

Research article

## The effects of creatine long-term supplementation on muscle morphology and swimming performance in rats

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### Abstract

Creatine (Cr) has been shown to increase the total muscle mass. The purpose of this study was to investigate the effect of Cr supplementation on muscle morphology and swimming performance, using an animal model. Each rat was subjected to exercise 15-minute period daily for the 12 weeks. The rats were randomly divided into four groups: no Cr supplementation (CON), no Cr supplementation and incomplete food intake (lacking lysine and methionine in diet for rats) (INCO), Cr supplementation  $1 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  (CREAT-I) and Cr supplementation  $2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  (CREAT-II). Three months later, all groups adult rats exercised in swimming pool chambers. Swimming time was recorded as minute for each rat. Following swimming performance period, the animals were killed by cervical dislocation and the gastrocnemius and diaphragm muscles were dissected. Serial slices of  $5\text{-}7 \mu\text{m}$  were allocated paraffin wax and histochemical staining procedure of cross-sections was carried out with heamatoxylin-eosin technics. All groups gained body weight at the end of 12 weeks but there was no statistical difference among them. Swimming time values were statistical difference between CREAT-II and CON group as well as between CREAT-I and CON group ( $p < 0.05$ ). In the INCO group was determined increased connective tissue cell of the muscle sample. In contrast, in the CREAT-I and CREAT-II group, the basic histological changes were large-scale muscle fibers and hypertrophic muscle cells. These results suggest that long-term creatine supplementation increased the number of muscle fibers and enhanced endurance swimming performance in rats.

**Key words:** Creatine, muscle hypertrophy, muscle morphology, exercise, swimming performance.

### Introduction

Phosphocreatine (PCr) plays a key role in energy provision to muscle cell. Dietary supplementation of creatine (Cr) has been shown to increase muscle levels of both Cr and PCr by 20–50% (Balsom et al., 1995; Green et al., 1996; Young and Young, 2007). Cr supplementation is thought to exert an ergogenic effect on activities that consist of short-duration, high-intensity muscular activity and activities that feature repeated bouts of high-intensity activity (Balsom et al., 1995; Canete et al., 2006; Jäger et al., 2008; Gotshalk et al., 2008). In addition, increases in fat free mass and resistance exercise performance have been attributed to creatine supplementation (Balsom et al., 1995; Kreider et al., 1998; Volek et al., 1999). Several studies on the ergogenic effects of creatine supplementation have resulted in mixed outcomes. Some studies have found that

creatine supplementation enhances performance in cycling, swimming, running, and weight lifting, while other studies have failed to show any difference between creatine supplementation and placebo in similar measures of performance (Terjung et al., 2000).

Endogenous Cr is synthesized by the liver, kidneys and pancreas from arginine and glycine. Creatine's phosphorylated form, PCr, is an important source of energy and ADP and acid buffer in skeletal muscle during activities of an intense nature (Lemon et al., 1995; Swynghedauw, 1989; Thomas and Hall, 1997). During high-intensity exercise PCr donates the phosphate to provide energy for the resynthesis of ATP, allowing for rapid ATP turnover and sustained maximal contractions during all-out exercise. However, an interaction between Cr supplementation and endurance training, resulting in increased citrate synthase levels in fast- and slow-twitch skeletal muscle of endurance-trained rats has recently been reported (Brannon et al., 1997). Exogenous Cr supplementation has been shown to increase the total Cr (TCr) content of skeletal, cardiac, and smooth muscle in both humans (Horn et al., 1999) and rats (Brannon et al., 1997). Skeletal muscle TCr has shown increase as much as 18% with exogenous Cr supplementation that are positively correlated with increases in muscle glycogen stores in humans (Green et al., 1996). Repetitive, high-intensity exercise performance improvements have been observed in humans with dietary Cr supplementation (Metzger et al., 1999; Swynghedauw, 1999; Buford et al., 2007). Cr depletion, however, has not been shown to affect endurance exercise performance in rats (Adams et al., 1995). Cr supplementation studies using different protocols and animal species, including humans, have been shown to increase body weight and change in the body composition (Virus et al., 1994; Mujika et al., 1996; 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 (Thomson et al., 1996; Stout et al., 1999; Rico-Sanz and Marco, 2000).

