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Aerobic training, but not creatine supplementation, alters the gluteus medius muscle\textsuperscript{1,2}

F. H. F. D’Angelis*, G. C. Ferraz*, I. C. Boleli*, J. C. Lacerda-Neto†, and A. Queiroz-Neto*\textsuperscript{3}

*Department of Animal Morphology and Physiology and †Department of Animal Surgery and Clinical Sciences, College of Agricultural and Veterinary Sciences, São Paulo State University, Jaboticabal Campus, Jaboticabal, SP, Brazil

ABSTRACT: The aim of the present study was to investigate the effect of oral supplementation of creatine on the muscular responses to aerobic training. Twelve purebred Arabian horses were submitted to aerobic training for 90 d, with and without creatine supplementation, and evaluated with respect to BW and BCS and to the area and frequency of the different types of muscle fibers in the gluteus medius. Supplementation consisted of the daily administration of 75 g of creatine monohydrate mixed into the ration for the 90 d of training. Physical conditioning was conducted on a high-performance treadmill, and training intensity was stipulated by calculating the velocity at which blood lactate reaches 4 mmol/L, determined monthly for each animal. The individual intensity of physical force at 80\% of aerobic threshold was established. Morphometry of gluteus medius muscle fibers was performed on frozen sections processed for histochemical analysis of myosin adenosine triphosphatase and immunohistochemistry of slow-contracting myosin. The results demonstrated that the animals maintained a moderate BCS without alteration of BW during the course of training, providing evidence of equilibrium between food intake and caloric expenditure during the study period. The present study demonstrated that aerobic training for 90 d caused hypertrophy of fiber types I (\(P = 0.04\)), IIA (\(P = 0.04\)), and IIX (\(P = 0.01\)), as well as an increase in the relative area occupied by type I fibers (\(P = 0.02\)) at the expense of type IIX fibers (\(P = 0.03\)), resulting in modifications of the contractile and metabolic characteristics of the gluteus medius muscle. It was not possible to show any beneficial effect from creatine on the skeletal muscle characteristics examined.

Key Words: Aerobic Training, Creatine, Gluteus Medius, Horses, Muscle Biopsy, Myosin ATPase

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Introduction

In recent years, special attention has been directed to the study of the adaptations that occur in skeletal striated muscle, emphasizing the modifications in its structural composition and biochemical characteristics in animals submitted to aerobic training programs. In a 3-mo program of submaximum training, Rivero et al. (1995a) showed significant hypertrophy of type I and IIA fibers and observed that endurance horses with better athletic performance have a larger aerobic capacity and proportionally lower anaerobic capacity in the

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3\textsuperscript{Correspondence: FCAV/UNESP, Via de Acesso Prof. Paulo Donato Castellane, 14884-900 (phone: 55-16-32092654; fax: 55-16-32024275; e-mail: aqueiroz@fcav.unesp.br).}
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and prolonged supplementation with creatine for 90 d, on muscle fibers in the gluteus medius. As a secondary objective, we tested the efficiency of the training protocol conducted exclusively on the treadmill in the physical conditioning of horses.

**Materials and Methods**

**Animals and Handling**

Twelve purebred Arabian horses were used, both males (five) and females (seven), with an initial BW of 391.0 ± 25.4 kg (mean ± SD) and mean age of 8.6 ± 3.3 yr. The animals selected for study were evaluated by clinical examination and laboratory tests, such as complete blood count and blood biochemistry. They had no signs or symptoms compatible with skeletal muscle lesions, and were deloused and dewormed with ivermectin (Ivotan LA, Intervet, São Paulo, Brazil) at intervals of 40 and 60 d, respectively.

The 12 horses were kept in one paddock for 8 mo of inactivity and then were divided randomly into two control groups (C₀ and CR₀). Both groups were subjected to aerobic training for 30, 60, and 90 d, but only one group (CR₀) received 75 g of creatine monohydrate (Vetnil, Louveira, Brazil) per animal per day for the same period (CR₃₀, CR₆₀, CR₉₀), whereas the other group served as the control group (C₃₀, C₆₀, and C₉₀) and received no supplementation. The animals were kept in a paddock of Brachiaria sp. grass, with ad libitum access to water and supplementation with Cyndon sp. hay and mineralized salt (Agromix, Jaboticabal, Brazil). Creatine was given individually in the stall, and mixed with half the daily commercial ration (Nu-triage Mix Guabi, Campinas, Brazil) followed by the rest of the ration to make it sure that all the creatine was ingested by the horse.

