Combined Exercise Training Positively Affects Muscle Wasting in Tumor-Bearing Mice

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ABSTRACT

RANJBAR, K., R. BALLARÒ, Q. BOVER, F. PIN, M. BELTRÀ, F. PENNA, and P. COSTELLI. Combined Exercise Training Positively Affects Muscle Wasting in Tumor-Bearing Mice. Med. Sci. Sports Exerc., Vol. 51, No. 7, pp. 1387–1395, 2019. Introduction: Cancer cachexia is characterized by loss of muscle mass and function. Increased protein catabolism, inflammation, impaired anabolism, and mitochondrial function markedly contribute to the pathogenesis of this syndrome. Physical activity has been suggested as a useful tool to prevent or at least delay the onset and progression of cancer-induced muscle wasting. Two main types of exercise can be adopted, namely, resistance and endurance training. The present study is aimed to investigate the effectiveness of a combined (resistance + endurance) exercise protocol in preventing/reverting cancer-induced muscle wasting. Methods: Mice bearing the C26 colon carcinoma have been used as a model of cancer cachexia. They have been exposed to combined exercise training during 6 wk (4 before tumor implantation, 2 during tumor growth). Climbing a 1-m ladder inclined at 85° has been used for resistance training, while aerobic (endurance) exercise has been carried out on the same day using a motorized wheel. Results: In C26-bearing mice, both muscle mass and strength are improved by combined training, while just the latter increased in exercised healthy animals. Such a pattern is associated with modulations of two markers of autophagy, namely, LC3B-I/II ratio, increased in sedentary tumor hosts and reduced in exercised C26-bearing mice, and p62, steadily increased in both sedentary and trained tumor-bearing animals. Finally, combined training is not able to modify PGC-1α protein levels, but it improves succinate dehydrogenase activity, both reduced in the muscle of the C26 hosts. Conclusion: The data reported in the present study show that combined training improves muscle mass and function in the C26 hosts, likely modulating autophagy and improving mitochondrial function; these observations suggest that combined exercise might become part of a multimodal approach to treat cancer cachexia. Key Words: CANCER CACHEXIA, AEROBIC TRAINING, RESISTANCE TRAINING, AUTOPHAGY, PGC-1α

The loss of muscle mass and function is one of the hallmarks of cachexia, a multifactorial syndrome that frequently complicates the management of cancer patients. The pathophysiologic mechanisms underlying muscle wasting in cachexia are complex and involve increased protein catabolism, downregulation of anabolic signals, and systemic inflammation (1). Alterations in energy metabolism involving mitochondrial dysfunction have been implicated as well (2,3).

Several therapeutic approaches are being proposed to counteract cachexia (1). In addition to drugs, physical activity has been suggested as a useful tool to prevent or at least delay the onset and progression of cancer-induced muscle wasting (4,5). However, although different training protocols could be adopted, their effects on the loss of muscle mass and function have not been completely elucidated yet. As an example, low-intensity endurance exercise has been suggested to exert beneficial effects on muscle wasting in cancer cachexia by triggering an anti-inflammatory response (1,2). By contrast, the possibility that resistance training can also achieve positive effects in cachectic subjects has been less documented (6). Reports in the literature show that resistance training alone might induce the expression of genes associated with muscle damage...
suggesting that caution should be used in adopting this type of intervention protocol in cancer patients. Few data in the literature suggest that the compromise to achieve the most positive effects from physical activity is a combination of resistance and endurance exercise, also known as combined training. Such an approach has been shown to improve body composition, systemic inflammation, and muscle protein synthesis in age individuals (7). Similarly, increased muscle strength and reduced C-reactive protein levels have been reported in both young and old physically inactive subjects practicing combined training (8). Recent reports show that in obese patients and in patients with type II diabetes, combined exercise improves anthropometric markers of adiposity, glycemia, and blood lipids more than aerobic or resistance training alone (9,10). Moreover, in young obese women, the addition of sprint intervals to a combined training protocol provided greater benefits than resistance activity only (9). Similar observations have also been reported in obese children (11). Finally, regular participation to combined exercise sessions has been shown to downregulate the inflammatory response more than aerobic exercise alone (12).