Short-term creatine supplementation results in an increase of muscle force and power output during intermittent exercise, even in the absence of resistance training (Greenhaff et al., 1993). Facilitated muscle phosphocreatine resynthesis (Greenhaff et al., 1994) and more rapid and efficient recovery periods (Greenhaff et al., 1993; Grindstaff et al., 1997) have been stated as proposed mechanisms for this ergogenic effect. However, a majority of studies suggest that creatine supplement-

ation does not improve endurance exercise capacity (Balsom et al., 1993; Engelhardt et al., 1998). The effect of creatine supplementation can be highly variable amongst individuals (Syrotuik et al., 2004) and low initial muscle creatine content has been found to be a prerequisite for maximum ergogenic effects (Greenhaff et al., 1994; Harris et al., 1992). Several studies reported that creatine may improve performance primarily during short-duration, high-intensity exercise. However, there was less evidence that long-term creatine supplementation enhanced exercise performance during moderate high-intensity prolonged endurance exercise.

The purpose of this study was to investigate the effect of Cr supplementation on muscle morphology and swimming performance, using an animal model. We hypothesized that long-term (12 weeks) Cr supplementation would increase skeletal muscle hypertrophy and enhance swimming performance in rat.

## Methods

### Study design

The Ethical Committee of Cumhuriyet University Medical School approved this study. Sixty 2-week-old newborn male Wistar Albino rats were kept in standard individual vivarium cages, for food intake and body weight determination, under a controlled light/dark (12/12h) cycle and temperature ( $22^{\circ}\text{C} \pm 1$ ) with free access to food and water. Each rat was subjected to swimming exercise 15-minute period daily for the 12 weeks. The rats were randomly divided into four groups: No Cr supplementation (CON,  $n = 15$ ), no Cr supplementation and incomplete food intake (lacking lysine and methionine in diet for rats) (INCO,  $n = 15$ ), Cr supplementation  $1 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  (CREAT-I,  $n = 15$ ) and Cr supplementation  $2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  (CREAT-II,  $n = 15$ ) (Table 1).

### Exercise training

Three months later, all groups adult rats exercised in swimming pool chambers at a water temperature of  $28^{\circ}\text{C}$ . Firstly, animals designated to exercise were familiarized with swimming conditions. Swimming tests were made in a square shaped glass water tank that was 50 centimeters in height, in width and filled to a depth of 40 centimeters with water. The uncoordinated movements and staying under the water for 10 seconds without swimming at the surface were accepted as the exhaustion criteria of the rats (Dawson and Horwarth, 1970) Swimming time was recorded as minute for each rat.

### Tissue processing and histological analysis

Following swimming performance period, the animals were killed by cervical dislocation and both the gastrocnemius and the diaphragm muscles were dissected. Each muscle was cut in the middle, transversally and fixed in 10% formalin solution. Tissues were embedded in paraffin wax. Later, serial slices of  $5\text{-}7 \mu\text{m}$  were allocated paraffin wax and let to dry at room temperature. Histochemical staining procedure of cross-sections was carried out with hematoxylin-eosin (HE) technique (Prophet et al., 1992). The slide images were obtained in a light microscope (Olympus BX50) connected to a computer.

### Image processing and quantitative measurements

Bright-field of sections were acquired using a camera, respectively, via a Olympus BX50. Image analysis was completed using imagebro software (Media Cybernetics, Silver Spring, MD, USA) with manual on-screen selection of functions. Montage images of hematoxylin and eosin stained sections were overlaid with a mask that randomly chose four  $400\text{-}\mu\text{m}^2$  microscopic fields per muscle cross section. These fields were used to determine manually the number of muscle fibers per defined area.

### Statistical analysis

All data are reported as means (SD). Statistical analysis was performed by Kruskal Wallis Varyans Analysis followed by a post-hoc Mann-Whitney U test. The level of  $p < 0.05$  was taken to indicate statistical significance. All statistical analyses were performed using computer software package (SPSS for Windows 13.0).

## Results

### Body weight

The body weight of all experimental animals was similar initially. However, all groups gained weight by the end of the experiment. At the 12<sup>th</sup> week, in relation to zero, CREAT-I group body weight increased the most by all the other groups while INCO group increased the least. In the CON, CREAT-I and CREAT-II groups, body weight increase were statistically significant as compared with INCO group ( $p < 0.05$ ). However, there was no statistical difference between CREAT-I and CON group ( $p > 0.05$ ) as well as between CREAT-II and CON group ( $p > 0.05$ ) (Figure 1).

