Aging attenuates muscle responsiveness to creatine supplementation, but not overload, in rat plantaris muscles

Naomi E. Brooks¹, Mark D. Schuenke², Robert S. Hikida³

¹Department of Physiological Sciences, Stellenbosch University, Stellenbosch, South Africa

²Department of Anatomy, University of New England, College of Osteopathic Medicine, Biddeford, Maine, USA

³Department of Biomedical Science, Ohio University College of Osteopathic Medicine, Athens, Ohio USA

Corresponding Author: Naomi Brooks

Department of Physiology
Stellenbosch University
Private Bag XI, Matieland
Stellenbosch 7602
South Africa

nbrooks@sun.ac.za

Tel: 27 (0)21 808 3155
Fax: 27 (0)21 808 3145

Running head: Creatine supplementation in aging skeletal muscle
Abstract

Creatine supplementation, combined with resistance training, is beneficial in increasing lean body mass and strength. Although creatine supplementation in elderly individuals is of interest for reducing sarcopenia, studies have produced equivocal results. In the present study, a compensatory hypertrophy model is used to compare the effects of creatine on hypertrophy in young and aging rats. METHODS: Young (5m) and aging (24m) male Fisher 344 rats were each randomly allocated to one of four treatment groups: 1) control (C); 2) creatine supplementation (Cr); 3) overloaded (O); or 4) overloaded plus creatine (OCR). O and OCR underwent bilateral surgical ablation of the gastrocnemius to functionally overload the plantaris muscle. Cr and OCR were supplemented with creatine monohydrate (4.45g/L in 5% dextrose) in their drinking water. Four weeks post-treatment, the plantaris muscle fiber type, CSA and myonuclear domain size were measured. RESULTS: Creatine alone significantly increased CSA in all fiber types of young (I:26%, IIA:28%, IIB/D:17%) but not aged animals (p < 0.05). Overload alone significantly increased CSA of all fibers in young (I:29%, IIA:35%, IIB/D:12%) and of type I (25%) and IIA (14%) fibers in aging animals (p < 0.05). OCR significantly increased CSA in all fibers of young animals (I:37%, IIA:38%, IIB/D:25%) but only I (27%) and IIA (14%) of old animals (p < 0.05).

CONCLUSION: These data indicate that creatine alone leads to hypertrophy in young animals but does not affect hypertrophy in aging muscle. Further research is needed to investigate the mechanisms underlying the unresponsiveness of aging muscle to creatine supplementation.
Introduction

Creatine supplementation is a popular dietary supplement with athletes. It has been shown to increase exercise performance (3, 19, 25) and strength (38). Creatine (C₄H₉N₃O₂) is a naturally occurring molecule in skeletal muscle and is both obtained in the diet and synthesized in the muscle. Creatine and phosphocreatine (PCr) are involved in coupling anaerobic metabolism with ATP demand (58). PCr can donate a high-energy phosphate to adenosine diphosphate to recreate adenosine triphosphate (ATP), especially at the onset of exercise and during intense contractions. Increased intramuscular levels of PCr may permit increased temporal buffering of ATP during high-intensity muscle contraction, and increased creatine levels may allow increased PCr re-synthesis (24). In so doing, creatine supplementation may also increase muscle mass and strength by allowing training at higher workloads (34). However, the mechanisms behind the enhancement of performance and increases in muscle strength by creatine supplementation have not been fully elucidated. It is thought that creatine exerts its effects through increasing myosin heavy chain mRNA and protein and this has been indicated, both in vivo (59) and in vitro (29, 30). Creatine may preferentially affect type II fibers (50); however, increases in fiber cross-sectional area of type I, IIA and IIAB muscle fibers have been noted after creatine supplementation combined with resistance training (56). Similarly, Willoughby and Rosene (59), demonstrated an increase of myosin heavy chain types I, IIA and IIX at both mRNA and protein levels, after 12 weeks of combined strength training and creatine supplementation in young individuals.

Aging is associated with reductions in skeletal muscle mass and strength (sarcopenia), which may lead to impaired mobility in older individuals. Despite a litany of research on sarcopenia, the underlying mechanisms remain to be elucidated. Comprehensive reviews of the potential mechanisms behind sarcopenia are available [eg (49)]. In brief, declines in muscle-specific stem cells (satellite cells); increases in myostatin, a negative regulator of muscle growth; changes in circulating hormone levels; and impairment of neuromuscular
function have all been proposed as mechanisms underlying sarcopenia. Of these proposed mechanisms, resistance training combined with creatine has been shown to significantly increase satellite cell proliferation in young males (41). Creatine supplementation combined with resistance training may also increase resting testosterone in young men (28). However, creatine supplementation does not appear to have a direct effect on myostatin (17). Furthermore, it is currently unknown whether these findings hold true in aging muscle.

