



ORIGINAL ARTICLE

Deleterious effects of immobilization upon rat skeletal muscle: role of creatine supplementation

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Received 16 July 2003; accepted 10 March 2004

KEYWORDS

Creatine;
Immobilization;
Skeletal muscle

Summary Aim: The aim of the study was to investigate the impact of creatine feeding ($5 \text{ g kg}^{-1} \text{ body weight day}^{-1}$) upon the deleterious adaptations in skeletal muscle induced by immobilization.

Methods: Male Wistar rats were submitted to hind limb immobilization together with three dietary manipulations: control, supplemented with creatine for 7 days (along with immobilization) and supplemented with creatine for 14 days (7 days before immobilization and together with immobilization). Muscle weight (wet/dry) was determined in the soleus (SOL) and gastrocnemius (GAS). The analysis of lean mass was performed by DEXA and myosin heavy chain (MHC) distribution by SDS-PAGE.

Results: After 14 days of creatine loading, immobilized SOL and GAS total creatine content were increased by 25% and 18%, respectively. Regardless of dietary manipulation, the immobilization protocol induced a decrease in the weight of SOL and GAS ($P < 0.001$). However, creatine feeding for 14 days minimized mass loss in the SOL and GAS ($P < 0.05$). Our findings also indicate that creatine supplementation maximizes the expected slow-to-fast MHC shift driven by immobilization ($P < 0.05$).

Conclusions: Previous creatine supplementation attenuates muscle wasting induced by immobilization. This effect is associated with the increment of intramuscular creatine content.

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Introduction

The deleterious effects of hypokinesia upon skeletal muscle have been intensely investigated for over 40 years. Generally, hypoactivity induces muscle atrophy, slow-to-fast twitch shift and a

decrease in force generation capacity.^{1–6} These adaptations have been observed in various conditions, such as immobilization,⁷ hind limb suspension³ and microgravity,⁸ although it is clear that other specific modifications in muscle function occur in these models.

Creatine is an organic compound that is directly involved in the muscle energy buffering system.⁹ When phosphorylated by creatine kinase, the

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resulting phosphocreatine can donate inorganic phosphate and energy in order to rephosphorylate ADP, thus providing the proper energetic environment to the skeletal muscle contractile apparatus.⁹ Creatine loading has also been widely studied due to its potential ergogenic effect in sports performance, for example in increased power output and muscle mass gain.⁹ In addition, recent studies have shown that creatine can enhance muscle functional capacity in patients with neuromuscular diseases or muscular dystrophies.^{10,11}

Experimental evidence indicates that creatine might modulate skeletal muscle tropism in specific conditions. For example, recent studies have shown that creatine feeding induces satellite cell proliferation¹² and expression of specific muscle transcriptional factors.¹³ Vierck et al.¹⁴ were able to show a direct effect of creatine on satellite cells in culture; it was observed that the cells differentiated earlier and to a greater level. In line with a stimulatory effect of creatine on satellite cells, Dangott et al.¹² demonstrated higher satellite cell mitotic activity in creatine-fed rats submitted to plantar skeletal muscle hypertrophy in the ablation model.

Another study conducted by Hespel et al.¹³ investigated the effects of creatine supplementation during immobilization and rehabilitation on the expression of muscle myogenic factors in humans. This study revealed an over expression of muscle regulatory factor-4 (MRF-4) in the creatine-fed group during recovery. These findings suggest that creatine can also modulate skeletal muscle plasticity by molecular mechanisms. Still the mechanisms underlying the beneficial effects of creatine loading on skeletal muscle remain largely unknown.

Given that creatine is involved in many processes that regulate skeletal muscle tropism, we aimed to investigate whether creatine loading is able to attenuate the deleterious effects of disuse. The identification and characterization of strategies prone to minimize the effects of skeletal muscle disuse, will certainly contribute to a better outcome of a large number of individuals subjected to hypokinesia.

