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Research article

CREATINE SUPPLEMENTATION INDUCES ALTERATION IN CROSS-SECTIONAL AREA IN SKELETAL MUSCLE FIBERS OF *WISTAR* RATS AFTER SWIMMING TRAINING

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ABSTRACT

Creatine has been shown to increase the total muscle mass. In this study, we investigated the effect of oral creatine monohydrate supplementation on cross-sectional area of type I, IIA and IIB fibers of gastrocnemius, *extensor digitorum longus* – EDL and soleus muscles from male *Wistar* rats subjected to swimming training for 33 days. Four groups were set up: sedentary with no supplementation (CON), sedentary with creatine supplementation (3.3 mg creatine per g *chow*) (CR), exercised with no supplementation (EX) and exercised with supplementation (CREX). The rats performed in a special swimming pool and swam five times a week for 1 hour each day, with a extra lead weight corresponding to 15% of their body weight. At the end of 33 days, skeletal muscles of the animals were dissected and the samples got immediately frozen using liquid nitrogen. Muscle samples were allocated to slices of 10 µm by a cryostat at -20ºC, which was followed by histochemical analysis in order to identify fiber types of the muscles, and morphometrical analysis to calculate the muscle fiber areas. All groups gained body weight at the end of 33 days but there was no statistical difference among them. The EX and CREX rats had a larger food intake than the sedentary groups (CON and CR), and the CREX group had a larger food intake than CR rats. The cross-sectional area of type I and IIA fibers of the soleus muscle, type IIA and IIB fibers of EDL muscle and type IIA and IIB fibers of the white portion of gastrocnemius muscle were greater in the EX and CREX groups in comparison to sedentary rats. In addition, these fibers were greater in the CREX rats than in the EX group. There was no change in the cross sectional area of type I fibers in EDL muscle among all groups studied. Our results suggest that creatine supplementation enhances the exercise related muscle fiber hypertrophy in rodents.

KEY WORDS: Creatine, skeletal muscle fiber, exercise, morphometry, histochemistry.

KREATİN ALIMININ YÜZME ANTRENMANI SONRASI *WISTAR* SIÇANLARIN İSKELET KASI LİFLERİNİN KESİT ALANIDAKİ DEĞİŞİKLİKLERE ETKİSİ

ÖZET

Kreatinin toplam kas kitlesini artırdığı gösterilmiştir. Bu çalışmada oral kreatin monohidrat alımının 33 gün süre ile yüzme antrenmanı yapan erkek *Wistar* sıçanların ekstansor digitorum longus –EDL ve soleus kaslarının tip I, IIA ve IIB liflerinin kesit alanı üzerine etkisini araştırdık. Dört grup oluşturuldu: ek gıda lamayan sedanter (CON), kreatin alan sedanter (3.3 mg kreatin her gr yiyecek başına) (CR), ek gıda almayan egzersiz yapan (EX) ve ek gıda alan egzersiz yapan (CREX). Sıçanlar özel yüzme havuzunda vücut ağırlıklarının %15'i ekstra yükle haftada 5 gün, her gün 1 saat yüzdürüldü. 33 günün sonunda hayvanların iskelet kasları disseke edilip hemen likit nitrojende dondurulduu. – 20° C'da bir kriostat kullanarak 10 µm kesitler alındı, takiben morfometrik (kas liflerinin alanı hesaplamak için) ve histokimyasal analizler (kas liflerini tanımlamak için) yapıldı. 33 günün sonunda bütün gruplarda vücut

ağırlığı arttı, fakat gruplar arasında istatistiksel farklılık yoktu. EX ve CREX sıçanlar sedanter gruplardan (CON ve CR) daha fazla yiyecek aldı ve CREX grup CR sıçanlardan daha fazla yiyecek tüketti. Beyaz gastroknemius kasının IIA ve IIB lifleri, EDL'nin IIA ve IIB lifleri ve soleusun tip I ve IIA liflerinin kesit alanları EX ve CREX gruplarda sedanter sıçanlarla karşılaştırıldığında daha büyüktü. EDL kasının tip I liflerinin kesit alanı çalışmadaki gruplar arasında değişiklik göstermedi. Sonuçlarımız kreatin alımının kemirgenlerde egzersizle ilişkili kas lif hipertrofisine katkı sağladığına işaret etmektedir.

