for function analysis of these stem cells (EndoCult-StemCell Technologies, Vancouver, Canada). After two days of incubation for selection of the EPC population, the non-adherent cells are again plated with specific medium EndoCult; 3) finally, after three days of culture, the third phase includes the count in inverted microscope attached to the camera (Nikon, NY, EUA) of the number of colony-forming units (CFU), after seven days at 37ºC, 5% of CO_2 with 95% of humidity. The results were expressed in percentage of the mean of the control of the number of colonies formed by $1x10^6$ of the plated cells.

Analysis of the capillary-to-fiber ratio

The soleus muscle was transversally cut, stained in formaldehyde at 6% for 24 hours and processed by dehydration, diafanization and paraffin bath at 60ºC. Subsequently, they were inserted in cassettes containing funded paraffin, which when solid, originated a paraffin block for each sample, in which 3 µm-thick cuts were made in microtome, submitted to slides mounting for histological analysis with PAS staining (*Periodic Acid Schiff*), for visualization of the capillary vessels.

The histological analysis was performed with use of the image analysis system Quantimet Leica®, (Leica, Cambridge, United Kingdom) through a blind study. The number of capillaries was analyzed with the image being enlarged 400x for measurements. According to established criteria, the diameter was the main parameter for capillaries identification, being considered capillaries, the vessels with diameter equal or smaller than 12 µm. Five fields of view of the slides of each sample were analyzed, in which the total number of muscle fibers and capillaries per field was quantified and from these values, the capillary-to-fiber ratio of each animal was calculated.

Statistical analysis

One-way ANOVA (group as independent variable) was used for the SBP, DBP, MBP, HR, activity of the citrate synthase enzyme, EPC function and capillary-to-fiber ratio data; and Tukey *post-hoc* test (Statistica software, StatSoft, Inc., Tulsa, OK, USA). Concerning analysis of exercise tolerance and VO_2 peak data, two-way ANOVA was used (group and time as independent variables) and Tukey *post-hoc* test. All experiments considered a $p < 0.05$ of significance. All results were presented as mean ± standard error of the mean (SEM).

RESULTS

Hemodynamic parameters: blood pressure and heart rate

The SBP, DBP and MBP values expressed in millimeters of mercury (mmHg) and HR expressed in beats per minute (bpm) in the post-ET period were summarized in table 1 for the SC, P1 and P2 groups.

Table 1. Hemodynamic parameters.

Groups	SBP, mmHg	DBP, mmHg	MBP, mmHg	HR, bpm
SC	127.6±3.9	97.6±10.3	113.5±7.3	344.8±12.1
P1	123.3±8.5	96.4±5.2	110.7±6.7	301.2±15.3*
P2	123.0±8.4	94.3±8.9	108.3±9.0	309.0±14.0*

Values expressed as mean ± SEM. Results of the systolic blood pressure (SBP), diastolic blood pressure (DBP), mean blood pressure (MBP) and heart rate (HR) were obtained post- ET period in control Wistar sedentary female rats (SC), Wistar female rats trained with protocol 1 (P1) and Wistar female rats trained with protocol 2 (P2). *P <0.05 vs. SC.

There was no difference in the BP among the three studied groups. However, HR significantly decreased after 10 weeks of swimming training in the P1 group (301.2 ± 15.3 bpm) and in the P2 group (309 ± 14 bpm), compared with the SC group (344.8 ± 12.1 bpm, $p < 0,05$).

Maximal exercise test

In the analysis of exercise tolerance, significant interaction of the factors group/time [$F(2.40) = 5.74$; $p = 0.008$] was observed. The groups SC, P1 and P2 presented the same physical performance in the pre-ET period. However, in the ost-ET period, there was significant performance improvement of the trained animals (P1 and P2) compared with the pre-ET period ($p < 0.001$). Moreover, the P1 group presented increase of 20.7% ($p < 0.001$) and group P2 increase of 29.4% ($p < 0.001$), both compared with the control group (figure 1A).