The use of a dietary supplement, such as creatine, to counteract sarcopenia is not unprecedented. Other nutritional interventions have produced positive results against sarcopenia. High protein diets may be beneficial for stimulating protein synthesis in the muscles of aging individuals (43). The use of creatine in elderly populations is of interest because of its potential to increase energy production, fat free mass and muscle mass (53). Combined, these effects would increase the ability of elders to perform everyday tasks such as rising from a seated position, walking, etc. However, older individuals appear to respond differently than young individuals to creatine supplementation (45). Short durations of creatine supplementation alone in elders have shown mixed results. Creatine supplementation for five days in aging population had no effect on isometric strength (31, 44). Similarly, five days of creatine supplementation had no effect on pedaling performance in cycling trained elderly subjects (60). Conversely, 5 days of creatine supplementation increased anaerobic power and work capacity of sedentary elderly subjects (60). Short term creatine supplementation has also produced increases in strength and fat free mass of elderly men (22) and women (21).

Longer term creatine supplementation combined with resistance training in the elderly has also demonstrated varied responses. The combined treatments resulted in either no extra benefit after 8 weeks of strength training and supplementation (4), small increases in muscle strength after 12 weeks (12) or moderate increases in strength and creatine levels after 14 weeks (9) beyond resistance training alone. It has recently been shown that low dose creatine
supplementation combined with protein supplementation and resistance training for 10 weeks increases lean tissue mass but not leg press strength (10). Furthermore, there was a greater response in creatine combined with protein supplementation than creatine alone or placebo. Conversely, neither creatine supplementation, protein supplementation, or a combination of protein and creatine was shown to provide further benefit beyond 16 weeks of isotonic resistance training alone in elderly subjects (11).

In light of these controversial results, an animal model of hypertrophy (surgical ablation/compensatory hypertrophy) was chosen to compare the effects of creatine supplementation on hypertrophy in young and aging rats. Surgical ablation is an animal model involving surgical removal of a muscle, thereby overloading its synergistic muscles and causing them to hypertrophy in compensation (2, 20, 48). The authors are aware of one other study that used this model to study creatine supplementation. Dangott et al. (15) showed no further hypertrophy in muscle fibers with creatine supplementation above compensatory hypertrophy alone. However, that study did not separate muscle into individual fiber types. This study was designed to investigate whether creatine supplementation alone, surgical ablation alone, or a combination of creatine supplementation and surgical ablation would lead to hypertrophy in individual fiber types of the plantaris muscle. Furthermore, the study examined whether there would be a different response in the young compared to the older animals after these experimental interventions. It was hypothesized that creatine supplementation would primarily increase CSA in fast fibers (IIA, IIB/D) and that this will be attenuated in aging muscle. The values for CSA and nuclear domain for the control animals only were also reported in Brooks et al. (8).
Materials and Methods

Animals

Young (5 months, N = 35) and aging (24 months, N = 27) adult male Fisher 344 rats from National Institute on Aging rodent colony (Harlan Labs, Indianapolis, IN) were used. Fisher 344 rats were selected, because their growth rate plateaus around 5 months of age. Relative to other strains, the mass of Fisher 344 rats does not increase as much with age. Animals were divided into eight groups: 1) young control (YC, N = 6); 2) young supplemented with creatine (YCr, N = 7); 3) young overloaded (YO, N = 7); 4) young overloaded and supplemented with creatine (YOCr, N = 7); 5) aging control (AC, N = 8); 6) aging supplemented with creatine (ACr, N = 9); 7) aging overloaded (AO, N = 9); and 8) aging overloaded and supplemented with creatine (AOCr, N = 9). All rats were housed at the Ohio University Animal Facility. They were housed individually and provided with free access to rat chow and water. Temperature was maintained at 22°C ± 2°C, and conditions consisted of a 12-hour:12-hour dark-light cycle. The experiments were approved by the IACUC of Ohio University.

Surgical Procedure

Animals were allowed 10 days to recover from shipping and to become accustomed to their surroundings prior to experimentation. At that point, four experimental groups (YO, YOCr, AO, AOCr) underwent surgical ablation surgery to induce overload by compensatory hypertrophy. Rats were anesthetized intraperitoneally by injection of either Veterinary Pentothol (~0.07mg/kg body weight) or a combination of Xylazine and Ketamine (0.01g/kg body weight xylazine; 0.01g/kg body weight ketamine). When there was no reflex response, an incision (~2.5cm) was made through the skin of the posterior compartment of the leg to expose the underlying muscle. The distal third of the gastrocnemius was removed bilaterally. Bilateral surgery ensured the animal would not favor the non-operated leg. In addition to the
plantaris, the soleus muscle was left intact. Analysis of the soleus can be found elsewhere (8). Care was taken to ensure blood supply and innervation to remaining muscles was not compromised. The incision was sutured with 4.0 black braided silk and the stitches were approximately 0.3cm apart, ensuring the incision was completely closed. After overnight recovery from the anesthetic, the animals were returned to their cages.

Creatine Supplementation

After returning to the cages, dietary creatine supplementation began for those in the creatine groups (YCr; ACr; YOCr; AOCr). Rats were given creatine monohydrate, 4.45g/L (Sigma Chemical Company, St Louis, MO) with a 5% dextrose solution (Sigma Chemical Company, St Louis, MO) in the drinking water, *ad libitum*. Consumption of creatine solution was measured daily. The groups not receiving creatine were given 5% dextrose in the drinking water. The dextrose solution was chosen because creatine uptake is increased when combined with a carbohydrate solution (23). Fluid levels for all rats were measured and replaced daily.Supplementation was continued for 4 weeks.

