©Journal of Sports Science and Medicine (2009) **8,** 51-57
http://www.jssm.org

Research article

Effects of voluntary wheel running on satellite cells in the rat plantaris muscle

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Abstract

This study investigated the effects of voluntary wheel running on satellite cells in the rat plantaris muscle. Seventeen 5-week-old male Wistar rats were assigned to a control (n = 5) or training (n = 12) group. Each rat in the training group ran voluntarily in a running-wheel cage for 8 weeks. After the training period, the animals were anesthetized, and the plantaris muscles were removed, weighed, and analyzed immunohistochemically and biochemically. Although there were no significant differences in muscle weight or fiber area between the groups, the numbers of satellite cells and myonuclei per muscle fiber, percentage of satellite cells, and citrate synthase activity were significantly higher in the training group compared with the control group ($p < 0.05$). The percentage of satellite cells was also positively correlated with distance run in the training group ($r = 0.61$, $p < 0.05$). Voluntary running can induce an increase in the number of satellite cells without changing the mean fiber area in the rat plantaris muscle; this increase in satellite cell content is a function of distance run.

Key words: Endurance training, muscle damage, hypertrophy, myonuclear, Pax7.

Introduction

Skeletal muscle satellite cells are located outside the sarcolemma and under the basal lamina of the muscle fiber (Mauro, 1961). They have the ability to generate new muscle fibers or to provide new myonuclei (Schultz and McCormick, 1994). After muscle damage, satellite cells are activated and proliferate for muscle repair and regeneration (Kadi et al., 2005). Therefore, they are considered important structures for the maintenance of normal muscle function.

It is thought that satellite cells are in a non-proliferative quiescent state in the resting condition and that they are activated by intense exercise. In fact, many studies have shown increases in the number of satellite cells after resistance training leading to muscle hypertrophy (Kadi et al., 2000, 2004; Roth et al., 2001; Mackey et al., 2007). Although the exact mechanism is still unknown, the activation of satellite cells by resistance training may be attributable to exercise-induced localized ultrastructural damage, segmental fiber damage, and the release of inflammatory substances or growth factors (Kadi et al., 2005). In addition, various types of prolonged exercise training also induce the activation and proliferation of satellite cells in skeletal muscle of both humans and animals, although the functional load on skeletal muscle may not be as strong as that seen with resistance training (Appell et al., 1988; Umnova and Seene, 1991; Charifi et al., 2003). For example, it was reported that 14 weeks of intermittent cycle ergometer training at 65~95% of the VO_2peak for 45 min increased the number of satellite cells in the vastus lateralis muscle in men aged 70 to 80 years, while hypertrophy was observed simultaneously in type IIA fibers (Charifi et al., 2003). Furthermore, there was a 2.5-fold increase in the number of satellite cells in rat gastrocnemius muscle after 6 weeks of treadmill running at a speed of 35 $m \cdot min^{-1}$ according to an increasing regimen, but the effect on muscle hypertrophy was not clear (Umnova and Seene, 1991). It is not known whether low-intensity exercise training, which does not result in muscle hypertrophy, can also induce the activation and proliferation of satellite cells in skeletal muscle, given sufficient exercise. In this regard, Putman et al. (1999) demonstrated that chronic low-frequency stimulation of fast-twitch muscle in the rat induced an increase in satellite cells. The low-frequency electrical stimulation can be considered as a highly standardized, reproducible protocol of maximum endurance training for muscle. However, their experimental model has not been examined under physiological conditions such as exercise training. Therefore, a suitable animal model should be used to determine whether exercise training can induce satellite cells in skeletal muscle in the absence of hypertrophy.

In animal studies, voluntary freewheel running is considered one of the most effective prolonged training models for inducing aerobic adaptation in rodent muscles, because the daily exercise duration is extremely long, although inter-individual variation is observed (Lambert and Noake, 1990). For example, 6 weeks of voluntary freewheel running increased the cytochrome oxidase activity in the plantaris muscles of the high-runner (> 11 $km \cdot day^{-1}$) group without increasing muscle weight (Rodnick et al., 1989). In addition, Ishihara et al. (1998) demonstrated that 8 weeks of voluntary wheel running in the unloaded condition did not increase the weight or fiber area of the plantaris muscle, regardless of the distance run. Therefore, unloaded voluntary running training constitutes a good model in which to study the effect of low-intensity, long-duration exercise training on satellite cells in skeletal muscle without associated muscle hypertrophy.