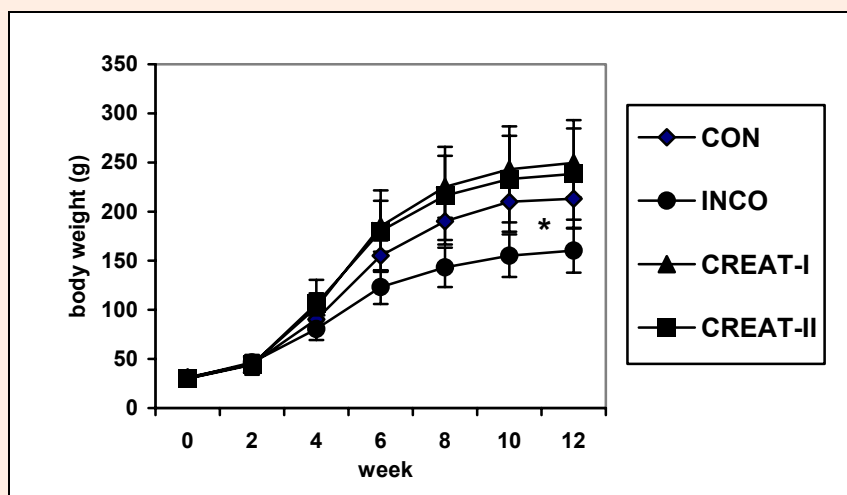
### Swimming performance

Three months later, all groups adult rats exercised in swimming pool chambers and recorded endurance swimming time as minute for each rat. Endurance swimming time for CREAT-II group was the most in the

**Table 1. Research design.**

Treatment	n	Week 0	Week 12	Week 13
CON	15	<sup>a</sup> Sucrose	Swimming performance	Tissue harvest
INCO	15	<sup>b</sup> Incomplete food	Swimming performance	Tissue harvest
CREAT-I	15	<sup>c</sup> Cr supplem started	Swimming performance	Tissue harvest
CREAT-II	15	<sup>d</sup> Cr supplem started	Swimming performance	Tissue harvest

CON = no Cr supplementation, INCO = no Cr supplementation and incomplete food intake, CREAT-I = Cr supplementation  $1 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ , CREAT-II = Cr supplementation  $2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ . <sup>a</sup> Sucrose placebo, <sup>b</sup> No Cr supplementation and incomplete food intake (lacking lysine and methionine in diet for rat), <sup>c</sup> Cr supplementation  $1 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  in 12 week, <sup>d</sup> Cr supplementation  $2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  in 12 week.



**Figure 1.** The effects of creatine (Cr) supplementation on body weight in rats. No Cr supplementation (CON,  $n = 15$ ), Incomplete food intake (INCO,  $n = 15$ ), Cr supplementation  $1 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  (CREAT-I,  $n = 15$ ), and Cr supplementation  $2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  (CREAT-II,  $n = 15$ ). All data are reported as means (SD). \* INCO group values were significantly lesser than all other group values ( $p < 0.05$ ).

other groups and for INCO group was at least in the other groups. Swimming time values were statistical difference between CREAT-II and CON group as well as between CREAT-I and CON group ( $p < 0.05$ ). In addition to this, swimming time values of CREAT-I and CREAT-II groups were statistically significant as compared with the INCO group ( $p < 0.05$ ). However, there was no significant difference between INCO and CON group ( $p > 0.05$ ) (Figure 2).

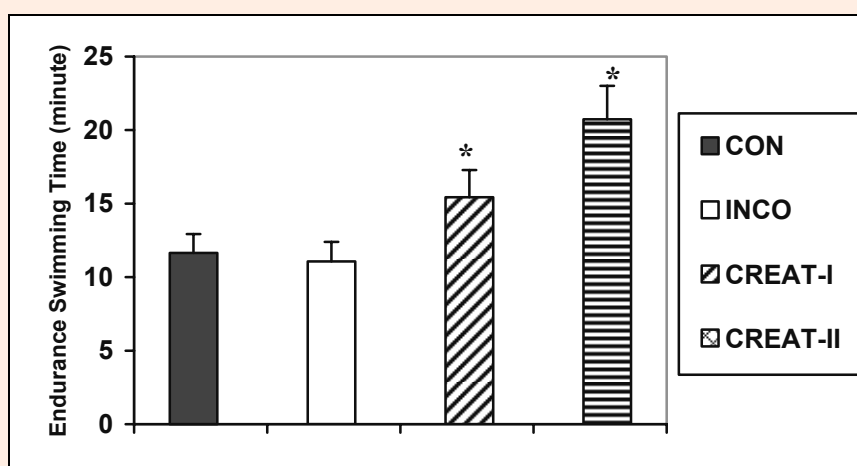
### Histological analysis

The histological changes in all groups were examined and results are given in Figure 3. In the diaphragm muscle tissue of the CON group did not show significant morphological changes (Figure 3a). The INCO group was observed similar to the CON group in terms of muscle fibers and nucleus structure. However, in this group was determined increased connective tissue cell of the muscle