Muscle Extraction

Four weeks after the surgical ablation and/or beginning of creatine supplementation, the animals were killed, and the plantaris muscles were prepared for analysis. Samples were mounted for cross-sectioning in a mixture of tragacanth gum and optimal cutting temperature (OCT) cutting medium (Sakura Fine Technical Co, Torrence, CA) and snap-frozen in methylbutane, cooled with liquid nitrogen. Samples were stored at –80°C until they were sectioned. Animals were subsequently euthanized by an anesthetic overdose, followed by heart removal.

Immunohistochemistry
Samples were cryosectioned at -20°C to thicknesses of 10μm for ATPase analysis and 6μm for immunohistochemistry for CSA measurement. Sections were placed onto cover slips coated with poly-L-lysine and stored at –40°C until the completion of immunohistochemical analysis.

**Cross-sectional Area & Myonuclei**

Using 6μm sections, the cell membrane and basal lamina were identified by immunostaining with antibodies for dystrophin (monoclonal anti-dystrophin antibody; Sigma-Aldrich, St Louis, Missouri) and laminin (goat polyclonal anti-laminin antibody; Santa Cruz Biotechnology, Santa Cruz, California), respectively. Sections were counterstained with hematoxylin to identify myonuclei. Sections were blocked with blocking serum, incubated with the dystrophin primary antibody (4°C) for 36 hours, washed with washing buffer, incubated in biotinylated secondary antibody (Vector, Burlingame, CA), washed, and labeled with the avidin-biotin complex (ABC) reagent conjugated with peroxidase. The sections were incubated with laminin antibody (22°C) for 2 hours and the same series of reactions repeated. The sections were then incubated in Nickel-enhanced DAB, stained with hematoxylin and dehydrated in a graded alcohol series, before clearing and mounting.

Cross-sectional area (CSA) was measured for each muscle fiber on a computer using a video captured microscope image of the cells stained with dystrophin and laminin and NIH Image software (v.1.62). Cross-sectional area was calculated for at least 300 fibers (from 3 random microscope frames ~0.56mm²), or all the fibers in the sample.

The myonuclei were counted at 1000X magnification and were identified due to their location inside the sarcolemma and the basal lamina. The total number per fiber type and total number per 100 fibers (%) were counted. Adjacent sections prepared for myofibrillar ATPase were matched with these fibers to characterize the fiber type and its properties.
Fiber Typing

Sections were stained for mATPase activity, using previously described techniques (7). In brief, serial sections were pre-incubated in a solution made to pH 4.15 or pH 4.45. These pH pre-incubations allowed characterization of Type I, Type IIA and Type IIB/D fibers.

Myonuclear Domain

For each fiber type, the Myonuclei/Cytoplasm (N/C) ratio was determined to estimate myonuclear domain (Myonuclear domain = Mean CSA/Mean myonuclei) as previously published (27). Myonuclear domain was compared between fiber types and for each group and age class.

Statistical Analysis

All statistical analyses were performed using Systat 11. A two-way analysis of variance (ANOVA) was used to compare the dependent variables (creatine and overload) across ages and muscle fiber types. Significance was set at p ≤ 0.05. Where significance was found for main effects, Fisher’s Least Significant Difference method was used for post-hoc analysis. One-way ANOVA was used to compare creatine consumption, muscle mass, and body mass across ages. T-tests were used for animal and muscle mass analyses. Significance was set at p ≤ 0.05. Where significance was found for main effects, Fisher’s Least Significant Difference method was used for post-hoc analysis.
Results

Fluid Consumption and Creatine Intake

Fluid intake is shown in Table 1. Older animals took in more creatine solution than young (p<0.05). For the duration of the study, all animals had consistently more creatine intake than recommended for loading dose (0.3g/kg body weight/day) and maintenance dose (0.03g/kg body weight/day) as advised in the literature by Kreider and colleagues (35).

Body Weight and Muscle Weight

Muscle mass was significantly heavier in the aging rats, regardless of the treatment group (Table 2). The within-subject difference in body mass between pre- and post-treatment showed both aging groups that underwent surgical overload (AO and AOGr) lost mass. Compared to the young overloaded animals (YO and YOGr), these differences were significant (Table 2). In both the young and aging animals, the plantaris muscle of OCr group was heavier than control or creatine (young 30% heavier and older 18% heavier; p<0.05) (Table 3). The ratio of plantaris weight/body weight was also greater in the OCr group than control of creatine in both young and aging animals. In each experimental group, older muscles were heavier than young muscles in all groups (Table 3).

Muscle Fiber Analysis: Young vs. Aging Control Muscle

The plantaris muscle was composed of predominantly fast fibers. Plantaris muscle from aging animals had a higher percentage type I fibers and a lower percentage type II fibers than young animals (Table 4). The percentage of type IIB/D fibers was similar between young and aging animals. For all treatment groups and ages, CSA data for type I, IIa, and IIB/D fibers are presented in figures 1a, 1b, and 1c, respectively. Fiber CSA was significantly larger in type IIA fibers of older rats, as compared to young rats (1822.98 ± 151.84 µm² vs. 1484.85 ± 93.34 µm²; Figure 1b). There were no other differences in CSA between young...
and older animals for any fiber type (p > 0.05). CSA for IIB/D was greater than I or IIA for all groups (p < 0.05). Myonuclear domain size significantly differed between fiber types within age groups of control animals (p < 0.05), except there was no difference between type I and IIA in YC (p = 0.717).