ANAHTAR KELİMELER: Kreatin, iskelet kas lifi, egzersiz, morfometri, histokimya.

INTRODUCTION

Phosphocreatine plays a key role in energy provision to muscle (Jenkins, 1998). It has been administered for therapeutic benefits in patients with AIDS, muscle diseases, neuropathies and post-surgery (Mihic et al., 2000). Creatine supplementation has been used mainly to increase muscle performance during exercise (Balsom et al., 1994; Greenhaff, 1995; Volek and Kraemer, 1996; Volek et al., 1997b; Tarnopolski and Martin, 1999). Phosphocreatine content in type II human muscle fiber is 5 – 15% higher than type I (Greenhaff et al., 1994). Following creatine supplementation the total creatine and phosphocreatine content raises in both fiber types, although there is a tendency for more increase in type II fibers (Casey et al., 1996).

Creatine supplementation studies using different protocols and animal species, including humans, have been shown to increase body weight and change in the body composition (Viru et al., 1994; Balsom et al., 1995; Mujika et al., 1996; Kreider et al., 1998; Managaris and Maughan, 1998; Engelhardt et al., 1998; Volek et al., 1999). The underlying basis of this weight gain is still unclear. It may be due to stimulation of muscle protein synthesis or water retention in the initial days of creatine supplementation. Other authors, however, reported contradictory results (Thompson et al., 1996; Terrillion et al., 1997; Stout et al., 1999; Rico-Sanz and Marco, 2000). The explanation for these discrepancies still remains to be clarified. In this study, therefore, we investigated whether the sole creatine supplementation changes the cross-sectional area of skeletal muscle fibers or it is associated with the addition of training in *Wistar* rats.

METHODS

Chemical and enzymes

All chemicals and enzymes used were obtained from Sigma Chemical Co., St. Louis, USA.

Study design

The National Animal Ethics Committee approved this study. Male *Wistar* rats (2-3 months old) were kept in standard individual vivarium cages, for food intake and body weight determination, under a controlled light/dark (12/12h) cycle and temperature (23º C ± 1) with free access to food and water. The rats were randomly divided into four groups: Sedentary with no supplementation (CON), Sedentary with creatine supplementation (CR), Exercised with no supplementation (EX) and Exercised with supplementation (CREX). The creatine-enriched diet consisted of normal rat chow supplemented with 3.3 mg creatine per g of diet (Advanced Nutrition Ltda, Brazil) according to Brannon et al. (1997).

Exercise training

EX and CREX rats exercised in swimming pool chambers at a water temparature of 32ºC. The protocol used was similar to that described by Rombaldi (1996). Briefly, the animals swam daily for 1 hour during 5 weeks carriying an extra-weight of lead corresponding to 15% of their body weight. During weekdays, except Wednesday, the rats swam with the extra-weight. This protocol has been considered as a predominantly anaerobic supra-maximal workload (Kokobum, 1990). The rats swam for 15 seconds and rested 15 seconds for another for 15 seconds consecutively during the first 30 minutes. Following 10 minutes interval of resting, the same protocol was repeated for another 30-minutes period. At Wednesdays, the rats swam for 1 hour long with no break.