Material and methods

Animals

Male adult Wistar rats, weighing 200–220 g, were obtained from the animal breeding facility of the Institute of Biomedical Sciences, University of Sao Paulo. The animals were maintained on a constant light-to-dark cycle (12 h:12 h) in metabolic cages,

and received water and food ad libitum. The animals were anesthetized by intra-peritoneal injection of a mixture of ketamine (30 g kg⁻¹ body weight) and xilazine (10 g kg⁻¹ body weight) before the immobilization procedure. The following groups were studied: control supplemented with saline (C; *n* = 6), supplemented with creatine during 7 days of immobilization (Cre7; *n* = 6) and pre-supplemented with creatine for 7 days before immobilization and also subsequently for 7 days after the onset of immobilization (Cre14; *n* = 6). In order to ensure that the contralateral leg was an adequate control reference, a fourth group of animals, non-immobilized age-matched controls (NI; *n* = 12) was studied. The experimental protocol (# 097/2002) was approved by the Institute of Biomedical Sciences Ethical Committee for Animal Research, University of Sao Paulo.

Supplementation protocol

All groups were fed with standard rat chow. A high dose of creatine monohydrate was administered daily via *gavage* (5 g kg⁻¹ body weight). In order to avoid gastrointestinal disturbance the daily dose was divided into three rounds. The control group (C) received the same volume of 0.9% saline.

Hind limb immobilization procedure

A mono-lateral hind limb immobilization was performed in all rats, with the exception of the NI group. The left limb of each animal was immobilized (I) with a cast on the total plantar extension.¹⁵ Gastrocnemius (GAS) and soleus (SOL) muscles remained in shortened positions for 7 days. As previously described,¹⁶ the contra-lateral leg that remained loose was utilized as a control for the immobilized leg of each animal. The legs were subdivided into: contra-lateral leg of control group (C-CL), contra-lateral leg of creatine-supplemented group (Cre7-CL), contra-lateral leg of creatine pre-supplemented group (Cre14-CL), immobilized leg of control group (C-I), immobilized leg of creatine-supplemented group (Cre7-I) and immobilized leg of creatine pre-supplemented group (Cre14-I). A fine-meshed steel net involved the cast to avoid chewing.¹⁶ Great care was taken to make sure that the cast did not cause ischemia.

Measurement of total creatine content

The SOL and GAS total creatine content were determined by reversed-phase high-performance liquid chromatography (HPLC) technique. Frozen

muscles were powdered in a stainless steel percussion mortar cooled in liquid nitrogen. The powder was homogenized in 0.4N perchloric acid at 0°C and aliquots of the homogenate were removed for protein determination. Samples were centrifuged at 3000 g for 1 min and the supernatant buffered to pH 7.0 with 0.2 mol l⁻¹ Tris and KOH, 40% weight per volume, in 60:40 mix. Further centrifugation at room temperature and 3000 g for 1 min was realized to remove the precipitate before 50 µl of the final supernatant was analyzed.¹⁷

The HPLC system consisted of auto-injector (SIL-10AD; Shimadzu, Tokyo-Japan), a liquid chromatograph (LC-10AD; Shimadzu, Tokyo-Japan) and UV-visible spectrophotometric detector (SPD-10A; Shimadzu, Tokyo-Japan). The column utilized was an Absorbosphere HSC18 (250 mm × 4.6 mm, 7 µm pore size; Alltech, Deerfield, USA). The column was perfused at 0.9 ml min⁻¹ with a mixture of Mg²⁺–Tris buffer containing MgSO₄ (4 mmol l⁻¹), Tris (4 mmol l⁻¹), KH₂PO₄ (22 mmol l⁻¹) and NaN₃ (0.2 mmol l⁻¹) and a solvent consisting of methyl alcohol: water mix, in a 60:40 volume per volume ratio at pH 6.05.¹⁷ To improve retention times for PCr and Cr, samples were isocratically using a phosphate buffer (90 mmol l⁻¹ KH₂PO₄, 10 mmol l⁻¹ K₂HPO₄ (pH 4.75) containing an ion-pairing agent (5.9 mmol l⁻¹ tetrabutylammonium hydrogen sulfate) that improves the retention of PCr.¹⁸ In order to minimize mislead results, since high phosphate breakdown is extremely rapid and would not represent the animal living content, according to Robinson and Loiselle,¹⁷ we report only the sum of Cr and PCr (Total creatine content).