**Creatine Supplementation**

Creatine supplementation had no significant effect on fiber type composition. Fiber CSA was significantly larger in young animals receiving creatine than young control rats, in all fiber types. Creatine supplementation was associated with a 26% increase in the CSA of type I fibers (1771.91 ± 325.96 µm² vs. 1315.08 ± 281.22 µm²; Figure 1a). Creatine also increased the CSA of type IIA and IIB/D fibers by 28% and 17%, respectively (2059.00 ± 115.28 µm² vs. 1484.85 ± 99.34 µm² and 3955.57 ± 264.98 µm² vs. 3284.22 ± 536.51 µm²; Figures 1b & 1c). In aging rats, creatine supplementation did not significantly affect fiber CSA for any fiber type (p > 0.05).

The effects of creatine supplementation on myonuclear domain were both age and fiber type specific. In type I fibers of young animals, the myonuclear domain size was not different between creatine supplementation and control animals (1458.47 ± 189.79 µm² vs. 1320.14 ± 298.32 µm²; Figure 2a). In young animals, the myonuclear domain sizes of type IIA and IIB/D fibers were significantly larger in creatine supplemented rats than in control rats (1667.02 ± 274.34 µm² vs. 1236.68 ± 239.61 µm² and 2386.83 ± 368.47 µm² vs. 1930.17 ± 405.09 µm²; Figures 2b and 2c, respectively). Similarly, creatine supplementation had no effect on myonuclear domain size in aging animals compared to control animals, regardless of fiber type (p > 0.05). Myonuclear domain was significantly different between fiber types within age groups of creatine-supplemented animals (p < 0.05), except there was no difference between type I and IIA in YCr (p = 2.66).
Overloaded Plantaris Muscle

In the overload group, young animals had significantly more type I fibers than control animals of a similar age (Table 4). There was no difference between the percentages of type I fibers in young and aging animals of the overload treatment. This was different from the other groups in which the older animals had higher percentages of type I fibers than the young animals (Table 4). Regardless of age, the percentage of type IIB/D fibers was lower in the overloaded group than in the control group.

Surgical overload significantly increased the CSA of fibers in the plantaris of young rats, regardless of fiber type (I 29%, 1841.68 ± 196.98 µm² vs. 1315.08 ± 281.22 µm²; IIA 35%, 2270.01 ± 142.84 µm² vs. 1484.85 ± 93.34 µm²; IIB/D 12%, 3747.01 ± 263.40 µm² vs. 3284.22 ± 536.51 µm²; figs 1a-1c). In the older animals, overload induced fiber hypertrophy of the slow-twitch fiber (25%; 1879.37 ± 212.47 µm² vs. 1413.42 ± 219.69 µm²) and IIA (14%; 2112.83 ± 226.32 µm² vs. 1822.98 ± 151.84 µm²) fiber types compared to the control group (Figure 1a-1b). However, CSA of type IIB/D fibers of aging animals were not significantly different between overloaded and control groups (p = 0.29; fig 1c). Despite seeing overload-associated hypertrophy in nearly all fiber types (except type IIB/D fibers of older rats), only type IIA fibers experienced a significant increase in myonuclear domain. This was true in young and older animals (1858.96 ± 287.91 µm² vs. 1236.68 ± 239.61 µm² and 1691.82 ± 145.18 µm² vs. 1408.73 ± 164.59 µm², respectively; Figure 2b). Overload did not cause any other significant changes in myonuclear domain size for any fiber type, irrespective of age (p > 0.05; Figures 2a-c). Myonuclear domain size was smallest in type I fibers; type IIA and IIB/D had significantly larger domain sizes than type I in both young and older animals. Myonuclear domain sizes were larger in type IIA fibers than type I fibers of older animals compared to young animals (p < 0.05). Myonuclear domain was significantly different between fiber types within age groups of overloaded muscle.
Overload and Creatine Supplementation

A combination of overload and creatine supplementation did not have the same influence on fiber type composition as overload alone. In young rats, the treatment group that underwent surgical overload and creatine supplementation (YOCr) had a significantly lower percentage of type I fibers than the treatment group that only underwent surgical overload (YO). Furthermore, the percentage of type I in the YOCr group was similar to young controls. In young rats, the overloaded plantaris had a significantly higher percentage of type I fibers than overloaded plantaris from rats that also received creatine. However, similar to the control group, older animals in the AOCr group had a significantly higher percentage of type I, and a significantly lower percentage of type IIA fibers than young animals (Table 4). By contrast, overload alone did not result in significant changes to fiber type composition in aging animals.

A combination of creatine and surgical overload produced significant hypertrophy in most muscle fibers. In young animals, the OCt treatment significantly increased fiber CSA in all fiber types, relative to young controls (37%, 2085.09 ± 224.12 µm² vs. 1315.08 ± 281.22 µm²; 38%, 2401.51 ± 199.17 µm² vs. 1484.85 ± 93.34 µm²; 25%, 4352.28 ± 519.12 µm² vs. 3284.22 ± 536.51 µm², respectively). In older animals, only type I and IIA fibers of the OCr group were significantly larger than those fibers in the aging control animals (27%, 1949.01 ± 180.77 µm² vs. 1413.42 ± 219.69 µm² and 14%, 2125.66 ± 205.74 µm² vs. 1822.98 ± 151.84 µm², respectively). Type IIB/D fibers of older animals in the OCr group did not change fiber sizes over controls (p = 0.698, Figures 1a-c).