Measurement of oxygen uptake peak

In the analysis of the VO_2 peak, significant interaction between the factors group/time [$F(2.40) = 3.59$; $p = 0.037$] was observed. Figure 1B shows that in the pre-ET period the groups SC, P1 and P2 presented the same mean level of VO_2 peak. In the post-ET period, there is maintenance of the VO_2 peak in the groups P1 and P2 compared with the pre-ET period. However, significant reduction of VO_2 peak can be observed in the group SC compared with the pre-ET period ($p < 0.05$). Moreover, the trained groups presented a VO_2 peak statistically higher in comparison with the VO_2 peak of the group SC in the post-ET period (SC: 67.6 ± 2.2; P1: 75.63 ± 2 and P2: 80.05 ± 2.4 $mL.kg^{-1}.min^{-1}$, $p < 0.05$ and $p < 0.01$, respectively *vs.* SC).

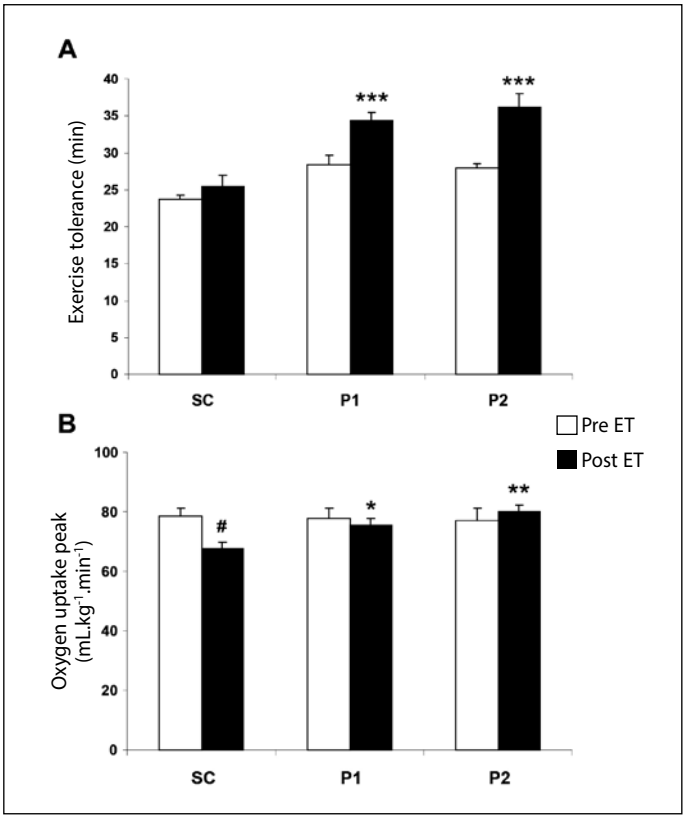

Figure 1. Markers of aerobic physical performance. Tolerance to maximum exercise evaluated by the duration time of the test (min) (A) and the pre and post- ET oxygen uptake (VO2) peak (B). The results are expressed as mean ± SEM. # $p < 0.05$ compared with pre-ET; * $p < 0.05$ compared with the SC post-ET; ** $p < 0.01$ compared with the SC post-ET; *** $p < 0.001$ compared with the pre-ET and SC post-ET. ET: exercise training.

Measurement of the citrate synthase enzyme activity

Figure 2 shows that there was increase of the activity of the citrate synthase enzyme in the soleus muscle of female rats trained with P1 (275.7± 24.6 $\mu mol.mL^{-1}.mg^{-1}$; $p < 0.05$) and P2 (385.4 ± 18.1 $\mu mol.mL^{-1}.mg^{-1}$; $p < 0.01$) compared with the group SC (188 ± 23.8 $\mu mol.mL^{-1}.mg^{-1}$).