In the present study, we tested the hypothesis

Received: 13 November 2008 / Accepted: 03 December 2008 / Published (online): 01 March 2009

that voluntary running will increase the number of satellite cells in the rat plantaris muscle, which is a well-recruited fast-twitch muscle during running (Jasmin and Gardiner, 1987), without causing hypertrophy.

Methods

Animals and treatment

The experimental procedures were approved by the Juntendo University Animal Care and Use Committee and followed the Japanese and American Physiological Society Animal Care Guidelines. Seventeen 5-week-old male Wistar rats were used in this study. A growing rat runs very well voluntarily, and the satellite cell frequency in a 2-month-old rat has stabilized at less than 5% of skeletal muscle nuclei during postnatal muscle growth (Charge and Rudnicki, 2004).

All animals used in this study were obtained from a licensed laboratory animal vender (Japan SLC, Shizuoka, Japan). The rats were maintained in an environmentally controlled room (22.0 ± 0.5 °C, 55.0 ± 5.0% relative humidity) under a fixed 12:12-h photoperiod cycle (lights on from 0900 to 2100). The animals were fed standard rat chow and water *ad libitum*. After a week of acclimation to the animal facility, the rats were assigned randomly to a control (n = 5) or training (n = 12) group. Each rat in the training group was kept individually in a stainless steel cage (19.5 × 27.5 × 3.0 cm) equipped with a unloaded running wheel (1 m·revolution^{-1}) for 8 weeks. The wheel revolutions were counted continuously throughout the experiment, and the number of revolutions indicated by the counter attached to the wheel was recorded at the same time each day.

Immunohistochemical analysis

All animals were anesthetized with pentobarbital sodium (50 mg·kg^{-1}) 48 h after the last training session to avoid the acute effects of training, and the plantaris muscle was removed and weighed. The plantaris muscle was studied because it is a primary ankle extensor and is activated during running (Jasmin et al., 1987). The plantaris muscles were embedded in Tissue Tek OCT compound and frozen in liquid nitrogen. Transverse 8-µm-thick sections were cut using a microtome (CM3050S, Leica, Wetzlar, Germany) at -20°C and mounted on glass slides. The numbers of myonuclei and satellite cells were counted, as described previously (Kojima et al., 2007). Briefly, immunofluorescence staining was used to detect satellite cells. Muscle cross-sections were air-dried and rinsed for 25 min in phosphate-buffered saline with Tween-20 (T-PBS, pH 7.4 and 0.1% Tween-20). T-PBS was used to rinse the sections between the incubation steps. After rinsing, the sections were fixed in 4% paraformaldehyde followed by methanol, for 15 min-each. Then, the sections were boiled using microwaves (BE-S160-W, Sharp, Tokyo, Japan) for 9 min in 0.01 M citrate buffer (pH 6.0) and blocked with 1% blocking regent (Roche Diagnostics, Penzberg, Germany) in T-PBS for 30 min. The sections were incubated overnight at 4°C with mouse monoclonal anti-pax7 antibody (1:500; undiluted tissue culture supernatant of hybridoma cells was obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA, USA) and rabbit polyclonal anti-laminin antibody (1:500; DAKO, Ely, UK) in the antibody solution (1% blocking regent). After rinsing, the sections were incubated for 1 h at room temperature with Cy3 anti-mouse IgG antibody (1:500; Jackson, Baltimore, MD, USA) and FITC-conjugated anti-rabbit IgG (1:200; Sigma-Aldrich, St. Louis, MO, USA) in antibody solution. Finally, the sections were stained with 4' ,6-diamidino-2-phenylindole (DAPI; 1:10,000; Sigma-Aldrich) in T-PBS for 5 min to stain the nuclei and were mounted using Vectashield (Vector Laboratories, Burlingame, CA, USA).

