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Co-ingestion of carbohydrate and whey protein induces muscle strength and myofibrillar protein accretion without a requirement of satellite cell activation

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ABSTRACT

Muscle development is controlled by the balance between muscle protein synthesis and protein degradation. Protein supplementation has been widely known to enhance muscle protein synthesis, and carbohydrate supplementation may attenuate protein degradation. The purpose of this study was to compare the effects of whey protein plus carbohydrate (CP), whey protein (WP), and placebo (PLA) supplements on resistance training adaptations. Two-month old rats were trained by ladder climbing every 3 days for 8 weeks. PLA, WP, or CP was given immediately after each exercise session. Non-exercise rats were used as a sedentary control (SED). Total body composition was assessed and blood samples were collected before, middle, and end of training. The flexor hallucis longus (FHL) was excised 24 h after the last exercise session. Following training, maximal carrying capacity was significantly greater in CP than PLA and WP. This improved training performance in CP paralleled an increase in total muscle and myofibrillar protein content. Muscle and fiber cross sectional areas (CSA) were significantly increased by exercise training, with a concomitant increase in myonuclear domain. CP significantly elevated IGF-1 protein expression over SED, but there were no significant differences in myostatin, Pax7, MyoD, or myogenin across treatment groups. There was also no difference in the number of total nuclei in each fiber CSA among groups. Corticosterone levels were significantly elevated in PLA and WP over 8 weeks of training, whereas this change in corticosterone over time was not observed in the CP group. The results suggest that the greater improvement of maximal caring capacity for CP compared with PLA and WP was associated with a greater increase in myofibrillar protein content. Satellite cell activation did not appear to contribute to the observed gains in muscle hypertrophy and strength.

1. Introduction

Skeletal muscle is a highly plastic tissue in response to exercise, inactivity, changes in hormonal environment, and nutritional supplementation. An increase in muscle size and muscle strength is of paramount importance for health as well as physical performance. Resistance exercise training is the most effective strategy to induce muscle development, while evidence exists indicating that post exercise nutrient supplementation may augment muscle training adaptations (Cribb and Hayes, 2006; Andersen et al., 2005; Cermak et al., 2012; Tarpenning et al., 2001; Naclerio and Larumbe-Zabala, 2016).

Muscle development is controlled by the balance between muscle protein synthesis and protein degradation. The initial enlargement of muscle mass is primarily attributable to pre- and post-translational mechanisms, which stimulate muscle protein synthesis and inhibit protein degradation while correspondingly increasing the myonuclear domain (quantity of cytoplasm/number of nuclei within that cytoplasm) (Petrella et al., 2006). According to the myonuclear domain theory (Allen et al., 1999), the area of cytoplasm that each nucleus can maintain is limited. Thus, expansion of the muscle cross sectional area (CSA) increases the demand of additional myonuclei once the myonuclear domain reaches a threshold level (Petrella et al., 2006). Satellite cells (SC) have been identified as a major source of new myonuclei (Rosenblatt et al., 1994; Rosenblatt and Parry, 1992). Myogenic regulatory factors (MRF), a superfamily of transcription factors, are composed of four members: MyoD, Myf5, myogenin, and MRF4. MyoD is

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predominantly upregulated during myogenic cell proliferation, and myogenin stimulates muscle differentiation (Berkes and Tapscott, 2005; Sharman et al., 2001). These MRFs can be controlled by growth factors, such as insulin-like growth factor (IGF) −1 and myostatin. IGF-1 serves as a potential candidate for promoting skeletal muscle hypertrophy. IGF-1 does not only activate the mammalian target of rapamycin (mTOR) signaling pathway contributing to protein synthesis, but also mediates the expression of MRFs to trigger SC proliferation and differentiation (Petrella et al., 2006; Allen and Boxhorn, 1989; Florini et al., 1991). Contrary to the actions of IGF-1, myostatin down-regulates muscle mass by inhibiting proliferation (Taylor et al., 2001) and differentiation (Langley et al., 2002) of SC and reducing muscle growth. While the role of SC is commonly accepted during skeletal muscle regeneration, it is still under debate as to their relationship to muscle hypertrophy during resistance training (Fry et al., 2014; Guerci et al., 2012).

Another important strategy to enhance muscle hypertrophy and strength is nutritional supplementation. The ability of protein supplementation post exercise to enhance protein synthesis is widely accepted (Churchward-Venne et al., 2012; Moore et al., 2009). Protein supplementation supplies the amino acid (AA) pool and regulates mRNA translation initiation via activating the mTOR signaling pathway (Morrison et al., 2008; Dickinson et al., 2011). During prolonged resistance training, protein supplementation has been found to increase the magnitude of muscle mass and strength development compared with the exercise stimulus alone (Willoughby et al., 2007). However, protein supplementation does not appear to attenuate protein degradation, which is also elevated during resistance exercise (Borsheim et al., 2002; Phillips et al., 1997). In addition, an increase in plasma cortisol during strenuous exercise may also counter an increase in muscle protein synthesis (Kraemer and Ratamess, 2005). Carbohydrate (CHO) supplementation has been shown to reduce the exercise-induced rise in cortisol (Tarpenning et al., 2001; Baty et al., 2007). Recently, we found that adding CHO to a post exercise protein supplementation accelerated muscle protein synthesis acutely (Wang et al., 2017). Moreover, several resistance training studies suggest muscle mass and strength development may be enhanced when a CHO/protein supplement is provided around the time of each exercise session compared with either provided alone (Bird et al., 2006; Miller et al., 2003). However, the few studies that have investigated the combined effects of CHO and protein supplementation on MPS have provided inconsistent results (Bird et al., 2006; Miller et al., 2003; Koopman et al., 2007; Staples et al., 2011). Therefore, the first aim of this study was to investigate, using a rat model, whether adding carbohydrate to a post exercise protein supplement would induce a greater muscle hypertrophy and strength development than protein supplementation alone. The second aim of the study was to determine the influence of exercise and post exercise nutrition on SC activation, and their involvement in muscle development during resistance exercise training. By studying rats rather than human subjects, diet and exercise training could be highly controlled and monitored, and sufficient muscle tissue obtained for the desired analyses.