To determine the daily quantities (2.5 to 3.8 kg/animal) of commercial diet furnished, the animals were evaluated monthly for BW and BCS using the protocol recommended by Henneke et al. (1983). This is a scoring system for the accurate comparison of stored body fat in horses, evaluated on a scale of 1 to 9, with 1 being extremely emaciated and 9 being extremely overweight.

The experimental protocol was approved by the University’s Institutional Animal Care and Use Committee.

**Physical Conditioning**

Physical conditioning of each animal was performed in a climate-controlled room, which contained a high-performance treadmill (Esteira Galloper 5500, Sahinco Ltda, São Paulo, Brazil). Before initiation of the training program, the horses were submitted to 30 d of adaptation to handling. The training program was conducted exclusively on the treadmill because Evans (2000) certified that the training on a treadmill is effective for the physical conditioning of horses. The velocity (intensity) was set at 80% of the velocity at which the blood lactate concentration reached 4 mmol/L ($V_{\text{lact4}}$). For determination of the $V_{\text{lact4}}$, the animals were submitted to an ergometric test, in which the velocity was increased (2, 4, 6, and 8 m/s) every minute, with the treadmill inclined at 6%. In this manner, regression analysis was used to determine the velocity at which blood lactate concentrations reached the aerobic threshold (4 mmol/L). At the end of each training period (30 d), a new ergometric test was conducted to establish a new $V_{\text{lact4}}$. The training velocity was thereby determined monthly for each animal.

The frequency of training consisted of the performance of exercises three times per week (alternate days). In the first month of training, each animal exercised for 10 km in a mean time of 50 min. In the second month, the distance run in each exercise was increased to 15 km, with a mean duration of 60 min. In the last month, all the horses exercised 20 km per session, with a mean duration of 80 min. In addition, a speed play type of training, which encompasses sudden, rapid, relatively short bursts of speed interspersed throughout the exercise bout, was instituted once per week with the aim of stimulating tissues not addressed by slower exercise and increasing the horse’s metabolic capabilities. This protocol was adopted because some authors (Ridgway, 1994; Evans, 2000) recommended strenuous training sessions as part of the endurance training to develop fitness for fast exercise.

**Biopsy of the Skeletal Striated Muscle Gluteus Medius**

A percutaneous muscle biopsy was performed according to the method of Lindholm and Piehl (1974) by using a 6.0-mm Bergström-type needle. Two muscle biopsies were taken before (left muscle) and after (right muscle) 90 d of training from each animal. The gluteus medius is a muscle frequently studied with respect to effects of training and detraining in athletic horses because this muscle shows the greatest activity and capacity for propulsion during locomotion and because it is also easy to access (Lindholm and Piehl, 1974). The fragments of gluteus medius muscle were removed at the same depth (60 mm), with the needle bevel in a caudal position, and frozen in prechilled hexane for approximately 40 s (Dubowitz, 1985), kept frozen in liquid N, and later stored in a deep freezer (Bio-freezer Forma Scientific; Instrucom Ind. Com. Ltda, São Paulo, Brazil) at −70°C until processed. The interval between obtaining and freezing biopsies was 3 min to avoid methodological problems due to the muscle shrinking that occurs when a biopsy is performed (Dubowits, 1985).