Lean mass determination

Lean mass (LM), which is mainly composed of muscle mass, was evaluated by dual energy X-ray absorptiometry as described before by Brommage¹⁹ using the pDEXA Sabre Bone Densitometer and Software (Norland Medical Systems[®]), both especially designed for small animals. The animals were scanned under anesthesia just before immobilization (basal scan—day 0) and after 7 days of immobilization (final scan—day 7). The lean mass changes were expressed in grams ($\Delta\text{LM} = \text{LM day 7} - \text{LM day 0}$).

Tissue samples

The muscles (GAS and SOL) were removed and weighed for relative mass determination. Immediately afterwards the muscles were quickly frozen, and stored at -70°C for posterior determinations of

MHC isoform distribution. To determine dry weight, a small aliquot of each muscle was weighed and then dried for 48 h at 60°C. The weight of adrenal glands was determined to estimate the level of stress in all experimental groups (C, Cre7 and Cre14) in relation to rats not submitted to immobilization (NI).

Myosin heavy chain analysis

The muscles were frozen in liquid nitrogen and stored at -70°C. Muscles samples preparation to electrophoretic separation were conducted as described before by Talmagde and Roy.²⁰ Proteins were separated by SDS-PAGE gels containing 30% vol/vol glycerol, 8% wt/vol acrylamide, 1% vol/vol glycine and 0.4% wt/vol SDS.²⁰ Stacking gels contained 40% vol/vol glycerol and 4% wt/vol acrylamide. After electrophoresis (275 V) for 20 h, gels were stained for 60 min in Coomassie Brilliant Blue and destained with 20% methanol and 5% acetic acid.²⁰ MHC bands were scanned and analyzed by the Kodak Digital Science™ 1D Image Analyses Software.

Statistical analysis

All values are expressed as mean ± SE. Differences among groups were determined by multivariate analysis of variance (ANOVA) with three levels for treatment—C vs. Cre7 vs. Cre14 and two levels for immobilization CL vs. I (3 × 2). Subsequently, a Tukey post hoc test was applied using Prism Graph Pad Software[®]. For comparison of NI and CI groups an independent *t*-test was utilized. The *P* value of confidence for statistical significance was established at 0.05.

Results

Total creatine content

After 14 days of creatine feeding, total creatine content of immobilized SOL of Cre14-I was increased by ~25% (*P* < 0.001) in comparison to C-CL and C-I. Compared with C-CL, immobilized GAS of Cre14-I also responds to supplementation protocol, however, in a less pronounced manner (18%, *P* < 0.001) (Table 1).

Mass parameters

Body weight after immobilization suffered a mild (but not statistically different) decrease until the 2nd day, after which it started to increase at the

Table 1 Body weight (g) and relative weight of adrenal glands (%) of NI, C, Cre7 and Cre14 groups.

	<i>n</i>	Body weight (g)	Weight of adrenal glands (%)
NI	6	239.8±8.1	0.032±0.003
C	6	232.7±10.1	0.038±0.004
Cre7	6	228.3±6.1	0.036±0.003
Cre14	6	238.4±6.2	0.036±0.003

Values are expressed as mean±SE. Non-immobilized group (NI; *n*=6), control group (C; *n*=6), supplemented with creatine during 7 days along with immobilization (Cre7; *n*=6) and Supplemented with creatine during 14 days (7 days before immobilization and along with immobilization) (Cre14; *n*=6).