Tissue processing

Following last exercise session, the animals were killed by cervical dislocation and the gastrocnemius (white portion), soleus and EDL (*extensor digitorum longus*) muscles were dissected. Each muscle was cut in the middle, transversally and one part was quickly frozen in tissue freezing medium (Jung-Germany). Serial slices of 10 μm were allocated in a cryostat at –20 ºC and mounted on glass slides and was let to dry at room temperature. Then, histochemical procedure of myofibril ATPase staining was carried out to identify the fiber types I and II. This method enables differential staining of muscle fibers by utilizing the varying sensitivities of their mATPase to acid or alkaline pH (Brooke and Kaiser, 1970). Utilizing

adjustment of pH in the pre-incubation to 4.6 produces a separation of different staining intensities: dark (type I), light (type IIA) and intermediate (type IIB) (Hämäläinen and Pette, 1993). The fiber type proportions from each muscle group were calculated by the formula suggested by Sullivan and Armstrong (1978), Armstrong and Phelps (1984). The slide images were obtained in a Zeiss (Germany) microscope connected to a computer; 5 to 20 fibers were randomly chosen for each section for measurement of cross sectional areas using the Image Tool software

Statistical Analysis

Statistical analysis was performed by one-way ANOVA followed by a post-hoc Tukey test. The level of $p < 0.05$ was taken to indicate statistical significance.

RESULTS

All groups gained weight by the end of the experiment. At the 33rd day, in relation to day zero, CON group increased body weight by 21.6%, CR by 17.6%, EX by 21.4% and CREX by 17.6% (Figure 1), those values were not statistically different among the groups.

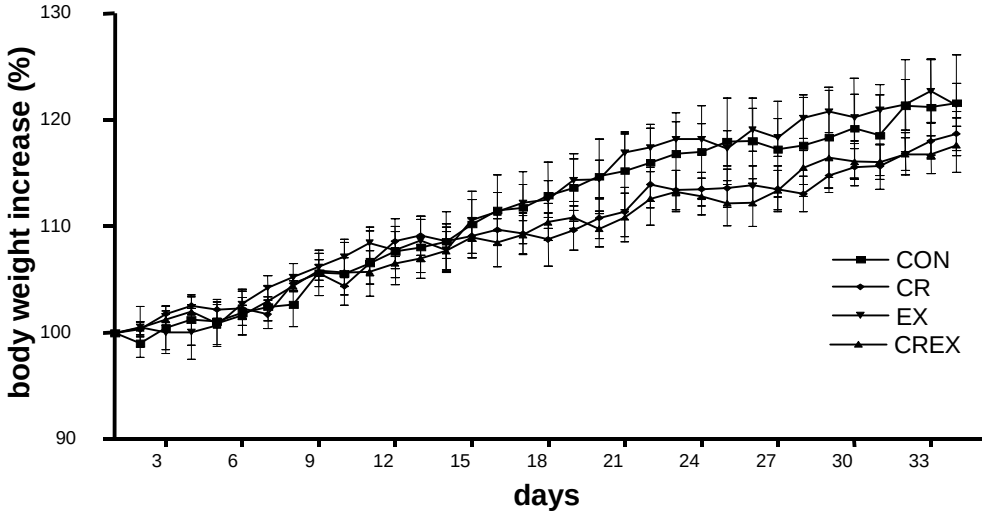

Figure 1. Body weight increases in percentage (%) of sedentary with no supplementation (CON, n = 9), sedentary with creatine supplementation (CR, n = 10), exercised with no supplementation (EX, n = 10) and exercised with supplementation (CREX, n = 11). Data are presented as mean ± SEM.

Food intake (Figure 2) was increased in the CON by 19.6%, in the CR by 25.3%, in the EX by 32.6% and in the CREX by 35.8%. The daily food intake was statistically different between EX and CREX as compared to CON and CR groups, respectively ($P<0.05$). However, there was no statistical difference between CON and CR ($P>0.05$) as well as between EX and CREX ($P>0.05$). Creatine intake was higher (41%) in CREX as compared to CR in the 33th day (Figure 3). The differences stated for food and creatine intake started at the second week and was maintained until the end of the experiment.

In the soleus muscle the proportion of type I and IIA muscle fibers was, in average, 90.5% and 9.5%, respectively. In the EDL muscle, type I was 9.5%, type IIA 21% and type IIB 69.4%. In the white portion of the gastrocnemius muscle, the proportion of type IIA and IIB was 70.6% and 29.4%, respectively (Figure 4C). A simple observation of the dye staining in the soleus muscle indicates the presence of types I and IIA muscle fibers with predominance of type I (Figure 4A). In the EDL muscle (4B), it was mainly type IIB followed by IIA and a lower proportion of type I muscle fibers. In the white portion of the gastrocnemius muscle, it was observed that type IIA fibers were more predominant than type IIB fibers (4C).