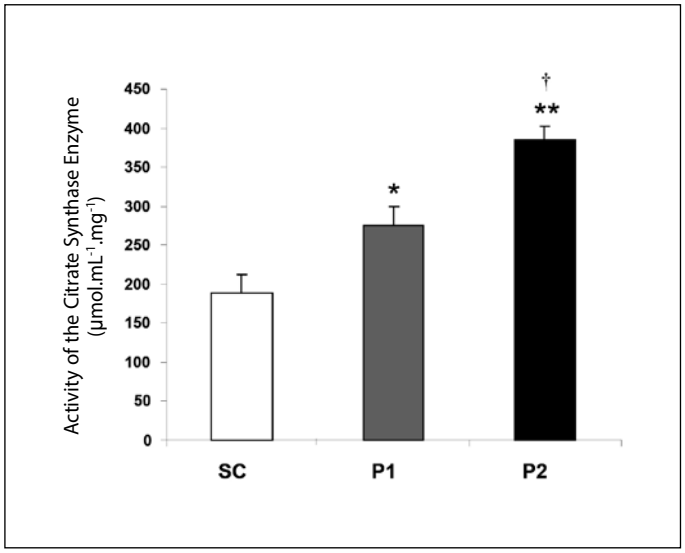

Figure 2. Effect of different volume of aerobic ET on the activity of the citrate synthase enzyme in the soleus muscle represented in values of µmol.mL-1.mg-1. Results are expressed as mean ± SEM. * $p < 0.05$ compared with the SC; ** $p < 0.01$ compared with the SC; † $p < 0.05$ compared with the P1. ET: exercise training.

Evaluation of the function of the endothelial progenitor cells

The number of bone marrow derived EPC colonies was altered by the different ET volume. As seen in figure 3, ET was able to improve the EPC function represented by the increase of 237% in the number of colony-forming units in the group P1 ($p < 0.001$) compared with the group SC. Interestingly, the increase in the ET volume increased even more the number of colonies, to 358%, in the group P2 ($p < 0.001$) compared with the group SC. The group P2 was different from the P1 with increase of 121% in the number of EPC colony-forming units ($p < 0.01$). The colony-forming units are presented in figure 3B.

Determination of the capillary ratio by skeletal muscular fiber

Morphological analyses after histological processing reveal important alterations in the skeletal muscular microcirculation induced by ET. Figure 4 presents the capillarization results obtained by PAS staining. The ET was effective in increasing 128% of the number of capillaries by the analysis of the capillary-to-fiber ratio in the group P1 ($p < 0.0001$) compared with the group SC. The increase of ET volume promoted angiogenesis even greater, with increase of 171% in the group P2 ($p < 0.0001$) compared with the group SC (SC: 0.7 ± 0.05; P1: 1.6 ± 0.06; P2: 1.9 ± 0.13 capillary-to-fiber ratio). The group P2 was different from the P1 with increase of 41% in the number of capillaries ($p < 0.0004$) per fiber.

DISCUSSION

In the present study, the effect of different aerobic ET volume on the EPC function of the bone marrow and metabolic and structural alterations of the skeletal musculature was assessed. The