The combined effects of creatine and overload differentially affected the myonuclear domains in the plantaris muscle of young and aging rats. For all fiber types, myonuclear domain was significantly larger in fibers of the YOCr group than in fibers from YC (I 12%, 1559.68 ± 201.39 µm² vs. 1320.14 ± 298.32 µm²; IIA 34%, 1934.58 ± 185.16 µm² vs. 1236.68 ± 239.61 µm²; IIB/D 18%, 2559.80 ± 510.02 µm² vs. 1930.17 ± 405.09 µm²).
Myonuclear domain was also significantly larger (14%, 1576.03 ± 247.18 µm² vs. 1408.73 ± 164.59 µm²) in type IIA fibers of the YOCr than in fibers of the YCr group. In older rats, the myonuclear domain was not significantly affected by combined creatine and overload, regardless of the fiber type (p > 0.05). Myonuclear domain was significantly different between fiber types within age groups of animals in the overload plus creatine supplementation groups, except there was no difference between type I and IIA in YOCr (p = 0.102).

The combination of creatine and overload also resulted in significantly greater fiber CSA than creatine alone in fiber types I and IIA, regardless of age. For the CSA of type IIB/D fibers, there was a trend toward significance between YOCr and YCr groups (p = 0.051). This trend was not apparent in the aging animals. The CSA of type IIB/D fibers were significantly larger in YOCr than in YO animals, and there was a similar trend in the CSA of type I fibers (p = 0.057). In aging animals, there were no significant differences between the CSA of AO for any fiber types.
Discussion

Creatine is a popular supplement with recreational and professional athletes. Research has shown that creatine supplementation, in combination with resistance training, can be beneficial in increasing lean body mass and strength [reviewed in (42)]. Only a few studies have looked at creatine supplementation alone in skeletal muscle tissue. Fewer still have examined the fiber type-specific effects of creatine supplementation on fiber CSA of aging muscle fibers. The current data demonstrate that the plantaris muscles of young and aging rats respond similarly to surgical overload. This is true for fiber type composition, cross-sectional area, and myonuclear domain size. However, the influences of creatine supplementation alone or creatine combined with overload surgery were age-specific.

Creatine Supplementation

The first specific aim of this research was to assess the efficacy of creatine supplementation in aging muscle. Data from the current study indicate creatine alone results in significant increases in fiber CSA and myonuclear domains of young animals. However, creatine supplementation alone had no effect on CSA or myonuclear domain for any fiber type in the plantaris of aging animals. There was also no effect of creatine alone on fiber type composition in young or aging rats. Few studies have examined the effects of creatine supplementation in the absence of exercise. In vitro, creatine alone has been shown to stimulate myosin heavy chain synthesis (29), and satellite cell proliferation and differentiation (55). These findings are in agreement with results in this study for YCr rats (creatine-induced increases in CSA and myonuclear domain). In vivo, creatine supplementation stimulated satellite cell activity, but this did not significantly alter fiber CSA (15). These data initially appear to contradict results reported here; however, Dangott and colleagues (15) did not separate their CSA measurements by fiber type. If creatine has a fiber type-specific effect, a failure to separate the data by fiber types could obscure significant effects. To this end,
creatine supplementation without exercise was found to increase expression of target genes such as mRNA of MHC I at rest and MHC IIa immediately after exercise, but did not enhance anabolic signals (17).

It is well documented that creatine supplementation may have beneficial effects for athletic performance in aging individuals. Studies in aging humans have shown varying results. Creatine supplementation combined with resistance training resulted in some level of increased lean tissue, strength, and endurance (10, 12, 21, 22), but none of these studies examined changes at the fiber level. The authors are not aware of any previous research that investigated fiber-specific changes subsequent to creatine supplementation in aging muscle.

In comparison to the effect of creatine supplementation on skeletal muscle of young animals, the results of this study showed no increase in hypertrophy of older animals. There are a number of possible explanations for this. Creatine supplementation significantly increases the myoprotective effects of carnosine and anserine in young, but not older animals (18). Additionally, Rawson and colleagues determined that creatine supplementation in young adults resulted in significantly greater muscle phosphocreatine concentration as compared to older adults (45). Non-supplementation studies also indicate that aging muscle is less capable of synthesizing or storing creatine. Compared to young skeletal muscle, aging muscle has fewer stores of PCr and Cr and a lower rate of PCr resynthesis (40). ATP production also decreases in the elderly (26, 33). This may contribute to the lack of response of aging muscle to creatine supplementation. Furthermore, a recent publication has profiled human “responders” and “non-responders” to creatine supplementation (51). A responder was characterized by low levels of creatine and phospho-creatine, greater amount of type II fibers, larger fiber CSA, and greater fat-free mass. Each of these characteristics is compromised in elderly individuals and may contribute to the age-associated lack of response to creatine: a reduction in type II fibers of aging muscle due to denervation-reinnervation and loss of type II fibers (36); decreases in fat-free mass and decreases in muscle fiber CSA (36).
**Overload Surgery**