A fluorescence microscope with a ×20 objective (DM5000B, Leica) was used to detect the satellite cells (Figure 1). Satellite cells, laminin, and myonuclei were visualized using N3 (546 nm), L5 (480 nm), and A4 (360 nm) fluorescence cubes, respectively. Images of the sections were obtained with a digital camera connected to the microscope and were stored on a personal computer. In each section, 10 different areas, which contained

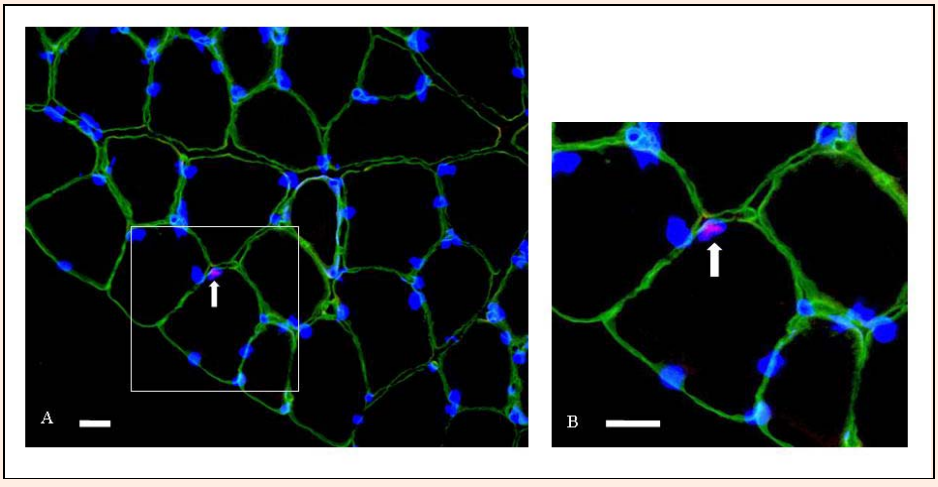

Figure 1. Identification of satellite cell and myonuclei. (A) A satellite cell was detected with triple staining for Pax7(red), myonuclei(blue) and laminin(green). (B) An area is viewed at a higher magnification. The arrow is showing a satellite cell. Bar = 20µm.

Table 1. Voluntary running distance, body weight and muscle weight of the plantaris muscle. Values are means (±SD).

	Control group (n = 5)	Training group (n = 12)
Voluntary running distance (m·day^{-1})		6570 (1773)
Pre-training body weight (g)	133.8 (5.1)	134.7 (7.2)
Post-training body weight (g)	303.9 (16.1)	259.5 (15.0) *
Muscle weight (mg)	300.8 (15.4)	300.6 (20.7)

* Significantly different from control group, $p < 0.05$.

1000~1200 fibers, 25~40 satellite cells, and 2400~3000 myonuclei, were chosen randomly for measuring myonuclei and satellite cells. The numbers of myonuclei and satellite cells per muscle fiber were calculated. For each section, the percentage of satellite cells was calculated as: [number of satellite cells/(total number of myonuclei and satellite cells)] × 100. The total area observed (sum of the 10 areas) was digitized on a computer attached to the fluorescence microscope (IM 500; Leica), and then the mean cross-sectional fiber area was determined by dividing the total area by the total number of fibers (IM 500, Leica). To determine the myonuclear domain, the mean fiber area was divided by the number of myonuclei per fiber.

Analysis of citrate synthase activity

The citrate synthase activity was determined based on the oxidative capacity, which is an index of the effect of endurance training. Muscle samples were minced and then homogenized in ice-cold homogenization buffer (100 mM potassium phosphate, 0.1 mM EDTA, pH 7.4). The homogenate was centrifuged at 400 × g for 15 min, and the enzyme activity in the supernatant was measured spectrophotometrically (U-2000; Hitachi, Tokyo, Japan) at 25°C, based on the method of Srere (1969). The assay was performed in duplicate, and the mean measurement was used to calculate the enzyme activity. The total protein concentration in the supernatant was determined using the Bradford (1976) technique.

Statistical analysis

All data are expressed as means ± SD. The statistical significance of differences between groups was analyzed using an unpaired t-test. Pearson's product correlations were used to determine the relationship between the distance run and the citrate synthase activity and between the distance run and the percentage of satellite cells. The results were considered significant at $p < 0.05$.

Results

Body and muscle weight

The body and muscle weights of both groups are shown in Table 1. After 8 weeks, the body weight in the training group was significantly lower than that in the control group ($p < 0.05$). There was no significant difference in muscle weight between the control and training groups.

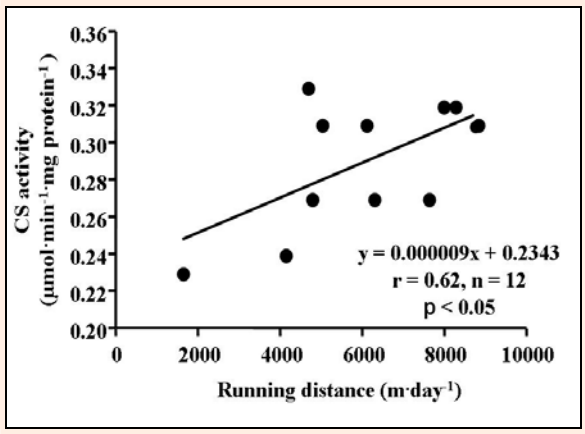

Figure 2. The relationship between daily voluntary running distance and the citrate synthase activity.