At present, very little is known about the possibility to adopt the combined training approach to improve muscle wasting in cancer cachexia. The present study was aimed to fill this gap. In particular, the hypothesis that the combination of endurance and resistance exercise may affect muscle protein degradation and functional impairment in experimental cancer cachexia has been tested. The results show that combined training positively affects the loss of muscle mass and strength in mice bearing the C26 tumor.

MATERIALS AND METHODS

Reagents. All reagents were supplied by Sigma-Aldrich (St. Louis, MO), unless differently specified.

Animals and treatments. Male BALB/c mice (6 wk old, 20 g) were provided by Charles River Laboratories, Calco, Italy. They were housed on a regular dark–light cycle (light from 08:00 to 20:00), with free access to food and water, and cared for in compliance with the Italian Ministry of Health Guidelines and the Policy on Humane Care and Use of Laboratory Animals (NIH, 1996). The experimental protocol was approved by the Bioethical Committee of the University of Turin. Mice were randomized into two groups, namely, sedentary and exercised (Ex), subsequently further divided into controls (C) and tumor hosts (C26). Tumor-bearing mice (n = 6) were subcutaneously inoculated between the shoulder blades with 5 × 10^7 C26 carcinoma cells. Control mice (n = 6) received saline subcutaneously. The animals were euthanized under isoflurane anesthesia at day 11 of tumor growth (Fig. 1). Several muscles and organs were rapidly excised, weighed, frozen in liquid N2, and stored at −80°C for subsequent analysis.

Combined exercise protocol. Combined exercise training included both resistance and aerobic sessions, repeated 4 d·wk⁻¹ during 4 wk before tumor implantation and 11 d after C26 cell injection (Fig. 1).

Resistance training was accomplished using a 1-m homemade ladder with 1.5-cm grid steps and inclined at 85°. Initially, mice were familiarized with the ladder by practicing climbing for 3 d. On the fourth day, the resistance-training regimen started loading the animal tail with a weight that was about 20% of the initial body weight and that gradually increased for control mice during the experimental period up to 80% of initial body weight. Tumor-bearing mice (days 0–11) were loaded as control mice, while the load weight was adjusted taking into account body weight loss (from day 8), not going above 50% of animal body weight at the end of the experimental period. The daily protocol consisted of a single repetition without weight as warm-up, followed by 3 sets of 2 repetitions at the prescribed loads. Rest periods (1 and 2 min) were provided between repetitions and sets, respectively. Outsource stimuli such as food reward or electrical stimulation were not adopted.

Aerobic training was carried out on the same day, after the resistance protocol, by using a motorized wheel. Before starting the experiment, mice were adapted to the motorized wheel for 5 d (3 × 10 min, 5 m·min⁻¹). During the experimental period, mice ran for 25 min ranging from 5 to 9 m·min⁻¹ speed. The daily protocol started with a 3-min warm-up and finished with a 3-min cooldown.

Grip force assessment. Muscle strength was assessed by the grip strength test as previously described (13) using a Panlab-Harvard Apparatus. The measurements were done in triplicate for each mouse, and the resulting mean was corrected for the initial body weight (day −28; Fig. 1). Grip strength was...
measured before the first training session on day −28, after 4 wk of exercise, the day before tumor inoculation (−1), and 2 h before sacrifice on day +11 (see Fig. 1).

**Western blotting.** Approximately 25 mg of tibialis muscle was homogenized in 80 mmol·L⁻¹ Tris–HCl, pH 6.8, containing 100 mmol·L⁻¹ dithiothreitol, 70 mmol·L⁻¹ SDS, and 1 mmol·L⁻¹ glycerol, with freshly added protease and phosphatase inhibitor cocktails; kept on ice for 30 min; and centrifuged at 15,000g for 10 min at 4°C. Then the supernatant was collected. Protein concentration was assayed according to Bradford, using bovine serum albumin (BSA) as a working standard. Equal amounts of protein (30 μg) were heat denatured in sample-loading buffer (50 mmol·L⁻¹ Tris–HCl, pH 6.8, 100 mmol·L⁻¹ dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol), resolved by SDS-PAGE, and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The filters were blocked with Tris-buffered saline containing 0.05% Tween and 5% nonfat dry milk and then were incubated overnight with antibodies (diluted in TBS containing 0.05% Tween and NFDM or BSA depending on manufacturer’s instructions) directed against LC3B (L7583), p62 (5114); Cell Signaling Technology Inc., Beverly, MA), PGC-1α (AB3242; Millipore, Temecula, CA), cytochrome c (556433; BD Biosciences, Franklin Lakes, NJ), and SDH (sc-377302; Santa Cruz Biotechnology, Dallas, TX). Peroxidase-conjugated IgG (Bio-Rad Laboratories) were used as secondary antibodies. Membrane-bound immune complexes were detected by an enhanced chemiluminescence system (Clarity Western ECL Blotting Substrates, Bio-Rad Laboratories) on a photon-sensitive film (Hyperfilm ECL; GE Healthcare, Little Chalfont, UK). Protein loading was normalized according to GAPDH expression (Santa Cruz Biotechnology). Band quantification was performed by densitometric analysis using the TotalLab software (NonLinear Dynamics, Quayside, UK).