A second specific aim of the current research was to examine the ability of aging animals to respond to a hypertrophic stimulus. Current results indicate that the slower fiber phenotypes of aging animals maintain their ability to hypertrophy in response to synergist ablation. This data also indicate that the overload-induced fast-to-slow fiber transition remains intact in older animals. These findings are in agreement with much of the literature related to compensatory hypertrophy of young muscle (47, 48). Relatively few studies have investigated compensatory hypertrophy in older animals. The young and aging animals in this study responded similarly to the overload surgery, demonstrating that older muscle maintains the ability to hypertrophy. These findings are in contrast to a few studies that found a blunted response to overload in elderly animals (1, 5, 16). These differences may be explained by the age difference in the “aging” group versus their elderly animals. Although the muscle fibers of the 24 month older rats did not exhibit age-associated atrophy, they did present other characteristics associated with aging, such as a slower fiber phenotype. In addition, the plantaris of the older groups had significantly higher muscle mass compared to the young groups. The increased muscle mass in the absence of fiber hypertrophy may reflect a reduction in muscle quality. Infiltrations of fat and noncontractile material are often noted in aging muscle (13, 14).

**Overload and Creatine Supplementation**

The third specific aim of this research was to examine the response of aging muscle to a combination of creatine and overload. Compared to the young control group, the OCr treatment resulted in significant hypertrophy and significant increase in myonuclear domain for all fiber types in the young plantaris muscle. In aging plantaris muscle, the combination of creatine and overload resulted in significant hypertrophy of types I and IIA fibers, but
myonuclear domain sizes were not significantly different between control animals and animals in the OCr group for any fiber types. Compared to creatine alone, OCr groups had significantly larger CSA of type I and IIA fibers, regardless of age.

The additional benefit of creatine in the OCr group over the O group was minimal. It is not immediately clear why the combination of creatine and overload did not result in significantly greater hypertrophy than overload alone. A combination of creatine supplementation and resistance training has previously been shown to result in larger fiber CSA (56, 57, 59). However, the present findings are in agreement with Young & Young (61), who demonstrated that creatine supplementation provided no additional hypertrophy beyond synergist ablation alone. It is possible that the overload model is not appropriate for examining the effects of creatine on resistance exercise. The proposed benefit of creatine supplementation is to increase PCr stores, thereby increasing the capacity to regenerate ATP stores during short bursts of exercise (6). In this scenario, the main benefit of creatine supplementation is to sustain high intensity work for a longer duration (39). In turn, the enhanced capacity to anaerobically train the muscle leads to increases in muscle mass and strength (34). In the synergist ablation model, the overload imposed upon the muscle is permanent, and therefore cannot be considered anaerobic exercise.

Creatine supplementation combined with overload surgery also seems to influence the shift in muscle phenotype to a slower phenotype. Interestingly, although both overloaded muscle and aging muscle demonstrated an increase in slow fibers, creatine supplemented animals of the aging group did not show that fast-to-slow shift in fiber types in either ACr or AOCr groups. This appears to show that the fiber type shift in aging animals can potentially be altered or reduced.

There is a different response between young and older animals with regards to fiber type shift in response to overload. The young animals have an increase in the percentage of both type I and type IIA fibers after overload, which is similar to previous reports (47, 48).
However, in the aging overload, and the young and aging OCr groups, there is a shift from IIB/D to IIA, without a shift from IIA to I. Although the young overload group had the greatest fast-to-slow shift in fiber phenotype, this was not significantly different from the percent type I fibers in the aging animals with overload alone. The aging animals had already undergone an age-related fast-to-slow fiber shift, and the overload did not produce an additional phenotype shift. Creatine appears to blunt the overload-induced fast-to-slow fiber transition in the YOCr group. However since creatine alone does not cause any significant shift in fiber type, we can only speculate that it must be an interaction between overload and creatine negated the fast-to-slow shift seen in the YO group. In addition, the young OCr group had the greatest percent increases in CSA indicating the greatest response to the interventions.

Myonuclear Domain

The final specific aim of this project was to examine the age-specific effects of creatine and compensatory hypertrophy on myonuclear domain size. Creatine supplementation alone resulted in a significant increase in myonuclear domain of types IIA and IIB/D fibers in young animals, whereas it had no effect on myonuclear domain size in aging animals. An increase in myonuclear domain implies that fiber size increased at a disproportionately higher rate than myonuclear addition. The results of this study indicate that creatine alone resulted in hypertrophy of young, but not aging, muscle fibers. Therefore, it is not surprising that the creatine-induced increase of myonuclear domain occurred only in the young animals. It is not clear why the myonuclear domain size of type I fibers did not significantly increase with creatine supplementation; however, fast-twitch fibers have relatively larger myonuclear domain sizes than slow-twitch fibers (54).