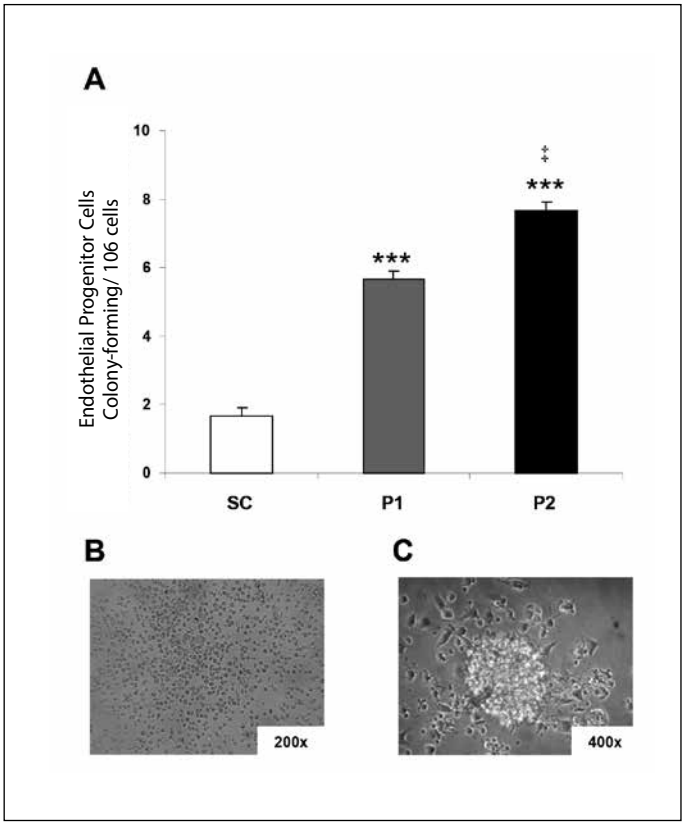

Figure 3. Effect of different volume of aerobic ET on the EPC function assessed by the capacity of formation of colony-forming unit. Illustrative photos of the second day of the EPC plaque process in which there is not colony formation yet (A) and on the seventh day already with the cell colony. The results are expressed as mean ± SEM. *** $p < 0.001$ compared with the SC; ‡ $p < 0.01$ compared with the P1. ET: exercise training; EPC: endothelial progenitor cells.

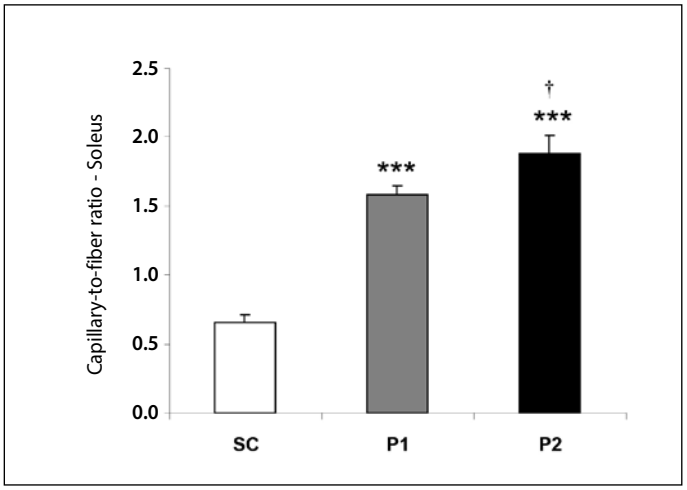

Figure 4. Effect of different volume of aerobic ET on the analysis of the capillary-to-fiber ratio by histological method through PAS (Periodic Acid Schiff) staining. The results were expressed as mean ± SEM. *** $p < 0.001$ compared with the SC; † $p < 0.05$ compared with the P1. ET: exercise training.

main results of the study show that the aerobic ET : 1) induced resting bradycardia with no alteration in the BP; 2) increased exercise tolerance; 3) maintained the VO_2 peak; 4) increased the activity of the citrate synthase enzyme; and 5) improved the EPC function of the bone marrow followed by increase of capillarization in the skeletal muscle dependent on the increase of ET volume.

The physiological adaptations which result from the aerobic ET provide greater capacity of the cardiorespiratory system in supplying oxygen and energetic substrates to the muscle tissue, and it uses them more efficiently during the ET[1-4].

Among the autonomic and hemodynamic adaptations which will influence the cardiovascular system, resting bradycardia consists in a good functional parameter to verify the adaptation and efficiency of the aerobic ET; therefore, it is considered an ET physiological marker. This adaptation is observed both in experimentation animals[20,21,23] and humans[24].