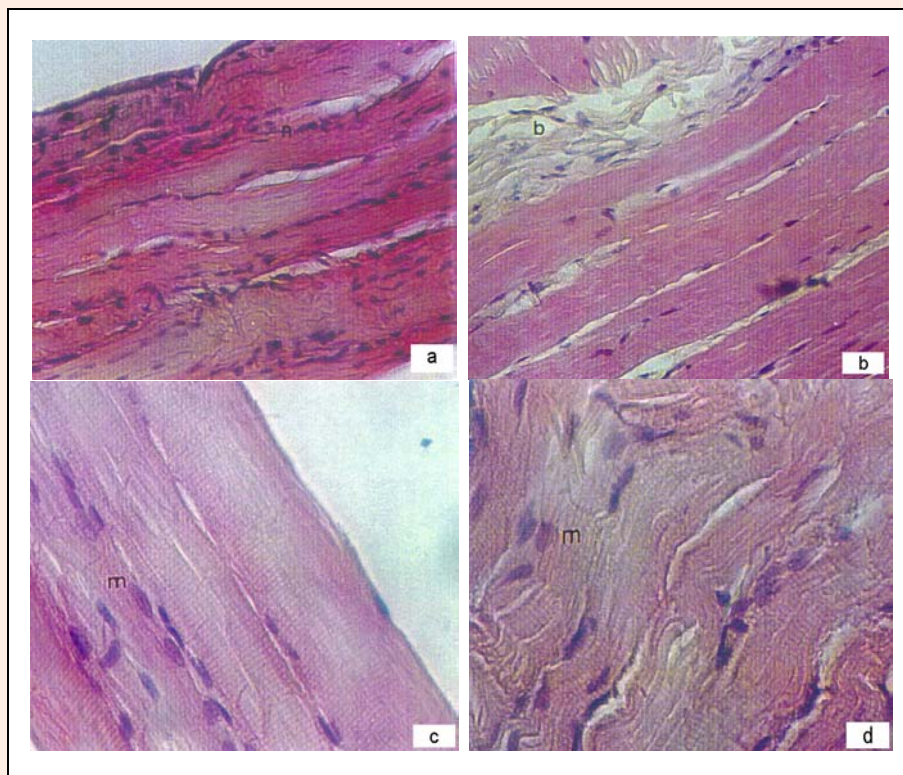
sample (Figure 3b). In contrast, in the CREAT-I and CREAT-II group, the basic histological changes were large-scale muscle fibers and hypertrophic muscle cells. The myonucleus of muscle cells were flat, basophilic and placed around the cytoplasm (Figure 3c, 3d). Furthermore, the oval-shaped mononuclear cells appeared particularly among the gastrocnemius muscle cells (Figure 3c). In the gastrocnemius muscle sample, the round-shaped myoblast cell was found among the hypertrophic muscle cells (Figure 3d).

### Quantitative measurements

The quantitative analysis indicated that the number of muscle fibers per defined area increased in creatine supplementation groups. The number of muscle fibers in CREAT-I and CREAT-II groups were statistically significant as compared with the CON group ( $p < 0.05$ ) (Figure 4).



**Figure 2.** The effects of creatine (Cr) supplementation on endurance swimming time performance in rats. No Cr supplementation (CON,  $n = 15$ ), Incomplete food intake (INCO,  $n = 15$ ), Cr supplementation  $1 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  (CREAT-I,  $n = 15$ ), and Cr supplementation  $2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  (CREAT-II,  $n = 15$ ). All data are reported as means (SD). \*Significantly different from CON values ( $p < 0.01$ ).