The concept of myonuclear domain implies that there is an optimal amount of genetic material to govern the structural proteins in that domain. It has been reported that if a fiber hypertrophies beyond 26%, myonuclei are added to maintain a constant myonuclear domain.
(32). The data reported here are in partial disagreement with these findings. In young animals, type I and IIA fibers from the YCr, YO, and YOCr treatment groups were all hypertrophied more than 26%. Of these, the myonuclear domain size of the IIA fibers was increased in all three treatments. The myonuclear domain size was also significantly increased in type I fibers of the YOCr group. In old rats, only type I fibers of the AOCr treatment were hypertrophied more than 26%. The myonuclear domain for these fibers was not different than the type I fibers of the OC treatment. The varying results may reflect inter-species (human vs. rat) differences in myonuclear domain maintenance. In addition, the surgical overload model may influence hypertrophy differently due to the permanency and severity of the overload stimulus.

In the current study the aging muscle shows an inability to respond to creatine and (in comparison with the young animals) a blunted response to overload. If creatine acts directly on satellite cells, as has been shown in cell culture (55), then the age-associated loss of responsiveness to creatine may be a result of dysfunctional satellite cells. In young individuals, creatine increased the quantity of both satellite cells and myonuclei in young males, contributing to enhanced growth (41).

Many age-related studies that have used a rat model have documented an increase in body mass throughout the animal’s lifespan. Fisher 344 rats were selected for this study, because the body mass of this strain plateaus. 24 months of age was selected for the aging group, because the NIH survival curves indicate a 50% survival for Fisher 344 rats at this age. Furthermore, 24-month Fisher 344 rats are analogous to 80-year old humans (46). Age-associated changes in fiber type and CSA often accompany animals in this age range (16, 37). While the skeletal muscle of the aging animals in this study did not exhibit some of the characteristics commonly associated with sarcopenia, the aim of this study was to determine whether the C/N characteristics and responsiveness to overload and creatine change during the
aging process. The data demonstrate that these age-associated changes did occur, despite the absence of age-associated declines of fast fiber types and fiber CSA.

Limitations

Interpretation of the present data is complicated by a few limitations of the study. First, creatine uptake by the muscle was not measured. Therefore, it is not clear whether age-specific results were due to the inability of aging muscle to take in creatine or to respond to the creatine. Previous research has shown that creatine transporters are not influenced by aging or activity (52). Secondly, the compensatory hypertrophy model is an involuntary model of extreme overload. Humans are incapable of voluntarily exercising at this level of intensity. Furthermore, creatine is believed to confer its effect on muscle by replenishing anaerobic energy stores, thereby allowing users to perform an additional repetition of resistance exercise. As such, creatine supplementation may result in greater gains if the training intensity can be increased throughout the training period. For example, significant increases in fat-free mass and strength were seen when creatine supplementation was coupled with resistance training that was modified to maintain maximal effort (9). It should be noted that in the Brose et al. study, fiber CSA was similar between creatine- and placebo-supplemented groups. Nevertheless, compensatory hypertrophy is a form permanent overload that cannot be modified incrementally and is a limitation of the study.

Summary

Given the prevalence of age-associated sarcopenia, the search for a preventative supplement is of vital importance. The data presented here indicate that creatine supplementation alone does not affect fiber hypertrophy in aging muscle. Furthermore, this study found that a combination of creatine and overload was no more effective than overload alone on hypertrophy in aging muscle. Further research is needed to determine the
mechanism(s) of creatine-associated hypertrophy in young muscle. Further research is also warranted to investigate the mechanisms underlying the unresponsiveness of aging muscle to creatine supplementation.
References


Figure Legends

Figure 1: Cross-sectional area for plantaris muscle in young and aging animals for all treatment groups. Open bars are control animals, light grey bars represent creatine supplemented animals, dark grey bars the surgically overloaded group and closed bars represent animals with overload surgery and creatine supplementation. Data are presented as mean ± SD for type I fibers (A), type IIA fibers (B) and type IIB/D fibers (C). * Significantly different than control treatment, within age group (p < 0.05); # Significantly different than creatine group, within age group (p < 0.05); $ Significantly different than same treatment in young rats (p < 0.05)

Figure 2: Myonuclear Domain size for plantaris muscle in young and aging animals for all treatment groups. Open bars are control animals, light grey bars represent creatine supplemented animals, dark grey bars the surgically overloaded group and closed bars represent animals with overload surgery and creatine supplementation. Data are presented as mean ± SD for type I fibers (A), type IIA fibers (B) and type IIB/D fibers (C) * Significantly different than control treatment, within age group (p < 0.05); # Significantly different than creatine group, within age group (p < 0.05); $ Significantly different than same treatment in young rats (p < 0.05)
Table 1: Fluid Consumption and Creatine Intake for Young and Aging Rats

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Aging</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fluid intake (ml/day)</td>
<td>Creatine intake (g/kg body wt)</td>
</tr>
<tr>
<td>Control</td>
<td>29 ± 6 ml/day</td>
<td></td>
</tr>
<tr>
<td>Creatine</td>
<td>30 ± 6 ml/day</td>
<td>0.41 ± 0.07g/kg</td>
</tr>
<tr>
<td>Overload</td>
<td>28 ± 4 ml/day</td>
<td></td>
</tr>
<tr>
<td>Overload+Creatine</td>
<td>28 ± 2 ml/day</td>
<td>0.42 ± 0.03g/kg</td>
</tr>
</tbody>
</table>

Data presented as Mean ± SD. \textsuperscript{a}p<0.05, old greater than young; \textsuperscript{b}p<0.05, greater than control group
Table 2: Weight of Young and Aging Animals