same rate as that of the NI group (Table 1). Food intake (data not shown) in all groups accompanied the body mass change profile. No statistical differences were observed in the weight of adrenal glands among all groups (Table 2). Furthermore, no differences were observed in the wet and dry weights of the SOL and GAS muscles from the NI group, as compared to the contra-lateral leg (C-CL). The immobilization markedly decreased relative wet weight of GAS and SOL muscles in all groups ($P<0.001$) (Table 2). No statistical difference was observed between Cre7-I and C-I relative mass, however, the relative muscle mass of the SOL and GAS of Cre14-I were 21% ($P<0.05$) and 15% ($P<0.05$) greater than C-I, respectively. As observed in relative muscle mass, the dry weight, mainly composed by muscle protein, suffered a marked decrease in SOL and GAS muscles of all immobilized legs ($P<0.001$). SOL of Cre14-I demonstrated a greater dry weight in comparison to SOL of C-I (Table 3) ($P<0.05$).

Lean mass determination

The longitudinal analysis by DEXA allowed us to follow the lean mass changes over time (Fig. 1). The Δ lean mass was markedly decreased (~70%) by immobilization in all animals (C-I, Cre7-I and Cre14-I; $P<0.001$). A slight lean mass loss reduction was observed in the Cre14-I as compared to C-I, although this decrease was not considered statistically significant ($P<0.1$). No statistical difference in lean mass change was noted between the NI and CL of all immobilized groups.

MHC distribution

The decrease in type MHC I induced by immobilization was observed in all SOL muscles of immobilized

Table 2 Muscle total creatine content (mmol kg⁻¹ dry weight) of NI leg, C-CL leg, Cre7-CL leg, Cre14-CL leg, C-I leg, Cre7-I leg and Cre14-I leg.

	<i>n</i>	Total creatine content	
		SOL	GAS
NI	6	88.7±9.0	113.3±11.4
C-CL	6	90.2±5.2	114.8±7.4
C-I	6	93.9±6.7	119.1±5.9
Cre7-CL	6	119.4±8.8 ^{a,b}	129.9±6.1
Cre7-I	6	104.1±6.8	122.0±7.0
Cre14-CL	6	122.3±7.2 ^{a,b}	138.4±9.8 ^a
Cre14-I	6	118.3±5.2 ^{a,b}	135.0±8.4 ^a

Values are expressed as mean±SE. Non-immobilized leg (NI; *n*=6), contra-lateral leg of the control group (C-CL; *n*=6), immobilized leg of the control group (C-I; *n*=6), contra-lateral leg of the Cre7 group (Cre7-CL; *n*=6), immobilized leg of the Cre7 group (Cre7-I; *n*=6), contra-lateral leg of Cre14 group (Cre14-CL; *n*=6) and immobilized leg of Cre14 group (Cre14-I; *n*=6).

^aDifferent from C-CL ($P<0.001$).

^bDifferent from C-I ($P<0.001$).

legs (C-I, Cre7-I and Cre14-I; Fig. 2- $P<0.001$), indicating a main effect of immobilization. While the C-CL, Cre7-CL and Cre14-CL displayed more than 90% of MHC I expression, C-I, Cre7-I and Cre14-I expressed approximately 84%, 78% and 80% MHC I expression, respectively. These changes were also accompanied by an increase in MHC IIa (Fig. 3) ($P<0.001$) (see also Fig. 4). Thus, immobilization led to a shift in MHC (slow-to-fast) in the postural SOL muscle. In addition, it is noteworthy that MHC I content of Cre7-I and Cre14-I legs were reduced in comparison to C-I, thus creatine loading further enhanced the MHC I to IIa shift in the SOL muscle induced by immobilization ($P<0.05$).

Discussion

In order to verify the role of creatine supplementation in skeletal muscle plasticity during disuse, we utilized a model of hind limb immobilization-induced hypokinesia. To estimate the level of stress, adrenal gland weight was determined in all immobilized groups and compared with rats not submitted to immobilization (NI). No statistical differences were observed among all groups, indicating that immobilized animals did not suffer significant additional stress. The lack of variation in weight gain and food intake indicated that the disuse model used in the present study did not interfere with developmental aspects of the animals.