**Succinate dehydrogenase enzymatic activity.** Tibialis muscles were homogenized (5% wt/vol) using an Ultra-Turrax Homogenizer (IKA-Works, Germany) in ice-cold 150 mM NaCl, 10 mM KH2PO4, and 0.1 mM EGTA and centrifuged for 5 min at 800g. The supernatant was collected, and total protein content was measured according to Bradford, using BSA as a working standard. Protein homogenates (50 μL) were incubated with 200 μL reaction buffer containing 10 mM Na-succinate, 50 μg·mL⁻¹ DCPIP, 10 mM phosphate buffer (pH 7.4), 2 mM KCN, 10 mM CaCl2, and 0.05% BSA. The absorbance at 600 nm was measured after 0, 3, and 20 min. The rate of absorbance decrease between 3 and 20 min was corrected for protein loading and used to calculate the relative succinate dehydrogenase (SDH) activity.

**Real-time PCR.** Total RNA was obtained from gastrocnemius muscles using the TRI Reagent following manufacturer’s instructions. RNA concentration was determined fluorometrically using the Ribogreen reagent (Invitrogen, Carlsbad, CA). Total mRNA was retrotranscribed using the i-Script cDNA synthesis kit (Bio-Rad Laboratories). Transcript levels were determined by real-time PCR using the SsoAdvanced SYBR Green Supermix and the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories). Primer sequences (forward and reverse) were as follows: LC3B, 5′-GCCGACCTTCGAAACCA-3′ and 5′-TCGGTTCTTATACCGGGGATT-3′; p62, 5′-CCCATGTCCTGGCA TTCTT-3′ and 5′-AGGGAAGCAGGAGGCTC-3′; atrogin-1, 5′-TCACGTCACATCCCTGAG-3′ and 5′-AGACTGCCGAGCTTGGAGA-3′; and MuRF-1, 5′-TGTCGAGGTCTGTCGTTTGCG-3′ and 5′-ATGGCCGTCCGATCATCCT-3′.

**Data analysis and presentation.** Data are expressed as mean ± SD, except for gene expression (mean ± SEM). The significance of the differences (P < 0.05) was evaluated by two-way ANOVA and Tukey’s post hoc test for multiple comparisons.

**RESULTS**

The onset and progression of cachexia induced in the host mice by the C26 tumor is consistent with previous report (14,15). Figures 2A and 2B report the effects of combined exercise, applied before and after tumor implantation, on body weight and muscle mass. The former is not modified by training activity in both healthy and tumor-bearing mice (Fig. 2A), and the same pattern can be observed for food intake (see Figure, Supplemental Digital Content 1, food intake during the experimental period, http://links.lww.com/MSS/B510). Combined exercise improves the loss of mass in the tibialis, but it affects the gastrocnemius only marginally, without reaching the statistical significance (C26, 386 ± 25; C26 Ex, 340 ± 60; data are mg% i.b.w.; see Fig. 2B). No effects of exercise can be observed on healthy animals (Fig. 2B). Muscle force significantly increases in exercised healthy animals at day −1 with respect to day −28, without further increases at day +11 (Fig. 2C). As for the C26 hosts, the marked reduction of muscle force observed in sedentary mice cannot be observed in the exercised group, where grip strength remains comparable with preimplantation levels (day −1; Fig. 2C).