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Aging</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight (g)</td>
<td>Weight (g)</td>
</tr>
<tr>
<td></td>
<td>Change in Weight</td>
<td>Change in Weight</td>
</tr>
<tr>
<td></td>
<td>(Post-Pre; g)</td>
<td>(Post-Pre; g)</td>
</tr>
<tr>
<td>Control</td>
<td>296.0 ± 31.7</td>
<td>416.8 ± 27.5</td>
</tr>
<tr>
<td></td>
<td>+22.8 ± 18.6</td>
<td>+23.8 ± 9.1</td>
</tr>
<tr>
<td>Creatine</td>
<td>324.9 ± 25.4</td>
<td>408.9 ± 19.7</td>
</tr>
<tr>
<td></td>
<td>+39.5 ± 14.4</td>
<td>+35.2 ± 8.3(^a)</td>
</tr>
<tr>
<td>Overload</td>
<td>302.6 ± 18.2</td>
<td>388.8 ± 23.2</td>
</tr>
<tr>
<td></td>
<td>+23.8 ± 7.3</td>
<td>-22.6 ± 12.2(^bc)</td>
</tr>
<tr>
<td>Overload+Creatine</td>
<td>302.3 ± 11.6</td>
<td>384.1 ± 18.0</td>
</tr>
<tr>
<td></td>
<td>+29.5 ± 7.6</td>
<td>-10.1 ± 9.5(^c)</td>
</tr>
</tbody>
</table>

Data presented as Mean ± SD. Change in weight from start of experimental procedure

\( ^a p < 0.001 \), greater than overload and OCr groups; \( ^b p < 0.05 \), less than OCr group; \( ^c p < 0.001 \), less body weight increase than young; \( ^d p < 0.05 \), heavier than creatine group.
Table 3: Plantaris Muscle Weight and Muscle Weight /Body Weight Ratio for Young and Aging Animals

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Aging</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plantaris Wt (g) Ratio</td>
<td>Plantaris Wt (g) Ratio</td>
</tr>
<tr>
<td></td>
<td>Wt/Body Wt</td>
<td>Wt/Body Wt</td>
</tr>
<tr>
<td>Control</td>
<td>0.236 ± 0.030</td>
<td>0.319 ± 0.040c</td>
</tr>
<tr>
<td></td>
<td>0.080 ± 0.011</td>
<td>0.077 ± 0.009</td>
</tr>
<tr>
<td>Creatine</td>
<td>0.255 ± 0.030</td>
<td>0.328 ± 0.037c</td>
</tr>
<tr>
<td></td>
<td>0.080 ± 0.009</td>
<td>0.080 ± 0.009</td>
</tr>
<tr>
<td>Overload</td>
<td>0.276 ± 0.050</td>
<td>0.358 ± 0.040c</td>
</tr>
<tr>
<td></td>
<td>0.091 ± 0.015</td>
<td>0.092 ± 0.010d</td>
</tr>
<tr>
<td>Overload+Creatine</td>
<td>0.308 ± 0.019&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.375 ± 0.037ac</td>
</tr>
<tr>
<td></td>
<td>0.102 ± 0.003b</td>
<td>0.098 ± 0.008b</td>
</tr>
</tbody>
</table>

Data presented as Mean ± SD  
<sup>a</sup> p < 0.05, heavier than control or creatine groups;  
<sup>b</sup> p < 0.05, higher ratio than control or creatine groups;  
<sup>c</sup> p < 0.05, old heavier than young;  
<sup>d</sup> p < 0.05, higher ratio than control or creatine groups
Table 4: Percent Fiber Type of Plantaris Muscle for Young and Aging Animals

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Aging</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type I</td>
<td>Type IIA</td>
</tr>
<tr>
<td>Control</td>
<td>8 ± 2^a,e</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>Creatine</td>
<td>6 ± 4^a,e</td>
<td>22 ± 4^f</td>
</tr>
<tr>
<td>Overload</td>
<td>19 ± 7^c</td>
<td>20 ± 5</td>
</tr>
<tr>
<td>Overload+Creatine</td>
<td>10 ± 4^a,d</td>
<td>24 ± 7^a</td>
</tr>
</tbody>
</table>

Data presented as Mean ± SD. ^a young significantly different than old within treatment p < 0.05; ^b significantly different than control within age group p < 0.05; ^c significantly different than control within age group p < 0.001; ^d significantly different than overload within age group p < 0.05; ^e significantly different than overload within age group p < 0.001; ^f young significantly different than old within treatment p < 0.001; ^g significantly different than overload within age group p < 0.001; ^h significantly different than creatine within age group p < 0.05; ^i significantly different than creatine within age group p < 0.001.
Figure 1a. Cross-sectional area of type I fibers across all treatment and age groups
Figure 1b. Cross-sectional area of type IIA fibers across all treatment and age groups.
Figure 1c. Cross-sectional area of type IIB/D fibers across all treatment and age groups
**Figure 2a.** Myonuclear domain of type I fibers across all treatment and age groups

RETRACTED October 2009
Figure 2b. Myonuclear domain of type IIA fibers across all treatment and age groups
Figure 2c. Myonuclear domain of type IIB/D fibers across all treatment and age groups