Table 3 Relative muscle wet weight (muscle weight–body weight ratio) and dry muscle weight of C-CL leg, Cre7-CL leg, Cre14-CL leg, C-I leg, Cre7-I leg and Cre14-I leg.

	Relative wet weight (%)			Dry weight (mg)		
	<i>n</i>	SOL	GAS	<i>n</i>	SOL	GAS
NI	12	0.50 ± 0.03	5.49 ± 0.13	12	25.8 ± 0.9	343.2 ± 14.1
C-CL	6	0.51 ± 0.02	5.44 ± 0.11	6	26.9 ± 0.7	335.3 ± 10.1
C-I	6	0.33 ± 0.02 ^a	3.91 ± 0.15 ^a	6	16.0 ± 0.8 ^a	225.7 ± 11.0 ^a
Cre7-CL	6	0.55 ± 0.01	5.57 ± 0.12	6	26.4 ± 1.5	326.9 ± 0.9
Cre7-I	6	0.38 ± 0.02 ^b	4.15 ± 0.13 ^b	6	18.5 ± 0.7 ^b	242.4 ± 12.3 ^b
Cre14-CL	6	0.53 ± 0.01	5.71 ± 0.12	6	26.2 ± 1.0	336.7 ± 18.9
Cre14-I	6	0.42 ± 0.02 ^{c,d}	4.63 ± 0.13 ^{c,d}	6	19.9 ± 1.5 ^{c,d}	245.4 ± 19.9 ^c

Values are expressed as mean ± SE. Non-immobilized leg (NI; *n* = 12), contra-lateral leg of the control group (C-CL; *n* = 6), immobilized leg of the control group (C-I; *n* = 6), contra-lateral leg of the Cre7 group (Cre7-CL; *n* = 6), immobilized leg of the Cre7 group (Cre7-I; *n* = 6), contra-lateral leg of Cre14 group (Cre14-CL; *n* = 6) and immobilized leg of Cre14 group (Cre14-I; *n* = 6).

^aDifferent from C-CL.

^bDifferent from Cre7-CL (*P* < 0.001).

^cDifferent from Cre14-CL (*P* < 0.001).

^dDifferent from C-I (*P* < 0.05).

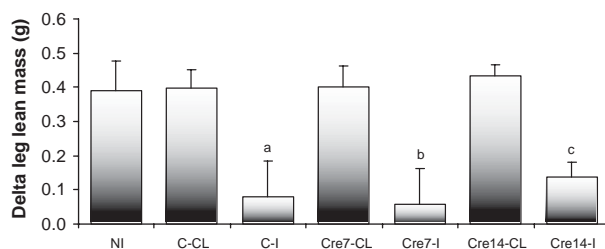


Figure 1 Leg lean mass evaluation (Δ LM) of C-CL leg, Cre7-CL leg, Cre14-CL leg, C-I leg, Cre7-I leg and Cre14-CL leg. Values are expressed as mean ± SE. Contra-lateral leg of the control group (C-CL; *n* = 6), immobilized leg of the control group (C-I; *n* = 6), contra-lateral leg of the Cre7 group (Cre7-CL; *n* = 6), immobilized leg of the Cre7 group (Cre7-I; *n* = 6), contra-lateral leg of Cre14 group (Cre14-CL; *n* = 6), immobilized leg Cre14 group (Cre14-I; *n* = 6) and non-immobilized leg (NI; *n* = 6). (Δ LM = Scan day 7–Scan day 0). (a) Different from C-CL (*P* < 0.001), (b) different from Cre7-CL (*P* < 0.001), (c) different from Cre14-CL (*P* < 0.001).

In order to refute the argument that the contra-lateral leg is not an adequate control reference, an age-matched group of animals that were not submitted to immobilization (NI) was utilized. Since no difference was observed in muscle mass (wet and dry) between NI and C-CL, as also demonstrated before,¹⁶ we concluded that the contra-lateral legs are appropriate controls.