Previous studies of our group demonstrated that the magnitude of the resting bradycardia depends on the type and intensity of the aerobic ET used. As observed, greater resting bradycardia in animals trained in swimming[20] than on treadmill[23] was found. In the present study, we observed that the groups submitted to the two ET protocols presented similar reduction in rest HR regardless of the exercise volume applied, when compared with its initial HR. The mechanisms through which this bradycardia occurs seem to be strongly influenced by the type of ET. In normotensive rats, this resting bradycardia resulting from the aerobic ET on treadmill is associated with reduction in the intrinsic HR[23], while the animals submitted to the swimming protocol seem to present resting bradycardia due to increase of vagal tonus[20].

The BP adaptation due to the ET seems to depend on the presence of risk factors and cardiovascular diseases. In fact, studies have shown that the BP response remains unchanged in normotensive humans and animals submitted to aerobic ET[20,21,23-25]. In the present study, consistent with other ones previously mentioned, there was no significant difference in BP between the trained and sedentary groups, showing hence that ET with swimming does not alter rest BP. Moreover, this effect does not seem to be intrinsic to the type of ET applied when compared with the modality on treadmill with swimming, nor to be species-dependent. Nevertheless, it can be conditioned to the level of initial BP, since hypertensive populations benefit from aerobic ET concerning its reduction[25].

In the skeletal muscle, improvement of physical performance is associated with improvement of oxidative capacity and angiogenesis[1-4]. Increase of aerobic work represented by higher exercise tolerance and VO_2 peak were observed in the groups P1 and P2, with more exacerbated responses in the group P2. Therefore, another important indication which demonstrates the optimization of the aerobic metabolism is the increase of the capacity of the oxidative system. This metabolic adaptation may be identified through many oxidative enzymes, which have also been used in the literature as markers of aerobic ET. The citrate synthase is one of the most important enzymes for the regulation of the metabolic ways of energy generation, since it catalyzes the first reaction of the Krebs cycle and consequently generates energy via aerobic metabolism[26]. In the present study, the activity of the citrate synthase oxidative enzyme in the soleus muscle increased 47% in the group P1 and 105% in the group P2 compared with the group SC, demonstrating that the increase of ET volume was able to induce higher muscular adaptation response to the necessary demands, strongly contributing to the increase of the observed physical performance.

Concomitantly, increase of the number of capillaries can be observed in the soleus muscle of the animals from the groups P1 and P2, being the angiogenesis more remarkable in the group P2. Some studies have shown that hypoxia and shear stress are

the most important stimuli which trigger the skeletal muscular angiogenesis induced by ET[1-4]. In the presence of hypoxia, an important increase in the transcription of the hypoxia-induced factor (HIF-1α) occurs, which, on its turn, stimulates the transcription of the vascular endothelial growth factor (VEGF). The initial imbalance of partial pressure of O_2, due to the VO_2 increase, stimulates the VEGF expression. and consequently, the proliferation of endothelial cells for the formation of new vessels[1-4].

Interestingly, the hypoxia and shear stress found in the skeletal musculature by the aerobic ET were described for being one of the most powerful physiological stimuli known for triggering the increase of the number and increasing the EPC function mediated by the synthesis of growth factors, especially the VEGF and nitric oxide (NO)[8,9,13-19]. Hypoxia and shear stress alter the microvascular endothelium casing the EPC mobilization to these regions. Their engagement is significantly high in the endothelium in that condition and the EPC exposure in those sites generates the proliferation and organization of cellular clusters, which align in the direction of the ischemic gradient and form cords like vessels. Thus, both the VO_2 and blood flow increase induced by the aerobic ET raise the VEGF and NO production, which have been said to be the main responsible for the migratory activity, EPC engagement and incorporation in the microcirculation, promoting repair and formation of new vessels [8,9,13-19].

Laufs *et al.*[13] were the first to show that the aerobic ET is able to influence on the number of EPC, where trained mice compared with sedentary ones resented significant increase of EPC in the bone marrow and circulation from the seventh day of ET, being kept for over a month of continuous activity. The researchers also showed that mice treated with NO synthase blocker (L-NAME) and knockout for this gene had their increase blocked, which suggests increase of NO-dependent EPC in response to aerobic exercise.