**Figure 3.** Histological appearance of the skeletal muscle in rat. (a) The tissue sample belonging to CON group (no Cr supplementation) diaphragm muscle in rat (haematoxylin–eosin (HE)-stained section, n = nukleous, ORIGINAL magnification X20). (b) The histological changes appear in the diaphragm muscle tissue sample of the INCO group. (HE-stained section, original magnification X20, fibroblast and collagen fibers are seen with increased connective tissue between muscle cells = b). (c) The myoblasts are seen between hypertrophic gastrocnemius muscle cells in the tissue sample belonging to CREAT-I group (Cr supplementation  $1 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ) (HE-stained section, original magnification X40, myoblast = m). (d) The tissue sample belonging to CREAT-II group (Cr supplementation  $2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ) diaphragm muscle in rat (HE-stained section, original magnification X40, the round-shaped myoblasts are seen between the hypertrophic muscle cells = m).

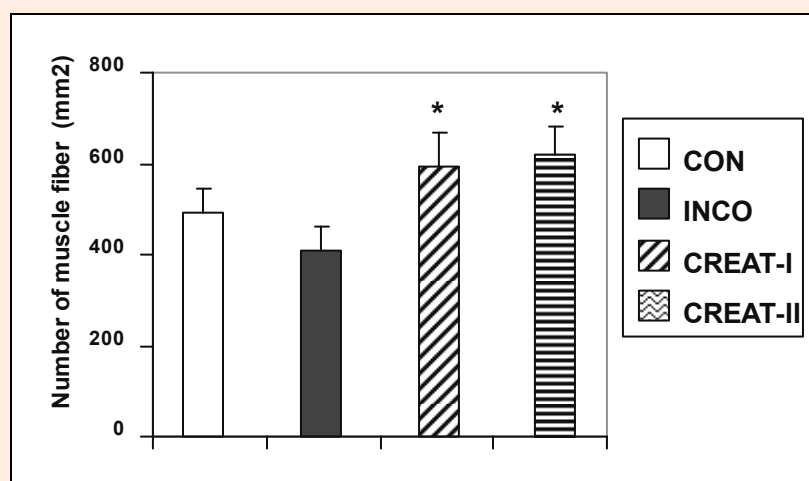
## Discussion

Creatine, a natural nutrient found in animal foods, is alleged to be an effective nutritional ergogenic aid to enhance sport or exercise performance. Research suggests that oral creatine monohydrate supplementation may increase TCr, including both FCr and PCr (Finn et al., 2001; Willoughby and Rosene, 2001). Some, but not all, studies suggest that creatine supplementation may enhance performance in high-intensity, short-term exercise tasks that are dependent primarily on PCr (Mujika et al., 2000; Theodorou et al., 1999).

The objective of this study was to determine whether dietary creatine supplementation enhanced skeletal muscle hypertrophy and endurance swimming performance in an animal model. Evidence is presented herein that creatine supplementation enlarges the magnitude of the cross-sectional areas of skeletal muscle fibers. Furthermore, the quantitative analysis indicated that the number of muscle fibers per defined area increased in creatine supplementation groups. In this study, in the no creatine supplementation group was observed increased connective tissue cell and wasn't hypertrophy when the histological changes were evaluated. On the contrary, in the creatine supplementation groups were seen skeletal muscle hypertrophy. This

effect, nevertheless, was not related to an increase in body weight. In the creatine supplementation groups, body weight increase were not statistically significant as compared with the control group. However, several researcher reported that creatine induces body weight gain in humans (Peeters et al., 1999; Mihic et al., 2000; 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). The underlying basis for this weight gain is still unclear. It may be due to stimulation of muscle protein synthesis (Flisinska and Bojanowska, 1996) or to increased water retention in the initial days of Cr supplementation (Juhn and Tarnopolsky, 1998; Killduf et al., 2004). Because the proportion of fat tended to decrease and lean tissue weight increased with Cr supplementation (Grindstaff et al., 1997; Gallo et al., 2006), the increase in body weight most likely reflects a corresponding increase in actual muscle mass and/or volume, a point that is particularly relevant for bodybuilders.