We observed, as described before,^{7,21} a marked muscle mass loss (wet and dry weight) in the SOL and GAS muscles as a result of immobilization for 7 days (Table 1). In addition, we observed a marked

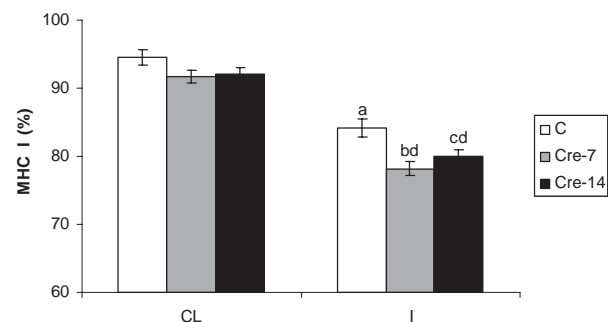


Figure 2 Myosin heavy chain I distribution of SOL from C-CL leg, Cre7-CL leg, Cre14-CL leg, C-I leg, Cre7-I leg and Cre14-I leg. Values are expressed as mean ± SE. Contra-lateral leg of the control group (C-CL; *n* = 6), immobilized leg of the control group (C-I; *n* = 6), contra-lateral leg of the Cre7 group (Cre7-CL; *n* = 6), immobilized leg of the Cre7 group (Cre7-I; *n* = 6), contra-lateral leg of Cre14 group (Cre14-CL; *n* = 6) and immobilized leg Cre14 group (Cre14-I; *n* = 6). (a) Different from C-CL (*P* < 0.001), (b) different from Cre7-CL (*P* < 0.001), (c) different from Cre14-CL (*P* < 0.001), (d) different from C-I (*P* < 0.05).

decline in the cross sectional area of immobilized SOL fibers (type I, 26%; type IIb, 23% and type IIa, 18%; data not shown) in comparison to C-CL. This result combined with the observation of a reduction in muscle weight, confirms that the hypokinesia model utilized was effective in promoting skeletal muscle atrophy.

A recent study conducted by Hespel et al.¹³ also investigated the effect of creatine supplementation during immobilization and rehabilitation in humans. These authors observed a mild decrease (~10%) in the cross-sectional area of quadriceps

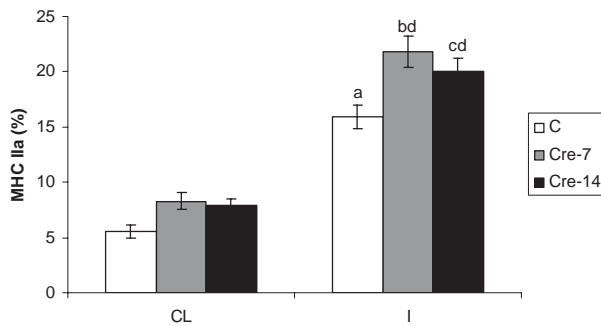


Figure 3 Myosin heavy chain IIa distribution of SOL from C-CL leg, Cre7-CL leg, Cre14-CL leg, C-I leg, Cre7-I leg and Cre14-I leg (%). Values are expressed as mean \pm SE. Contra-lateral leg of the control group (C-CL; $n=6$), immobilized leg of the control group (C-I; $n=6$), contra-lateral leg of the Cre7 group (Cre7-CL; $n=6$), immobilized leg of the Cre7 group (Cre7-I; $n=6$), contra-lateral leg of the Cre14 group (Cre14-CL; $n=6$) and immobilized leg of the Cre14 group (Cre14-I; $n=6$). (a) Different from C-CL ($P<0.001$), (b) different from Cre7-CL ($P<0.001$), (c) different from Cre14-CL ($P<0.001$), (d) different from C-I ($P<0.05$).