Furthermore, Adams *et al.*[14] observed that patients with myocardial ischemia presented systemic EPC increase within 24-48 h after maximum exercise. The increase found in the number of EPC was related to higher plasma VEGF concentration. Studies have shown that the VEGF interaction with its main angiogenic receptor, the VEGFR2, type tyrosine kinase receptor, promotes activation of a intracellular signaling cascade mainly mediated by the PI3K/Akt way, which stimulates the expression of genes responsible for the bone marrow EPC mobilization to the peripheral blood[8,9,17,18,27]. Further studies in healthy individuals confirmed the ET role in the promotion of angiogenesis and improvement of vascular function due to improvement in EPC bioactivity dependent on the VEGF and NO increase[8,9,15-19].

The EPC profile can also be influenced by the ET intensity. Thus, Strong physical exercise, as the ones performed by marathoners, did not present any alteration in the EPC concentration[28], while a recent study published by Goussetis *et al.*[29] demonstrated increase of 10 times of the EPC after 246 km of foot race and that his mobilization mediated by exercise was associated with the release of VEGF in the circulation. Similarly to this study, Möbius-Winkler *et al.*[30] showed EPC increase in the circulation of healthy individuals from the 210 min of strong exercise, indicating hence increase of these cells which depend on the time of performance of the exercise.

Corroborating the data in the literature, our results also mention improvement in the EPC parameters in response to ET. Moreover, we presented for the first time that such improvement in the EPC function depends on the increase of the training volume, since addition of 121% was observed in the EPC function in the group P2 compared with the P1. Besides that, increase in the number of capillaries dependent on the ET volume was observed.

CONCLUSION

In conclusion, these results suggest that the bone marrow, as the main EPC reservoir, is influenced by different ET volume, being possibly responsible for the higher physical performance observed when greater EPC endogenous mobilization occurs, being also active participants in the muscular angiogenesis process induced by the aerobic ET.

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REFERENCES

1. Holloszy JO, Coyle EF. Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. J Appl Physiol 1984;56:831-8.
2. Gustafsson T, Puntschart A, Kaijser L, Jansson E, Sundberg CJ. Exercise-induced expression of angiogenesis-related transcription and growth factors in human skeletal muscle. Am J Physiol Heart Circ Physiol 1999;276:679-85.
3. Amaral SL, Papanek PE, Greene AS. Angiotensin II and VEGF are involved in angiogenesis induced by short-term exercise training. Am J Physiol Heart Circ Physiol 2001;281:H1163-9.
4. Prior BM, Yang HT, Terjung RL. What makes vessels grow with exercise training? J Appl Physiol 2004;52:1119-28.
5. Rafii S. Circulating endothelial precursors: mystery, reality and promise. J Clin Invest 2000;105:17-9.
6. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cell for angiogenesis. Science 1997;275:964-7.
7. Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999;85:221-8.
8. Urbich C, Dimmeler S. Endotehlial progenitor cells: Characterization and role in vascular biology. Circ Res 2004;95:343-53.
9. Eguchi M, Masuda, H, Asahara T. Endothelial progenitor cells for postnatal vasculogenesis. Clin Exp Nephrol 2007;11:18-25.
10. Gnecchi M, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res 2008;103:1204-19.

11. Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, et al. Neovascularization of ischemic myocardium by human bone marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med 2001;7:430-6.

12. Kalka C, Masuda H, Takahashi T, Kalca-Moll WM, Silver M, Kearney M, et al. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci USA 2000;97:3422-7.

13. Laufs U, Werner N, Link A, Endres M, Wassmann S, Jürgens K, et al. Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis. Circulation 2004;109:220-6.