**Histochemistry**

Histochemical analysis was utilized to identify the type I, IIA, and IIX muscle fibers. The histochemical method used was an adaptation of metachromatic
staining for mATPase activity in myofibers as described by Olgivie and Feeback (1990), and also used some steps from Guth and Samaha (1970) and Ennion et al. (1995). Five cryosections, semi-serial 12-μm-thick cross-sections were analyzed in each sample of the gluteus medius muscle. The sections were kept at room temperature for 30 to 40 min to dry and adhere to slides. They were then fixed for 6 min at room temperature (22 to 25°C) in buffered 5% formalin, pH 7.2, containing 0.17 M NaCl, 336 mM saccharose, and 0.13 M sodium cacodylate (Guth and Samaha, 1970). After successive washings in 21 mM Tris buffer, pH 7.8, containing 3.4 mM calcium chloride (pH adjusted with 5 N HCl; Guth and Samaha, 1970), the sections were preincubated in acid medium (pH 4.52 to 4.55) containing 52 mM potassium acetate and 17.7 mM CaCl₂ for 5 to 6 min at 22 to 24°C (Olgivie and Feeback, 1990). They were washed using the same buffer and procedure described above, and incubated according to the procedure described by Ennion et al. (1995), in basic medium (pH 10.50 to 10.55) containing 40 mM glycine, 20 mM CaCl₂, and 2.5 mM ATP (Sigma, St. Louis, MO) at 37°C for 25 min. Next, the sections were washed rapidly in distilled water and incubated in 1% CaCl₂ for 3 min, washed in distilled water, stained with 1% toluidine blue, dehydrated rapidly in a series of increasing concentrations of ethanol, cleared in xylene, and mounted in Entellan (Olgivie and Feeback, 1990).

Muscle fibers were identified based on the following staining: type I (light blue), type IIA (medium blue), and type IIX (dark blue), as shown in Figure 1, according to the classification of Serrano and Rivero (2000). The confirmation that different colors and fibers types correlate dependably was done by immunohistochemical analysis that differentiates type I from type II fibers. The subtypes of the type II fibers were confirmed based on the size of the cross-sectional area (CSA) because it is known that type IIX has a larger area (Rivero et al., 1993a,b).

Immunohistochemistry

Two 12-μm-thick serial cross-sectional cryosections were obtained from the same samples used for histochemical studies, and both were prepared simultaneously. The sections were submitted to the indirect immunohistochemical (peroxidase antiperoxidase) method to stain fibers containing slow-contracting myosin. The sections were incubated with the primary monoclonal antibody antislow myosin (Clone NOQ7.5.4D; Sigma) and with secondary antibody goat anti-mouse immunoglobulin G conjugated with peroxidase (Sigma). The antigen–antibody complexes were visualized by incubation with diaminobenzidine (0.2 mg/1.5 mL of 0.1 M phosphate buffer, pH 7.2). Slow-contracting fibers (type I) and fast-contracting fibers (type II) were identified by golden-brown staining in the former and the absence of staining in the latter (Figure 1).

Morphometry of Muscle Fibers

The variables examined were CSA of each type of fiber, relative frequency of each type of fiber per microscopic field analyzed, and relative cross-sectional area that a fiber type occupied in the biopsy specimens. The mean CSA of the fibers was calculated in micrometers squared from the measurement of 100 cross sections of each type of fiber. Mean frequency was obtained as the percentage of the total number of fibers present per microscopic field analyzed, whereby four fields measuring 585,000 μm² selected randomly from histological sections were examined. The relative cross-sectional area was determined as a percentage according to Rivero et al. (1993a). The morphometric data were obtained from photomicrographic images of the histological sections (Camedia Olympus 95-98 ME, Olympus, São Paulo, Brazil) and evaluated by an image-analyzing program (Image Pro Plus, Cybernetica, São Paulo, Brazil).

Statistical Analyses

Body weight was studied using a factorial split plot in time design (3 × 2 + 1) considering three periods of training (30, 60, and 90 d), two treatments (animals trained, with and without creatine supplementation), and one additional treatment related to the untrained horses. The ANOVA was performed considering training split in periods of training. The means were compared by Tukey’s test at P < 0.05.

Regarding the morphometric analysis, the paired Student’s t-test (P < 0.05) was applied to compare the means of the different fiber type variables, before and after the training period (C₀ and C₉₀; Cᵣ₀ and Cᵣ₉₀). The Student’s t-test for nonpaired samples (P < 0.05) was used to compare the means of the groups that were submitted to the training protocol and either did or did not receive creatine supplementation (C₉₀ and Cᵣ₉₀); also, means of the groups that were not submitted to the training protocol and did not receive creatine supplementation (C₀ and Cᵣ₀). Friedman’s nonparametric test was used to analyze BCS, and the values are reported as medians.