The data for cross-sectional areas of these muscle fibers are presented in the Table 1. The magnitude of the areas of type I and IIA fibers in soleus muscle were not different between the CR and CON groups. However, in the EX group, the muscle fiber areas for type I and type IIA were increased by 10.8% and 16.9% as compared to CON and by 16.0% and 20.8% as compared to CR, respectively. The combination of exercise and creatine supplementation (CREX) increased the areas of type I and IIA fibers by 25.5% and 29.4% as compared to CON, by 31.5% and 33.8% as compared to CR, and by 13.3% and 11.4% as compared to EX, respectively.

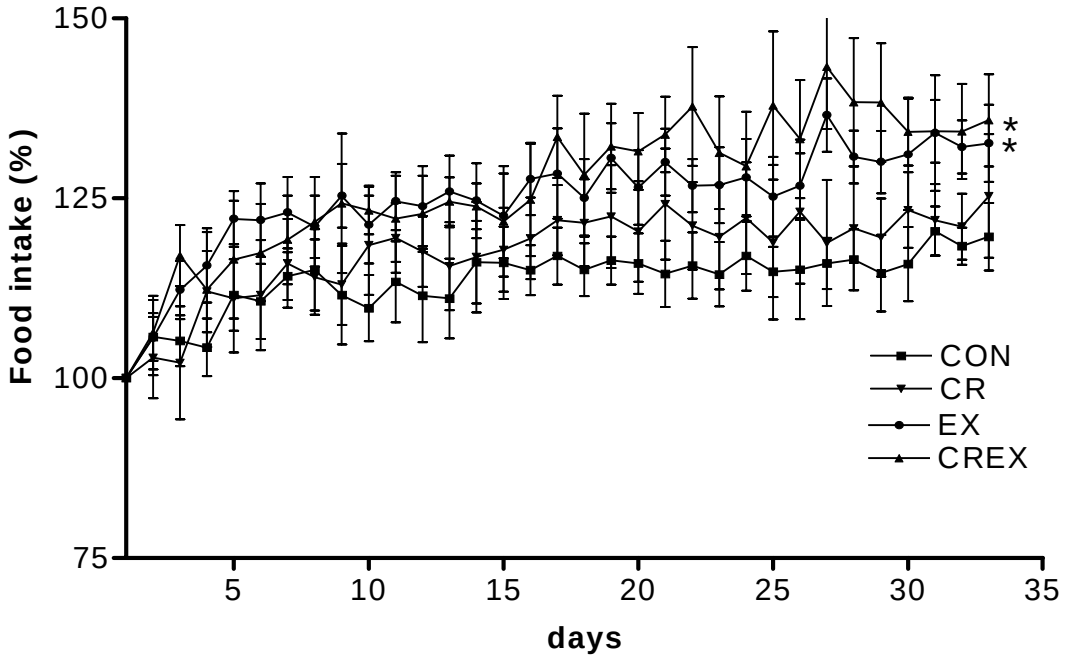

Figure 2. Food intake increases in percentage (%) along 33 days in the sedentary with no supplementation (CON, n = 9), sedentary with creatine supplementation (CR, n = 10), exercised with no supplementation (EX, n = 10) and exercised with supplementation (CREX, n = 11). * Statistical significance ($p < 0.05$) as compared to CON and CR. Data are presented as mean ± SEM.