14. Adams V, Lenk K, Linke A, Lenz D, Erbs S, Sandri M, et al. Increase of circulating endothelial progenitor cells in patients with coronary artery disease after exercise-induced ischemia. Arterioscler Thromb Vasc Biol 2004;24:684-90.

15. Yang Z, Wang JM, Chen L, Luo CF, Tang AL, Tao J. Acute exercise-induced nitric oxide production contributes to upregulation of circulating endothelial progenitor cells in healthy subjects. J Hum Hypertens 2007;21:452-60.

16. Hoetzer GL, Van Guilder GP, Irmiger HM, Keith RS, Stauffer BL, De Souza CA. Aging, exercise, and endothelial progenitor cell clonogenic and migratory capacity in men. J Appl Physiol 2007;102:847-52.

17. Wahl P, Block W, Shmidt A. Exercise has a positive effect on endothelial progenitor cells, which could be necessary for vascular adaptation processes. Int J Sports Med 2007;28:374-80.

18. Shantsila E, Lip G. Endothelial function and endothelial progenitors: possible mediators of the benefits from physical exercise? Eur J Cardiovasc Prev Rehabil 2009;16:401-3.

19. Walther C, Gaede L, Adams V, Gelbrich G, Leichtle A, Erbs S, et al. Effect of increased exercise in school children on physical fitness and endothelial progenitor cells: a prospective randomized trial. Circulation 2009;120:2251-9.

20. Medeiros A, Oliveira EM, Gianolla R, Casarini DE, Negrão CE, Brum PC. Swimming training increases cardiac vagal activity and induces cardiac hypertrophy in rats. Braz J Med Biol Res 2004;37:1909-17.

21. Fernandes T, Hashimoto NY, Magalhães FC, Fernandes FB, Casarini DE, Carmona AK, et al. Aerobic exercise training-induced left ventricular hypertrophy involves regulatory microRNAs, decreased angiotensin-converting enzyme-angiotensin II, and synergistic regulation of angiotensin-converting enzyme 2-angiotensin (1-7). Hypertension 2011;58:182-9.

22. Srere PA. Studies on purified citrate-enzymes: metabolic interpretations. Biochem Soc Symp 1968;27:11-21.

23. Evangelista FS, Martuchi SE, Negrão CE, Brum PC. Loss of resting bradycardia with detraining is associated with intrinsic heart rate changes. Braz J Med Biol Res 2005;38:1141-7.

24. Katona PG, McLean M, Dighton DH, Guz A. Sympathetic and parasympathetic cardiac control in athletes and nonathletes at rest. J Appl Physiol 1982;52:1652-7.

25. Fernandes T, Magalhães FC, Roque FR, Phillips MI, Oliveira EM. Exercise training prevents the microvascular rarefaction in hypertension balancing angiogenic and apoptotic factors: role of microRNAs-16, -21, and -126. Hypertension 2012;59:513-20.

26. Siu PM, Donley DA, Bryner RW, Alway SE. Citrate synthase expression and enzyme activity after endurance training in cardiac e skeletal muscles. J Appl Physiol 2003;94:555-60.

27. Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, et al. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI3-kinase/Akt pathway. J Clin Invest 2001;108:391-7.

28. Adams V, Linke A, Breuckmann F, Leineweber K, Erbs S, Kränkel N, et al. Circulating progenitor cells decrease immediately after marathon race in advanced-age marathon runners. Eur J Cardiovasc Prev Rehabil 2008;15:602-7.

29. Goussetis E, Spiropoulos A, Tsironi M, Skenderi K, Margeli A, Graphakos S, et al. Spartathlon, a 246 kilometer foot race: effects of acute inflammation induced by prolonged exercise on circulating progenitor reparative cells. Blood Cells Mol Dis 2009;42:294-9.

30. Möbius-Winkler S, Hilberg T, Menzel K, Golla E, Burman A, Schuler G, et al. Time-dependent mobilization of circulating progenitor cells during strenous exercise in healthy individuals. J Appl Physiol 2009;107:1943-50.