Citrate synthase activity

The citrate synthase activity in both groups is shown in Table 2. The citrate synthase activity in the training group was significantly higher than that in the control group ($p < 0.05$). Furthermore, the citrate synthase activity was positively correlated with the distance run in the training group ($r = 0.62$, $p < 0.05$; Figure 2).

Mean muscle fiber area, myonuclei per muscle fiber, and myonuclear domain

The mean muscle fiber area, myonuclei per muscle fiber, and myonuclear domain in both groups are shown in Table 2. The mean muscle fiber area did not differ significantly between the control and training groups. The number of myonuclei per muscle fiber was significantly higher in the training group ($p < 0.05$), while the myonuclear domain was significantly smaller in the training group ($p < 0.05$).

Satellite cells per muscle fiber and the percentage of satellite cells

The satellite cells per muscle fiber and percentage of satellite cells in both groups are shown in Table 2. There were significantly more satellite cells per muscle fiber in

Table 2. Citrate synthase activity and histological properties of the plantaris muscle. Values are means (±SD).

	Control group (n = 5)	Training group (n = 12)
Citrate synthase activity (μmol·min^{-1}·mg protein^{-1})	.25 (.01)	.30 (.03) *
Mean fiber area (μm^2)	7063 (419)	7195 (969)
Number of myonuclei per muscle fiber	2.03 (.14)	2.48 (.40) *
Myonuclear domain (μm^2)	3489 (350)	2983 (389) *
Number of satellite cells per muscle fiber	.021 (.004)	.032 (.007) *
Percentage of satellite cells (%)	1.03 (.17)	1.27 (.15) *

*Significantly different from control group, $P < 0.05$. The percentage of satellite cells (%) = [number of satellite cells/(total number of myonuclei and satellite cells)] × 100.

the training group ($p < 0.05$), and the percentage of satellite cells was significantly higher in the training group ($p < 0.05$). Furthermore, the percentage of satellite cells was positively correlated with the distance run ($r = 0.61$, $p < 0.05$; Figure 3).

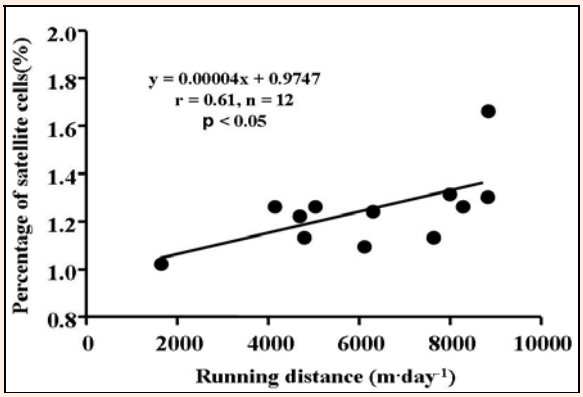

Figure 3. The relationship between daily voluntary running and the percentage of satellite cells.
The percentage of satellite cells (%) = [number of satellite cells/(total number of myonuclei and satellite cells)] × 100.

Discussion

The effects of resistance training on satellite cells were well studied but there are a few studies about the effects of endurance training on satellite cells. A previous study in human shows that the amplitude of the increase in satellite cell number following an high intensity interval training is within the same range as that reported in response to strength training (Charifi et al. 2003). Verney et al. (2008) also demonstrat the effects of 14 weeks of concurrent lower body endurance and upper body resistance training on vastus lateralis and deltoid muscles of elderly men and they show that satellite cell pool increases similarly in both muscles, mainly in type II muscle fibers. It is very interesting that satellite cells can respond to a training intensity much lower than that used in resistance training because the power developed at VO_2peak is about 25% of the maximal power (Charifi et al., 2003; Kadi et al., 2005). However, these human studies investigated effects of high intensity interval training to elderly men and the training intensity seems to have been relatively high as an endurance training. In addition, fiber area of vastus lateralis muscle which was mainly recruited by the endurance training was significantly increased although aerobic capacity was also increased.