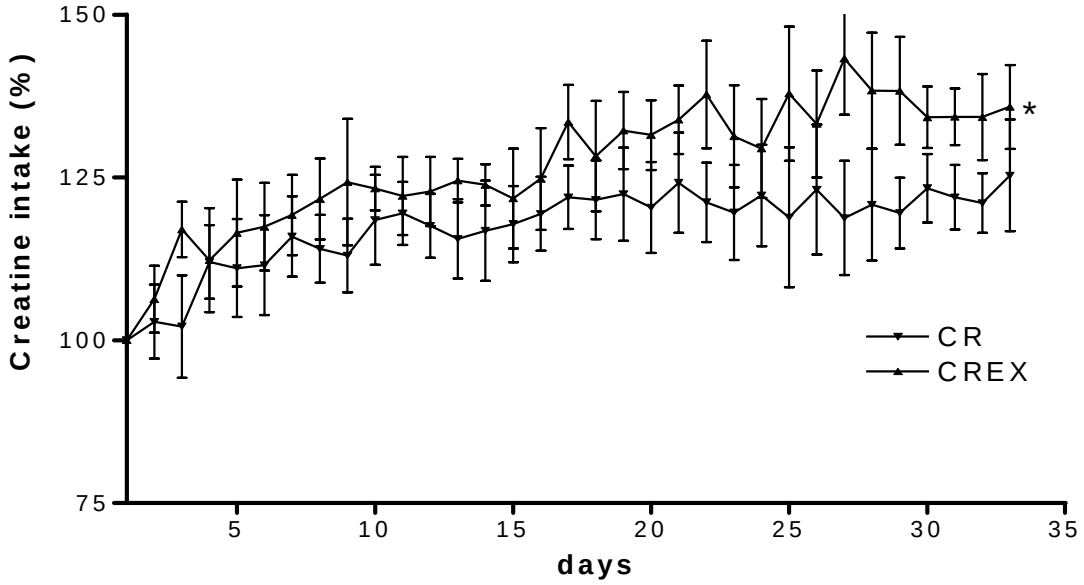

Figure 3. Percentage (%) of creatine intake along 33 days in the sedentary with creatine supplementation (CR) (n=10) and exercised with creatine supplementation (CREX) (n=11). Data are presented as mean ± SEM. *Statistical significance ($p < 0.05$) as compared to CR

In EDL muscle the magnitude of the cross-sectional areas of type IIA and IIB fibers were not different between CR and CON. In the EX group, these muscle fibers areas were increased by 27.7% and 19.3% as compared to CON and by 20.3% and 9.7% as compared to CR, respectively. The combination of exercise and creatine supplementation (CREX) increased the areas of type IIA and IIB muscle fibers by 41.9% and by 36.6% as compared to CON, by 33.7% and by 25.6% as compared to CR, and by 11.1% and 14.4% as compared to EX, respectively. The magnitude of the type I fibers in EDL muscle were not altered by either exercise or creatine supplementation (Table 1).

Table 1. Cross-sectional area (μm^2) of type I, IIA and IIB fibers from soleus, *extensor digitorum longus* - EDL and gastrocnemius (white portion) muscles of sedentary with no supplementation (CON), sedentary with creatine supplementation (CR), exercised with no supplementation (EX) and exercised with supplementation (CREX). Data are presented as mean (SEM). * $p < 0.05$ as compared to CON and CR. § $p < 0.05$ as compared to EX.

Muscle-Fiber type	CON (n=9)	CR (n=10)	EX (n=10)	CREX (n=11)
***Soleus*-I**	3833 (97) (n=100)	3659 (96) (n=90)	4246 (80) * (n=95)	4812 (68) *, § (n=93)
***Soleus*-IIA**	3553 (117) (n=25)	3436 (136) (n=21)	4152 (94) * (n=30)	4597 (128)*, § (n=33)
EDL-I	1715 (56) (n=82)	1760 (65) (n=93)	1829 (47) (n=97)	1864 (39) (n=99)
EDL-IIA	1988 (69) (n=60)	2111 (52) (n=58)	2540 (69) * (n=65)	2822 (91) *, § (n=71)
EDL-IIB	3331 (71) (n=104)	3623 (70) (n=111)	3977 (104) * (n=90)	4552 (90) *, § (n=90)
***Gastrocnemius*-IIA**	4473 (79) (n=104)	4612 (72) (n=105)	5064 (125) * (n=67)	5831 (89) *, § (n=102)
***Gastrocnemuis*-IIB**	2770 (40) (n=48)	2791 (36) (n=63)	3342 (38) * (n=55)	3698 (26) *, § (n=48)