Results

The main finding of this study was the lack of a significant effect of creatine supplementation on muscular responses to endurance training.

Mean BW did not differ significantly (P = 0.749, 0.075, and 0.873 for 30, 60, and 90 d, respectively) between the groups over the course of training (Table 1). Similarly, BCS did not show a significant difference between groups and among times of training.

In relation to CSA (Table 2), a comparison between groups C₀ and C₉₀ showed that there was a significant increase in the CSA of the three types of fibers (P = 0.041, 0.044, and 0.010 for types I, IIA, and IIX, respec-
Figure 1. Serial sections of gluteus medius muscle biopsy from a horse submitted to 90 d of aerobic training and creatine supplementation. A) Histochemistry for the analysis of myosin adenosine triphosphatase activity. Fiber type I = light blue; type IIA = medium blue; and type IIX = dark blue. Panel B = immunohistochemistry for slow-contracting myosin. Fiber type I = brown, and type II = absence of staining; 200×.

Effectively) with submaximal training of 90 d. In relation to the groups CR₀ and CR₉₀, there was no significant difference between the groups for the CSA of type I fibers (P = 0.062). There also was a significant increase in CSA of type IIA (P = 0.005) and type IIX fibers (P = 0.042). When we compared the C₀ with the CR₀, the analysis did not show any significant difference for CSA of any fiber types (P = 0.346, 0.676, and 0.953 for types I, IIA, and IIX, respectively). Regarding the comparison between C₉₀ and CR₉₀, the analysis similarly did not show any significant difference for CSA of any fiber types (P = 0.386, 0.569, and 0.112 for types I, IIA, and IIX, respectively).

An analysis of frequency percentage for the different types of fibers (Table 2) in animals of groups C₀ and C₉₀ showed no significant difference for type I (P = 0.125) and IIA fibers (P = 0.748), whereas frequency for type IIX fibers diminished significantly (P = 0.008) with training. Comparing groups CR₀ and CR₉₀, no significant change was noted in the frequency of any of the fiber types with training and creatine supplementation for 90 d (P = 0.136, 0.512, and 0.193 for types I, IIA, and IIX, respectively). When we compared the C₀ with the CR₀, the analysis did not show any significant difference for frequency of any fiber types (P = 0.551, 0.625, and 0.784 for types I, IIA, and IIX, respectively). The same finding was noted when comparing the C₉₀ with the CR₉₀ (P = 0.934, 0.687, and 0.851 for types I, IIA, and IIX, respectively).

Comparison of RCSA values between groups C₀ and C₉₀ (Table 2) showed a significant increase (P = 0.003) for type I fibers but no significant change for type IIA
and 0.789 for types I, IIA, and IIX, respectively). The same finding was noted when we compared CR90 and CR0 did not show any significant difference for RCSA = 0.062), and no significant difference for type IIA fibers (P = 0.0985, 0.123, and 0.67 for types I, IIA, and IIX, respectively).

The comparison between uncorrelated groups C0 and CR0 revealed a significant increase in RCSA for type I fibers (P = 0.022), a significant decrease for type IIX fibers (P = 0.002), and no significant difference for type IIA fibers (P = 0.062).

The comparison between uncorrelated groups C0 and CR0 did not show any significant difference for RCSA of any fiber types (P = 0.0985, 0.112, and 0.67 for types I, IIA, and IIX, respectively). The same finding was noted when we compared C90 and CR90 (P = 0.765, 0.123, and 0.789 for types I, IIA, and IIX, respectively).