The effects of combined exercise in healthy animals are not confined to the skeletal muscle because a slight but significant reduction of both liver and spleen mass with respect to control values can be observed (Table 1; see Table, Supplemental Digital Content 2, absolute tissue weight, mg, http://links.lww.com/MSS/B511). By contrast, none of the C26-induced changes such as spleen hypertrophy and fat tissue depletion can be reverted by training (Table 1; see Table, Supplemental Digital Content 2, absolute tissue weight, mg, http://links.lww.com/MSS/B511). Tumor mass is not modified by combined exercise (Table 1).

The beneficial effects of exercise have been ascribed to modulations of systemic inflammation and muscle protein metabolism (16). As for this latter, particular emphasis has been put on the lysosomal–autophagic proteolytic system (17,18). To investigate this point, the expressions of two molecules accepted as markers of autophagy have been assessed. The results shown in Figure 3 conform to previous observations (15). Indeed, although the levels of microtubule-associated...
protein 1 light chain 3B isoform I (LC3B-I) remain comparable in healthy and tumor-bearing mice, both the lipidated form (LC3B-II) and the ratio between the two isoforms, which are considered reliable markers of autophagosome formation, are significantly elevated in the muscle of the C26 hosts with respect to controls. Combined exercise significantly reduces LC3B-II/I ratio, although not the absolute LC3B-II levels, in the muscle of C26-bearing mice, whereas no effects can be observed in exercised healthy animals (Fig. 3A). Moreover, p62 levels have been evaluated as a measure of substrate
sequestration into autophagosomes. Consistent with previous data (15), p62 expression increases in the muscle of sedentary C26 hosts and is not modified by combined exercise (Fig. 3B). LC3B mRNA levels increase in the muscle of sedentary C26 hosts and are partially reduced in exercised tumor hosts (Fig. 3C). As for p62 transcript, its levels remain high in both sedentary and exercised C26-bearing mice (Fig. 3C).

To assess if proteasome-dependent proteolysis has also been affected by the combined exercise protocols, transcript levels of two markers of this degradative systems that strongly increase in the muscle of C26-bearing mice, namely, Atrogin-1 and MuRF-1, have been evaluated in the gastrocnemius and found substantially unchanged by exercise, even if the former shows a reduction that is borderline to significance (Figs. 4A and 4B), somehow paralleling the marginal effect exerted by exercise on gastrocnemius mass (Fig. 2B).

Aerobic exercise also improves energy metabolism by enhancing oxidative metabolism and stimulating mitochondrial biogenesis (2,19). Consistent with previous findings (20), the protein levels of peroxisome proliferator activated receptor (PPAR)-γ coactivator-1α (PGC-1α), the master regulator of mitochondrial biogenesis, decrease in the muscle of the C26-bearing mice. Such reduction is not efficiently prevented by combined exercise, although a trend toward improvement

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can be suggested and no differences can be observed between sedentary and exercised healthy mice (Fig. 5A). To better assess the effects of combined exercise on the mitochondrial compartment, cytochrome c and SDH levels have been evaluated as a measure of mitochondrial content. Both proteins remain comparable among the four experimental groups, even if a trend toward increase, that does not reach significance, could be suggested in exercised healthy mice (Figs. 5B and 5C). By contrast, the reduction of SDH activity is significant versus control values in the muscle of sedentary tumor-bearing mice but not in exercised C26 hosts (Fig. 5D), indicating a preservation of mitochondrial function in the latter.

DISCUSSION

The occurrence of cachexia is an important complication in the management of cancer patients, worsening their quality of life and reducing both tolerance to antineoplastic regimens and survival. Because there is actually no available effective treatment for such a syndrome, the search for therapeutic options is very active. Encouraging data have been obtained on experimental models of cancer cachexia, showing that different strategies can be used to interfere with the progression of body and tissue wasting. As for human cachexia, there are several ongoing clinical trials, most of them based on nutritional and pharmacological approaches (21); at present, however, the available results do not provide any winning evidence.