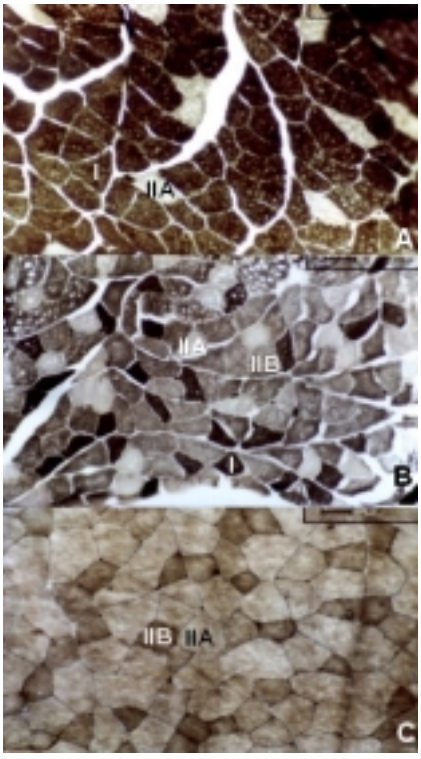

Figure 4. Reprasantative examples of transverse sections (10μm) of soleus (A), *extensor digitorum longus* – EDL (B) and gastrocnemius – white portion muscles (C) from sedentary group with no supplementation (CON), stained for myofibrilar ATPase activity after acid pre-incubation at pH 4.6. Labels provided for type I (slow), IIA (intermediary) and IIB (fast). Magnify of 200X.

Gastrocnemius muscle type IIA and IIB fibers areas were not different between CR and CON. Exercise enlarged the magnitude of the type IIA and IIB fibers areas by 13.2% and 20.6% as compared to CON and by 9.80% and 19.70% as compared to CR, respectively. The combination of exercise and creatine supplementation (CREX) increased the areas by 30.4% and 33.5% as compared to CON, by 26.4% and 32.5% as compared to CR and by 15.1% and 10.7% as compared to EX, respectively (Table 1).

DISCUSSION

Evidence is presented herein that both creatine supplementation and exercise training enlarges the magnitude of the cross-sectional areas of skeletal muscle fibers. This effect, however, was not related to an increase in body weight. Although several groups reported that creatine induces body weight gain in humans (Harris, 1992; Volek et al., 1997a; Engelhardt et al., 1998; Peeters et al., 1999; Kelly and Jenkins, 1998; Mihic et al., 2000; Volek et al., 1999; Mujika et al., 2000; Volek et al., 2001), others did not show any significant change in body mass in humans (Redondo et al., 1996) or Sprague-Dawley rats (Brannon et al., 1997; McKenna et al., 1999; McMillen, 2001). These discrepancies may result from protocol differences, doses of creatine, type and duration of the exercise. The administration of creatine may cause an alteration in the proportion of

the lean and fat mass only. The hind limb corresponds to 8% of the total body weight of which 71% of that amount composed of muscle tissue (Armstrong and Phelps, 1994). Hence, an increase of 30% in the cross-sectional area of these muscles is not enough to provoke an increment in the total body weight. The increases in food intake of the EX and CREX groups might compensate for the energy demand required during a physical activity (Figure 2). Since creatine was added to the diet, the increase in creatine intake by the CREX (Figure 3) was due to the higher food intake.