**Discussion**

In relation to the effects of creatine supplementation, it was not possible to show any beneficial effect on the skeletal muscle characteristics examined. In humans, investigators have reported that creatine supplementation increases fat-free mass (Vandenberghe et al., 1997; Volek et al., 1997; Kreider et al., 1998). The former authors suggested that this substance exerts an anabolic action on the skeletal muscle, causing an increase in cell volume. Volek et al. (1997) speculated that the BW gain was due mainly to an increase in total body water because creatine is an osmotically active substance that may induce the influx of water into the cell. Nevertheless, in a recent study, Powers et al. (2003) found that supplementation resulted in water retention without altering fluid distribution. In the present study, no significant increase in BW was seen. These findings agree with those of Schuback et al. (2000), who noted that horses would have to gain a considerable amount of BW to show a significant increase. Another possible reason to explain our findings could be the difference in the absorption mechanisms between humans and horses.

| Table 1. Mean body weight (±SE) and median body condition score of purebred Arabian horses, untrained and after aerobic training for 30, 60 and 90 d, with and without creatine supplementation |
|----------------------------------|-----------------|-----------------|-----------------|-----------------|
| Variables                        | C0 + CR0        | C30             | CR30            | C60             |
| No. of horses                    | 6 + 6           | 6               | 6               | 6               |
| BW, kgb                         | 391.0 ± 10.4    | 390.7 ± 6.5     | 404.5 ± 8.0     | 382.8 ± 6.6     |
| BCSc                            | 5               | 5               | 5               | 5               |

| Table 2. | Mean (±SE) cross sectional area, frequency, and relative cross sectional area of each fiber type in the biopsy specimens of the gluteus medius muscle in purebred Arabian horses submitted to aerobic training for 90 d, with and without creatine supplementation |
|----------------------------------|-----------------|-----------------|-----------------|-----------------|
| Variables | C0              | C90             | CR0             | CR90            |
| No. of horses | 6               | 6               | 6               | 6               |
| Mean cross sectional area, μm²b |                 |                 |                 |                 |
| Fiber type I                    | 1,938.0 ± 302c  | 2,891.5 ± 104d  | 2,402.6 ± 359e  | 3,252.8 ± 384e  |
| Fiber type IIA                  | 2,719.8 ± 338c  | 3,775.4 ± 255d  | 2,502.6 ± 375e  | 4,126.5 ± 538d  |
| Fiber type IIX                  | 4,410.7 ± 630c  | 7,252.8 ± 754d  | 4,357.6 ± 621c  | 5,865.5 ± 261d  |
| Frequency, %b                   |                 |                 |                 |                 |
| Fiber type I                    | 17.0 ± 2.7c     | 25.3 ± 3.3c     | 16.1 ± 1.9c     | 24.7 ± 3.6c     |
| Fiber type IIA                  | 37.0 ± 2.0c     | 36.5 ± 2.5c     | 37.6 ± 1.5c     | 36.3 ± 1.0c     |
| Fiber type IIX                  | 46.0 ± 1.2c     | 38.2 ± 2.1c     | 46.3 ± 2.3c     | 39.0 ± 3.6c     |
| Relative cross sectional area, %b|                 |                 |                 |                 |
| Fiber type I                    | 9.8 ± 0.6c      | 15.2 ± 1.7d     | 11.7 ± 0.9c     | 17.9 ± 1.7d     |
| Fiber type IIA                  | 29.6 ± 0.9c     | 27.8 ± 1.1c     | 28.5 ± 1.7c     | 32.3 ± 2.5c     |
| Fiber type IIX                  | 61.2 ± 3.1c     | 57.0 ± 3.3c     | 59.8 ± 3.1c     | 49.8 ± 3.0d     |

*ac = animals before training; CR0 = animals before training and supplementation with creatine; C90 = animals trained for 90 d and not supplemented with creatine; CR90 = animals trained and supplemented with creatine for 90 d.

bComparison between uncorrelated groups (C0 and CR0; C90 and CR90) for each type of fiber (within row) indicated that means did not differ significantly, according to Student’s t-test for unpaired samples.

cdMeans followed by different letters differ (P < 0.05) for comparison between correlated groups (C0 vs. C90 and CR0 vs. CR90) for each type of fiber (within row), according to Student’s t-test for paired samples.
Results of the BCS and BW showed that all horses maintained a moderate BCS without alteration of BW, providing evidence of equilibrium between food intake and caloric expenditure throughout the experiment.

The findings of the present study demonstrate that aerobic training for 90 d causes hypertrophy of the three types of fibers studied. These results agree with those of Tyler et al. (1998), who associated this effect to the high-intensity of the training protocol.