An easy way to improve muscle mass and function in physiological condition is represented by exercise, suggesting that this same approach could be proposed to treat muscle wasting associated with different chronic diseases. Despite this intriguing hypothesis, however, the effect of physical activity on cancer cachexia has been explored only marginally (22,23). In the present study, the effects of a combined resistance–endurance exercise protocol on functional parameters and skeletal muscle adaptations in tumor-bearing mice have been investigated. The main findings are that combined training started 4 wk before tumor implantation positively affects muscle mass and function, partially by modulating autophagy and improving mitochondrial function.

Previous data show that treadmill exercise delays cancer cachexia in mice, and that both endurance training and resistance training reduce inflammation in tumor-bearing rats (24–26). Consistently, voluntary wheel running has been reported to prevent cachexia and increase survival in mice hosting the C26 tumor (27), and also to improve muscle wasting in animals exposed to cisplatin (28). Despite these encouraging results, however, several factors such as anemia, cardiac dysfunction, and/or chronic fatigue may limit the exercise capacity in the cancer hosts (23). Indeed, data obtained on mice bearing the C26 tumor show that low-intensity endurance exercise started the day after tumor implantation does not improve, rather worse cancer-induced muscle wasting. Coupling exercise to erythropoietin (EPO) administration results in prevention of anemia, rescue of oxidative myofiber atrophy, inhibition of the oxidative–glycolytic myofiber shift, and enhanced PGC-1α expression (2). Similar results have also been obtained in mice implanted with the Lewis lung carcinoma, which are partially protected from the loss of both muscle mass and strength by combining endurance exercise with the administration of eicosapentenoic acid (13). The results reported in the present study show that combined training before and during tumor growth is able to reduce the loss of muscle force and mass in the C26 hosts, in the absence of any additional treatment. Such observation suggests that the different pathways addressed by the two exercise modes likely synergize, eventually improving muscle metabolism. Of particular interest is the observation that combined training does not modulate tumor growth, differently from previously reported data on tumor-bearing mice voluntary running on a wheel (29). Along this line, it is unlikely that reduced tumor burden-related factors may account for the protective effects exerted by combined training. Another peculiar aspect of the present study is that training starts 4 wk before tumor implantation and is continued until the day before sacrifice (see Fig. 1). Such a protocol generates a sort of preconditioning, allowing the animals to adapt to exercise-induced stress well before the superimposition of tumor growth. Consistent with this hypothesis, we previously demonstrated
that endurance exercise alone does not rescue tumor-induced muscle wasting in any of the tested experimental setting (the day after tumor implantation or 6 wk before (2). However, if exercise is started the day after tumor implantation, muscle mass depletion is even worsened, suggesting that in addition to be ineffective, exercising the animals only while the tumor is growing might be deleterious. The present study does not allow to draw conclusions about the possibility that combined exercise can do better than endurance training if applied just during the tumor growth period. Despite interesting, this result cannot be achieved in the present experimental setting because the training procedure is quite complicated and the right adaptation level would be reached when muscle wasting is overt, which is too late to be effective (see also Khamoui et al. [5]).

Exercise is known to affect muscle mass and function by impinging on both local (myofiber metabolism) and systemic (inflammation) factors. In this regard, the anti-inflammatory effects of exercise are well known, as observed in healthy subjects (30) and cancer hosts (31). On the other side, muscle mitochondrial dysfunction has been reported to play a crucial role in the onset and progression of cachexia (32). Endurance exercise, in particular, leads to enhanced PGC-1α expression, likely promoting mitochondrial biogenesis and oxidative metabolism (33). In addition, genes involved in mitophagy, the process in charge of clearing damaged mitochondria, are also induced by endurance training (34). In this regard, the regulation of mitophagy has been proposed to depend also on PGC-1α (35). Consistently, the decreased expression of both PGC-1α and molecules involved in mitochondrial dynamics has been shown to be associated with reduced mitochondrial content in ApcMin/+ mice, a genetic model of cancer cachexia (36). The general idea derived from the present study is that the positive effects achieved by combined exercise in the C26 hosts do not likely derive from inhibition of systemic inflammation, although this has been just broadly inferred by the lack of modulations of cancer-induced spleen enlargement. Further studies investigating the propensity of inflammatory cells to release cytokines after combined training will allow to better clarify this point. On the other side, combined training reduced LC3B-II/I ratio (tibialis) and LC3B gene expression (gastrocnemius), only suggesting, in the absence of a flux experiment, that it proved effective in modulating autophagic sequestration.