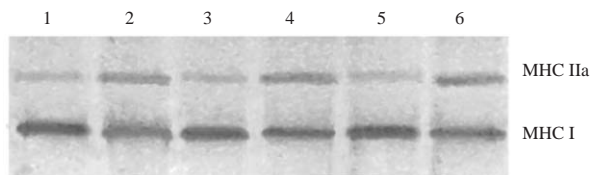


Figure 4 Representative SDS-PAGE of SOL muscle from C-CL leg, C-I leg, Cre7-CL leg, Cre7-I leg, Cre14-CL leg and Cre14-I leg. Lane 1, C-CL leg; Lane 2, C-I leg; Lane 3, Cre7-CL leg; Lane 4, Cre7-I leg; Lane 5, Cre14-CL leg and Lane 6, Cre14-I leg.

after 2 weeks of immobilization in both creatine and placebo groups, although no changes were observed in the fiber cross-sectional area after immobilization. Conversely, in the rat hypokinesia model (used in the present study), a severe loss of muscle mass and fiber cross sectional area was observed after 1 week of immobilization. It should be pointed out that, in contrast to the rat model, immobilization in humans is not performed in the totally shortened position, a condition that is well recognized to be important for accelerating the rate of muscle atrophy.^{7,15} This may, at least in part, explain the apparent discrepancies between our study and the study conducted by Hespel et al.¹³ who were not able to show a protective effect of creatine load upon muscle mass loss under hypokinesia.

To ensure the effectiveness of the creatine loading, we used a high dose (5 g kg^{-1} body weight day^{-1}). This dosage has been utilized pre-

viously and is shown to increase free creatine (30%), phosphocreatine (15%) and total creatine (20%) levels in the rat SOL in just 5 days of creatine feeding.²² In humans, lower doses have also been found to be effective for boosting up intramuscular creatine content.^{9,13,23} The supplementation protocol adopted in our study successfully increased total creatine content in SOL and whole GAS (red and white portions). As expected, SOL (mainly composed by slow-twitch fibers) showed higher creatine uptake capacity after creatine loading as compared to GAS. Op't Eijnde et al.²² also demonstrated a significant increase only in the red portion of GAS, while the white portion of GAS showed mild increment (7%) in creatine content. Accordingly, other studies utilizing muscles which are composed by a mixture of fast and slow twitch fibers (red and white portions) such as vastus lateralis also showed an increase (20%) in creatine content after creatine loading.¹³

The present study demonstrates for the first time, to our knowledge, that previous creatine loading (Cre14) minimizes skeletal muscle mass loss in a hypokinesia rat model. Creatine supplementation only during immobilization (Cre7), however, failed to promote the same response. The creatine uptake by skeletal muscle is modulated by many factors, such as the intramuscular baseline level of creatine,²³ muscle activity,²³ insulin sensitivity²⁴ and extracellular sodium concentration²⁵. Muscle inactivity and insulin resistance observed during immobilization contributes to decrease creatine uptake capacity. This decreased creatine uptake capacity may compromise the effect of creatine supplementation upon skeletal muscle mass during immobilization (Cre7). This might explain the lack of protection against muscle mass loss observed in Cre7. It could be hypothesized that creatine loading in association to normal muscle activity is required for the attenuation of muscle wasting induced by disuse.

Our results demonstrated that the effective minimization of muscle wasting observed in the Cre14 is associated to the increment of muscle creatine content. One of the proposed mechanisms for the effect of creatine on skeletal muscle mass gain includes higher mitotic activity in satellite cells.¹² Also in line with a relationship between creatine and satellite cells, it has been recently shown that creatine activates the satellite cell differentiation program in vitro.¹⁴ It is possible that creatine, in addition to a satellite cell mitotic effect, is also able to activate signaling pathways, protecting the skeletal muscle cell against proteolysis.