Hypertrophy of type I myofibers as a response to aerobic exercise also was observed by Serrano and Rivero (2000). These authors, who used long-duration training of much lower velocity (half the velocity producing a blood lactate concentration of 2 mmol/L) in Andalusian mares, did not find notable modifications of CSA of myofibers IIA and IIX after 3 mo of training, and argued that this period of training would not be sufficient to cause conversion of type IIX fibers to type IIA fibers, and then of type IIA fibers to type I fibers. Indeed, the method used in this experiment does not permit the discrimination of the IIA and IIXA fibers. However, as both fiber types are considered fast-contracting fibers, the method does not represent a limitation to the interpretation of the results regarding the effect of training on the area occupied by slow-contracting and fast-contracting fibers.

Serrano et al. (2000), using a method identical to that of Serrano and Rivero (2000), except with Andalusian stallions, found an increase in the size of type IIA fibers after a 3-mo training of long duration and low intensity; however, CSA of fibers type I and IIX did not show any significant difference. The differences observed in the present study in comparison with the literature cited above could be related to the greater intensity of physical force (80% of \( V_{\text{lact}} \)) to which the animals were submitted in our study.

Previous studies indicate that purebred Arabian horses competing in endurance events possess type I and IIA fibers in the gluteus medius muscle with greater CSA than in horses considered to be of moderate performance (Rivero et al., 1993b). In that regard, Rivero et al. (1995a) found significant hypertrophy of type I and IIA fibers in deep samples (60 mm) of gluteus medius muscle in purebred Arabian horses submitted to a 3-mo program of endurance training. The present study showed that the animals with better morphometry results had greater frequencies and area occupied by type I and IIA fibers after 3 mo of aerobic training, and that they also were better in athletic shape (greater values of \( V_{\text{lact}} \); data not shown) according to the ergometric test. The horses that showed less frequency and areas occupied by type I and IIA fibers displayed a poorer performance (lower values of \( V_{\text{lact}} \); data not shown) in the exercise tests. These findings reconfirm that horses with excellent athletic performance in an aerobic training program have greater frequencies of slow-contracting, oxidative muscle fibers.

Aerobic training for 90 d did not significantly alter the frequency of type I and IIA fibers, but did significantly decrease the frequency of fibers of type IIX. This decrease was not observed in the group treated with creatine, probably due to the larger standard deviation observed in this group. Considering that type I and IIA fibers have an oxidative metabolism, our data suggest that the training program used caused an increase in the aerobic potential of the muscle studied at the expense of glycolytic potential.

In animals trained for 90 d, the relative area occupied by type I fibers was greater than that in untrained animals, whereas type IIA and IIX fibers remained constant. The increase in area occupied by type I fiber could be a result of the hypertrophy noted in the trained horses. In turn, the maintenance of the same area occupied by type IIX fibers, despite the occurrence of hypertrophy, was due to the reduced frequency of this type of fiber. Muscle fibers exhibit a high capacity of adapting structurally and metabolically as a result of training (Rivero et al., 1995b; Serrano and Rivero, 2000). Our study revealed an increase in the relative area occupied by type I fibers, indicating that there was a modification of metabolic potential leading to an increase in muscle oxidative metabolism. It can therefore be established that this type of aerobic training induces improvement in aerobic capacity and physical conditioning of the animals.

Again, the results of morphometric evaluations of muscle fibers in the present study indicate an adaptive response of the gluteus medius muscle in aerobic training, increasing the oxidative capacity of the muscle.

The present study demonstrated that treadmill-based aerobic training for 90 d at 80% aerobic threshold induced hypertrophic growth of myofibers. It caused an increase in the area occupied by type I fibers at the expense of type IIX fibers, resulting in modifications of the contractile and metabolic characteristics of the gluteus medius muscle. However, it is important to emphasize the need for additional studies to determine whether myofiber hypertrophy was accompanied by increases in capillary and mitochondrial density, which could ensure the improvement of the aerobic oxidative potential of the training protocol adopted. Regarding the effect of creatine, no beneficial effect from the supplementation with this substance was detected on the skeletal muscle characteristics examined.

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