Numerous studies have examined the effects of short-term creatine supplementation (5-7 days) on exercise performance. As described in a number of reviews, the majority of initial studies suggested that creatine supplementation can significantly increases



**Figure 4.** Morphological analysis of the skeletal muscle in rat. Number of muscle fibers per unit area (mm<sup>2</sup>) (n = 4 fields per animal). No Cr supplementation (CON, n = 15), Incomplete food intake (INCO, n=15), Cr supplementation 1 g·kg<sup>-1</sup>·day<sup>-1</sup> (CREAT-I, n = 15), and Cr supplementation 2 g·kg<sup>-1</sup>·day<sup>-1</sup> (CREAT-II, n = 15). All data are reported as means (SD). \* Significantly different from CON values (p < 0.05).

strength, power, sprint performance, and/or work performed during multiple sets of maximal effort muscle contractions (Hultman et al., 1996; Jäger et al., 2008; Williams et al., 1998). The other studies have supported these observation. For example, Volek et al. (1997) reported that creatine supplementation (25 g·day<sup>-1</sup> for 7 days) resulted in a significant increases in the amount of work performed during five sets of bench press and jump squats in comparison to a placebo group. Urbanski et al. (1999) reported that creatine supplementation (20 g·day<sup>-1</sup> x 5 days) increased maximal isometric knee extension strength and time to fatigue. Moreover, Wiroth et al. (2001) reported that creatine supplementation (15 g·day<sup>-1</sup> x 5 days) significantly improved maximal power and work performed during 5 x 10-sec cycling sprints with 60-sec rest recovery in younger and older subjects. These findings and many others support prior reports indicating that creatine supplementation can improve performance when evaluated in controlled laboratory and testing settings.

Inconclusive data have been published on the effect of Cr supplementation on swimming performance. An improvement in performance parameters was seen in some studies (Grindstaff et al., 1997; Peyrebrune et al., 1998), but not in others (Burke et al., 1996; Thompson et al., 1996). In this study, we were determined that the creatine supplementation increased endurance swimming performance in rats. To our knowledge, this is the first study to report the effects of creatine supplementation on the endurance swimming performance in rats. The average endurance swimming time of all rats was found 14.7 ± 6.0 minutes (range 9 to 23). Contrary to our findings, Young and Young (2007) reported that creatine supplementation did not augment muscle hypertrophy or force production in rat. In the study of Thompson et al. (1996) on trained swimmers, the daily ingestion of only 2 g Cr for 14 days may have been insufficient to increase the muscle stores of total Cr. On the other hand, the tendency toward worse 25- and 50-m performance times of competitive swimmers after Cr supplementation (20

g/day over 5 days) was suggested to be due to an increase in hydrodynamic resistance which is associated with the body weight gain caused by Cr ingestion (Burke et al., 1996; Juhn and Tarnopolsky, 1998). In elite competitive male swimmers, Cr supplementation (9 g/day for 5 days) had no effect on performance in a single 50-yard sprint but significantly improved performance in a repeated sprint set of 8 x 3 50 yards at intervals of 90 s (Peyrebrune et al., 1998). In conclusion, the results published so far suggest that Cr supplementation has either no or even an adverse effect on single-sprint swim performance but may have a slight, favorable effect on repetitive swimming performance. Consequently, an increased rate of PCr recovery after Cr supplementation may be relevant for muscle performance during high intensity, intermittent exercise and might allow harder training units, mostly in explosive sports disciplines, but possibly also in endurance sports. Van Leemputte and co-workers (1999), however, reported that in their studies, Cr supplementation (20–25 g·day<sup>-1</sup> for 2–5 days) had no effect on PCr resynthesis rate but accelerated muscle relaxation during intermittent brief isometric muscle contractions, which also may contribute to the ergogenic action of Cr. More research is required to solve this controversy. To conclude, Cr supplementation may improve muscle performance in three different ways: by increasing the muscle stores of PCr which is the most important energy source for immediate regeneration of ATP in the first few seconds of intense exercise; by accelerating PCr resynthesis during recovery periods; and by depressing the degradation of adenine nucleotides and possibly also the accumulation of lactate during exercise. The results published so far provide both a rational explanation and promising results for an ergogenic action of Cr in intermittent, supramaximal exercise.

## Conclusion

In conclusion, long-term creatine supplementation increased muscle hypertrophy (but not body weight) and

enhanced endurance swimming performance in rats. In addition, it is clear that creatine supplementation enhances the potential to perform high intensity exercise much like carbohydrate loading enhances the potential to perform endurance exercise to exhaustion. However, the underlying basis for this increasing performance of skeletal muscle is still unclear. Therefore, further research is required to examine the physiological and biochemical mechanism of increasing performance of skeletal muscle.

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### Key points

- There is no study about the effects of creatine long-term supplementation on muscle morphology and swimming performance in rats.
- Long-term creatine supplementation increase muscle hypertrophy (but not body weight) and enhance endurance swimming performance in rats.
- The quantitative analysis indicated that the number of muscle fibers per defined area increased in creatine supplementation groups.

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