The purpose of this study was to clarify the effect of low-intensity, long-duration exercise training on satellite cells in skeletal muscle without associated muscle hypertrophy. As expected, the voluntary running model enhanced citrate synthase activity but caused no changes in absolute muscle mass or fiber size. The main finding was that voluntary running significantly increased the number of satellite cells in the plantaris muscle and that the increase in satellite cells was positively correlated with the distance run ($r = 0.61$, $p < 0.05$). To our knowledge, this is the first study to show an increase in the number of satellite cells in rat skeletal muscle with voluntary running training without muscle hypertrophy.

Increase in satellite cells with voluntary wheel running

Many studies have shown increases in the number of satellite cells following resistance training that leads to muscle hypertrophy (Kadi and Thornell, 2000; Kadi et al., 2004; Mackey et al., 2007; Roth et al., 2001). An increase in the number of satellite cells is reported to be associated with fibroblast growth factor (Sheehan and Allen, 1999) and insulin-like growth factor-I (IGF-I) (Charge and Rudnicki, 2004). As resistance training increases IGF-I secretion, it also promotes satellite cell proliferation and fusion and causes muscle hypertrophy. However, IGF-I secretion is regulated mainly by serum testosterone and growth hormone, which are determined more by exercise intensity than exercise volume (Hawke, 2005). The purpose of prolonged endurance training, as in our study, is not to cause muscle hypertrophy, but rather to increase the oxidative capacity. Indeed, previous studies have demonstrated that voluntary wheel running did not affect the plantaris muscle mass or fiber size (Ishihara et al., 1998; Rodnick et al., 1989). In our study, the absolute muscle weight and mean muscle fiber area determined using a simple method did not differ significantly between the control and training groups, although the citrate synthase activity was increased in the training group. Therefore, the exercise intensity of voluntary wheel running training in this study was insufficient to induce muscle hypertrophy. Ishihara et al. (1998) also demonstrated that 8 weeks of voluntary running on an unloaded wheel did not increase the weight or fiber area of the plantaris muscle regardless of the distance run, whereas running on a wheel in the loaded condition did. In our study, the running wheel was unloaded, and the results were consistent with the previous study. Given that voluntary running does not result in an increase in IGF-I mRNA expression in hind limb muscles (Matsakas et al., 2004), we speculate that the increase in the number of satellite cells observed in this study was not associated with hypertrophy involving the hormonal mechanisms observed in resistance training.

Verney et al. (2008) have recently suggested that satellite cell population in the human skeletal muscle might be genetically determined. However, in our study model, the same strain, gender and age of rats were obtained from a licensed laboratory animal vender and they were randomly assigned to either a control or training group. In addition, their voluntary running activities in the training group were between about 2 and 9 km·day^{-1} and it was very similar to previous studies and rats in the training group would not be especially talented as high runners (Ishihara et al. 1998; Rodnick et al., 1989). Therefore, it is thought that there was no difference of genetic factors between the two groups and that the involvement of genetic factor in the regulation of satellite cell content could be ruled out in this study model.

The activation of satellite cells may also involve exercise-induced localized ultrastructural damage,

segmental fiber damage, and the release of inflammatory substances or growth factors (Kadi et al., 2005). Myofiber damage leads to the release of nitric oxide, which mediates the release of active hepatocyte growth factor from the heparin sulfate chains on the extracellular matrix and surrounding myofibers (Hawke, 2005). In the present study, it is not clear whether physical activity per se or muscle injury induced by exercise training secondarily stimulated satellite cell division; nevertheless, running may markedly stimulate satellite cells.

For example, McCormick and Thomas (1992) showed that 10 weeks of progressive treadmill running doubled the satellite cell mitotic activity and doubled the number of damaged fibers in the soleus muscle of rats. In addition, Umnova and Seene (1991) reported that the number of satellite cells increased 2.5-fold with local muscle damage after 6 weeks of treadmill training. It has been reported that voluntary wheel running in the mouse causes eccentric contractions, damaging the hind limb muscles (Werning et al., 1990). Furthermore, regardless of strain of mice, hind limb muscles are damaged and repaired with voluntary wheel running (Irintchev and Wernig, 1987). Although we did not investigate whether the voluntary wheel running caused muscle damage, we believe that it would have occurred, as in the previous studies. Therefore, the muscle damage induced by daily voluntary running may stimulate an increase in the number of satellite cells.