It has been reported that creatine feeding is able to increase water retention, due to its osmotic

property²⁶ and some evidences suggest that the cell hydration state is a major factor in controlling protein turnover.^{27,28} In hepatocytes, an increase in cell hydration state (swelling) acts as an anabolic signal, stimulating protein synthesis and attenuating protein breakdown. In fact, it has been already suggested that water retention induced by the increase in creatine content might function as an anabolic proliferative signal, which would involve MAPK signaling pathway that plays a pivotal role in protein synthesis regulation.²⁹ Although no effort has been made to address the effects of swelling in skeletal muscle, Parise et al.³⁰ showed that short-term creatine supplementation presented an anti-catabolic action in men, indicating a direct mechanism of creatine upon skeletal muscle protein turnover. Our results demonstrate that pre-creatine feeding (Cre14) attenuates SOL dry weight (mainly composed by muscle protein) loss, which is in line with the idea that creatine supplementation involves reduced muscle protein wasting.

The lean mass loss (indirectly determined by DEXA) in all immobilized legs confirms the results obtained from muscle weight (wet and dry). Furthermore, DEXA analysis also showed a similar muscle mass in the NI vs. C-CL groups, reinforcing that the contralateral leg might be used as a reference control. DEXA analysis also showed a tendency (although not statistically significant, $P < 0.1$) of the pre-supplemented group (Cre14-I) to present a decreased muscle wasting induced by immobilization (C-I). Although DEXA analysis has great potential to be used as a non-invasive method to address loss of muscle mass,¹⁹ our results suggest that the sensitivity of this method is not suitable to follow the effect of creatine during immobilization. This is probably due to the fact that DEXA only allows analysis of the whole leg. It is well known that muscle length during immobilization determines muscle atrophy or growth.¹⁵ Since certain muscles (such as the *Tibialis anterior*) in the leg, in our model, are immobilized in a full stretching position (conditioning that stimulates muscle longitudinal growth), the effect of creatine supplementation is likely to be underestimated.

As previously described, hypokinesia leads to a slow-to-fast MHC shift.^{31–33} These studies have shown that the proportion of MHC IIa isoform in SOL increases after hindlimb suspension.^{31–33} To characterize the effect of disuse and creatine loading upon the MHC profile, we chose a postural muscle (SOL), which predominantly expresses the MHC I isoform. Our findings are in accordance with the previous data. Furthermore, in the present study, the combination of immobilization and creatine loading had an additive effect on MHC

shift towards to the fast phenotype observed during immobilization. The mechanism by which creatine determines the MHC phenotype is still unknown, but it may include the modulation of intra-cellular signaling pathways involved in slow twitch phenotype determination, such as the calcineurin pathway.

The fiber phenotype was affected by creatine supplementation in immobilized SOL, however, its adaptive role remains to be elucidated. Since postural maintenance requires tonic contractile activity, the slow-to-fast shift in postural muscles, observed in disuse conditions, might represent a deleterious adaptation. Other clinical conditions such as hyperthyroidism clearly show that shift to the fast phenotype is associated with diminished muscular endurance.³⁴ We speculate that the increase in slow-to-fast shift observed in the immobilized SOL of creatine-fed rats could also lead to early fatigue. Functional studies should be conducted in the future to determine the role of the slow-to-fast shift driven by creatine supplementation in rats in skeletal muscle inactivity.

Since pre-treatment with creatine before immobilization attenuates the muscle wasting, we suggest that creatine supplementation might be a suitable strategy for individuals who will be submitted to hypokinesia, particularly athletes in the pre-operative phase involved in osteo-articular injuries. In such conditions, to ensure surgery success, which is followed by a prolonged post-operative immobilization, an intense muscle reinforcement program should be initiated prior to surgery. Therefore, creatine supplementation during the muscle reinforcement program might be a viable dietary manipulation to maximize muscle mass gain before surgery and also minimize muscle wasting during immobilization.

Acknowledgements

We would like to thank FAPESP for financial support (#03/00431-0). We also gratefully acknowledge the contribution of Mr. Yuri Borges for the creatine content determination, Mr. Vagner Raso for statistical analysis, Mr. Antonio Garcia Soares and Mr. Luiz Carlos Carnevali Júnior for the outstanding technical support.

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