**FIGURE 5**—Densitometric analysis and representative blots of PGC-1α (A), cytochrome c (B), SDHA (C), and SDH total enzymatic activity (D) in tibialis muscles of controls (C; n = 6), tumor bearers (C26; n = 6), exercised controls (Ex; n = 6), and exercised tumor bearers (C26 Ex; n = 6). Protein data (mean ± SD) are expressed as percentages of controls. SDH activity data (mean ± SD) are expressed as the ratio between absorbance and total protein content. Significance of the differences: *P < 0.05; **P < 0.01 vs controls. ***P < 0.05 vs C26. ****P < 0.05 vs C Ex.
This observation is in line with the restoration of muscle function and with the improvement of muscle mass, although this latter is only marginal in the gastrocnemius. In addition, it is consistent with previous data obtained in mice bearing the C26 tumor exposed to voluntary wheel running (27) or to exercise and EPO (2); in both experimental settings, LC3B-II protein levels are reduced with respect to untreated tumor-bearing mice. The association of exercise with EPO also normalized Bnip3 expression, suggesting an improvement of mitochondrial clearance (2).

The combination of exercise with EPO or eicosapentenoic acid was able to increase PGC-1α expression (2,28), and similar results were induced in healthy adults by combined exercise (37). In the present study, however, just a trend toward increased PGC-1α levels could be observed in the muscle of the C26 hosts exposed to combined training. In this regard, no data are actually available in the literature describing the effectiveness of combined exercise modality on PGC-1α expression in conditions associated with muscle wasting. Although combined exercise does not increase the expression of molecules involved in mitochondrial biogenesis (PGC-1α) or abundance (cytochrome c and SDH), it is able to partially prevent the reduction of SDH activity induced by tumor growth, suggesting that mitochondrial function is preserved in exercised versus sedentary tumor-bearing mice. This observation is consistent with previous reports showing that exercise coupled to EPO administration preserves the number of oxidative fibers in the tibialis muscle of C26 hosts (2). Similarly, combined exercise in healthy volunteers exposed to 2 wk of knee brace-inducted disuse has been shown to result in increased activity of muscle citrate synthase and cytochrome c (37). The possible improvement of mitochondrial function exerted by combined exercise likely plays a role in the restoration of muscle function, and it might also contribute to the maintenance of tibialis mass, as suggested by previous studies (36).

On the whole, the present study shows that a protocol based on combined training started before tumor implantation partially protects tumor-bearing mice from the loss of muscle mass and function in the absence of any additional pharmacological treatment. The mechanisms by which the combination of the two exercise modes achieves such effects are still unclear. The endurance component is likely responsible for preserving SDH activity, suggesting a prevention of cancer-induced energy deficit that could result in improved muscle function, consistent with previous observations (5). On the other side, the resistance component of the training protocol used in the present study might have induced an anabolic signal, as suggested by data in the literature reporting increased muscle IGF-1 mRNA expression (5). Finally, both resistance and endurance training could have downregulated autophagy (38). Although the results presented here sound interesting, the study suffers from some limitations. As stated before, it is not possible to understand if combined training must be adopted before tumor implantation to be effective. Although this is highly relevant, because the institution of such training in cancer patients would necessarily start when the tumor is already established, the same limitation applies to other treatment options. As an example, the protective action against cachexia exerted by soluble Activin receptor relies on its administration before and after tumor implantation (39).

Another important point is that the present data do not generalize the effectiveness of combined exercise on muscle mass. Indeed, significant changes can be observed in the tibialis, but the gastrocnemius response appears to be marginal, likely due to intragroup variability. In this regard, further studies will clarify this point. Other weaknesses of the study are the lack of autophagic flux measurements and the presence of a single experimental point (day 11 after C26 implantation), which could have masked intermediate changes in PGC-1α expression. In addition, further investigations are needed to clarify the molecular mechanisms underlying the positive effects of combined exercise in tumor-bearing mice. Despite these limitations, however, the present results suggest that the exercise mode adopted here might be a useful component of a multitask approach to treat cancer cachexia.

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The authors declare no conflict interests. Authors declare that the results of the present study do not constitute endorsement by the American College of Sports Medicine and that the data are presented clearly without inappropriate manipulation.

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