Changes in domain size and endurance training

Although the fiber area did not differ between the control and training groups in this study, voluntary wheel running significantly increased the number of myonuclei. As a result, the myonuclear domain size was significantly smaller in the training group. One possible explanation for the ability of endurance training to induce an increase in the number of myonuclei is related to a change in fiber type. The number of myonuclei is influenced by fiber type: muscle fibers of slow muscle have smaller myonuclear domains than those of fast muscle (Roy et al., 1999). In the plantaris muscle of cats, slow fibers have 25 to 50% more myonuclei in a longitudinal section and a smaller average myonuclear domain size compared with fast fibers (Allen et al., 1995). Voluntary wheel running is considered one of the most effective types of exercise training to increase oxidative capacity in rodents (Rodnick et al., 1989); it can increase the percentage of fast-twitch oxidative glycolytic fibers and decrease the percentage of fast-twitch glycolytic fibers in the rat plantaris muscle (Ishihara et al., 1991). Although we did not determine muscle fiber type in the plantaris muscle, it is possible that the voluntary wheel running training in our study transformed fast-twitch glycolytic muscle fibers into slow or oxidative fibers, as in previous studies. Therefore, the increase in the number of myonuclei with endurance training could be related to changes in muscle fiber type and the concomitant increase in the oxidative capacity of fast type fibers in the plantaris muscle. Alternatively, an increase in muscle nuclei in the fast fibers may be a prerequisite for a fast-to-slow fiber transition (Putman et al., 2000).

In addition, in this study, the percentage of satellite cells was positively correlated with the distance run. Satellite cell activation occurs in response to various protocols that enhance contractile activity. Putman et al. (1999) showed that low-frequency electrical stimulation of fast-twitch muscles in the rat led to a time-dependent increase in satellite cell content and myonuclear density, although the experiment did not involve physiological conditioning such as exercise training. Briefly, they showed that the percentage of satellite cells increased from 3.8% in control muscle to 7.9, 11.5, and 13.8% in muscles stimulated for 5, 10, and 20 days, respectively, with the transformation of existing fast fibers into slower fibers. In our study, the percentage of satellite cells was positively correlated with the distance run ($r = 0.61$, $p < 0.05$; Figure 3); the greater the total distance run or time spent exercising, the greater the percentage increase in satellite cells. In addition, citrate synthase activity was significantly higher in the training group, and the increase was also a function of the distance run ($r = 0.62$, $p < 0.05$; Figure 2). Combined, these results indicate a relationship between an increase in satellite cell number and amount of exercise or muscular oxidative capacity. However, we did not determine muscle fiber types and regional differences (e.g. distal, middle, or proximal) in the plantaris muscle and did not analyze a slow type of muscle like the soleus muscle. Further investigation is required to elucidate the muscle fiber type and region specific adaptation of satellite cells with endurance training.

New myonuclei arise by the incorporation of proliferating satellite cells to maintain the myonuclear domain size, and an increase in the number of myonuclei generally occurs with increased muscle fiber area (Kadi and Thornell, 2000). Resistance training was shown to increase the muscle fiber area, yet the number of myonuclei was unchanged (Hikida et al., 1998; Kadi et al., 2004). Kadi et al. (2004) explained that until a certain limit of hypertrophy is reached, an increase in the area of muscle fibers could occur without the addition of new myonuclei. If endurance training were to result in the opposite adaptation to the phenomenon seen in resistance training, it may be possible that the satellite cells and myonuclei change first. Further investigation is required to elucidate the adaptation of satellite cells and myonuclear domain that occurs with endurance training.

Conclusion

The present study showed that voluntary running can induce the proliferation of satellite cells without changing the mean fiber area in the rat plantaris muscle; this increase in satellite cell content is a function of the distance run. This study found that endurance training can be used as preconditioning to increase muscle volume and to improve muscle function. Further study is necessary to clarify the mechanisms of satellite cell proliferation and differentiation following endurance training.

Acknowledgments

Our research was partly supported by Grants-in-Aid for Scientific Research (16300212 and 12480011 to H.N.) and High Technology Research Center Grants for Juntendo University from the Ministry of

Education, Culture, Sports, Science, and Technology of Japan.

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Key points

- There is no study about the effect of voluntary running on satellite cells in the rat plantaris muscle.
- Voluntary running training causes an increase of citrate synthase activity in the rat plantaris muscle but does not affect muscle weight and mean fiber area in the rat plantaris muscle.
- Voluntary running can induce an increase in the number of satellite cells without hypertrophy of the rat plantaris muscle

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