The proportion, size and identification of type I and IIA fibers in the soleus (Figure 4A) and EDL muscles (Figure 4B) were similar to those reported by others (Brooke and Kaiser, 1970; Sullivan and Armstrong, 1978; Armstrong and Phelps, 1984; Eddinger et al., 1985; Desypris and Parry (1990). In the white portion of the gastrocnemius muscle, type IIA (71%) and IIB (29%) fibers were most frequent than type IIA (Figure 4C). The proportion and size of these fibers, however, are inconsistent with those studies in which greater proportion of type IIB (87.2%) than IIA (12.8%) muscle fibers are reported (Sullivan and Armstrong 1978; Armstrong and Phelps 1984). In view of these results two hypotheses can be formulated. One, this could be typical for *Wistar* rats, considering that most studies were performed using *Sprague-Dawley* rats. Second, the portion of the gastrocnemius muscle used here may have a different fibers distribution, which could not be representative of the whole muscle. This hypothesis remains to be tested.

Creatine itself was not able to promote significant modification of the cross-sectional areas. This result was similar to that reported by Brannon et al. (1997). Exercise, on the other hand, raised fiber cross-sectional area significantly, as it is well known that regular and forced muscle contraction promotes hypertrophy whereas little or no activity leads to atrophy (Van Der Meulen et al., 1974; Booth and Gollnick, 1983). The applied exercise protocol assumed predominantly anaerobic due to the extra-weight of 15% of the body weight. Under this physical activity, type II muscle fibers are mostly recruited (Saltin and Gollnick, 1983). In fact, as a response to the applied training protocol the cross-sectional areas of type IIA and IIB muscle fibers enlarged. Type I fibers from soleus muscle was slightly increased whereas in the EDL muscle did not change. This could be due to the fact that these muscles are differently recruited. Soleus muscle, which is mostly involved in maintaining the posture, is more efficiently recruited during swimming than EDL muscle. In addition, the proportion of type I fibers in the soleus muscle is higher than in the EDL muscle, which is richer in type II and poorer in type I fibers. The applied exercise protocol is anaerobic, which may also help to explain these observations.

The combination of creatine and exercise had an additive effect on cross-sectional areas of all muscle fibers studied, except for type I fibers from EDL muscle. Our findings corroborate the work of Volek et al. (1999) who did a study using heavy resistance exercise. Also, Brannon et al. (1997) reported similar results in *plantaris* muscle of rats by determination of the dry weight. The physiological and biochemical mechanisms by which creatine and exercise combination induce additional increase in the cross-sectional area is not fully known (Bessman and Savabi, 1988) and it was not investigated in this study. Some studies have shown an increase in the heavy chain myosin and actin synthesis either *in vitro* and *in vivo* as well as in the total RNA (Ingwall et al., 1972; 1974; Ingwall and Wildenthal 1976). Bessman and Savabi (1988) suggested that changes in metabolism also should be considered to explain these observations. These authors argue that an increase in the phosphocreatine provides energy to protein synthesis resulting in muscle hypertrophy. Another hypothesis is focused on cell volume. Since creatine is an active osmotic compound (Volek et al., 1997b), increasing content of it inside the cell causes water influx and, leads to swelling of the cell. This enlargement in cell volume inhibits protein breakdown and stimulates glycogen synthesis (Häusinger et al., 1994; Hue, 1994; Lang, 1995). In addition, cell swelling also inhibits glycolysis and stimulates the flux of substrates through the pentose phosphate pathway (Häussinger et al., 1994). This metabolic pathway provides NADPH, which is important to protect the cell against oxidative stress and also substrates for lipogenesis. Furthermore, there is formation of ribose 5-phosphate, that is a component of purines and pyrimidines, which are required for cell proliferation. Finally, cell volume modifies the cytoskeleton, whereby cell swelling stabilizes the microtubule network, stimulates actin polymerization, and increases mRNA for β-actin and tubulin (Häussinger et al., 1994). All these possible hypothesis remains to be tested.

CONCLUSION

In our study, sole supplementation of creatin did not modify cross-sectional area of the hind limb muscle fibers. However, the combination of creatine supplementation with exercise training had an additive effect on increasing the cross-sectional areas of type I, IIA and IIB fibers of hind limb muscles in *Wistar* rats.

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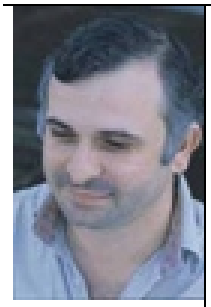

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