2. Material and methods

2.1. Animals

A total of 31 male *Sprague-Dawley* rats were obtained at approximately 2 months of age from Charles River (Wilmington, MA). The reason male rats were selected is because the resistance exercise protocol used in this study was established and validated using this gender. The rats were singly housed in their cages in order to monitor diets and feeding patterns. PVC pipes were used as an environmental enrichment in each cage. Rats were provided standard laboratory chow (Prolab RMH 1000 5P07) and water ad libitum. The percentage of macronutrients provided in the laboratory chow based on calories provided were 18.6% protein, 15.8% fat, and 65.6% carbohydrate. The temperature of the animal room was maintained at 21 °C. A reverse artificial 12 h of dark-light cycle was set with the light phase from 8:00 pm to 8:00 am. Because rats are nocturnal, reversing the light cycle in the animal room was done so that the rats were awake and active during the day when they were being trained. The rats were provided one week of acclimation to their new light cycle prior to starting their exercise familiarization program. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Austin and conformed to the guidelines for the use of laboratory animals published by the United States Department of Health and Human Resources.

2.2. Familiarization

The resistance exercise training program employed consisted of ladder climbing. The ladder was 1 m high with 2 cm grid steps on an incline of 85°. Following one week of acclimation to their new environment, each rat underwent ladder climbing with three repeated sessions separated by one day between each session to familiarize them with the exercise protocol. During these three sessions, rats carried no weights. Rats also completed three practice sessions of climbing separated by one day between each session with 50, 60, and 70% of their body mass attached to their tails, respectively. The weight was attached at the base of the tail with foam tape (3 m Conan) and a Velcro strap. Rats were encouraged to climb by lightly tapping their tails with a bottlebrush. At the end of each familiarization session, the rats were also familiarized with the blood sampling procedure used during training as described below, but with only a minor amount of blood collected. This was done to reduce stress on the rats during actual blood sampling.

2.3. Experimental design

Following familiarization with the ladder climbing, rats began a high-intensity progressive resistance exercise regimen according to Hornberger and Farrar (Hornberger and Farrar, 2004). In brief, the initial climb consisted of carrying a load equal to 75% of the rat's body weight. Upon successful completion of climbing the ladder with this load, an additional 30 g weight was added to the tail of the rat and the climb repeated after 2 min of rest. This procedure was repeated until a load was reached, which prevented the rat from completing the climb. In the subsequent training session, the first 4 ladder climbs consisted of 50%, 75%, 90%, and 100% of maximum load, respectively. Then, an additional 30 g load was progressively added until a new maximal carrying capacity (indicative of strength) was established. The training regimen was repeated every 3 days for 8 weeks, which consisted of a total of 20 exercise sessions. Personnel who trained the rats were blinded to the treatments. Whey hydrolysate (WP = 0.5 g/kg) (#8360, Hilmar Ingredients, Hilmar, CA), whey plus carbohydrate (WP = 0.5 g/kg, CHO = 1.2 g/kg), or placebo (PLA = 8 ml/kg water) was provided by intubation immediately after each exercise regimen. Food was withdrawn 3 h before and after each exercise session with water provided ad libitum. Seven non-exercised rats were used as a sedentary group and received an intubation of water (8 ml/kg) during the times of training. The day before the 1st exercise session, and the days after the 9th and 19th exercise sessions the rats were fasted for 3 h and body mass obtained. Body composition was then determined via dual-energy X-ray absorptiometry (DEXA). Blood was collected 22–24 h after the 1st, 10th, and 20th exercise sessions. Following the last blood collection, rats were anesthetized to remove the flexor hallucis longus (FHL) from both legs and the epididymis adipose tissue. Muscle and adipose tissue weights were obtained quickly. Then, the muscle from the left leg was embedded in optimal cutting temperature (OCT) medium and frozen in cooled isopentane for later determination of fiber cross sectional area (CSA), quantification of myonuclei, and myonuclear domain. The FHL from the right leg was freeze clamped in liquid nitrogen, and stored at −80 °C for later determination of protein expression of IGF-1, myostatin, Pax 7, MyoD, and Myogenin, and myofibrillar and total protein content.

2.4. Body composition

DEXA (enCORE 14.10, GE Healthcare, Madison, WI) was used to determine the body composition of each rat. On the day of testing, rats were fasted for 3 h and then anesthetized via intraperitoneal injections of ketamine (70 mg/kg) and xylazine (7 mg/kg). Each rat was then placed in a supine position with attention to body alignment on a platform grid. The forelimbs and hindlimbs of the rat were placed perpendicular to the long axis of the body. The tail was positioned in a left curve toward the head so the entire body was contained within the scan area. Body mass, bone mineral density, fat mass, lean mass, and percent body composition were obtained.

2.5. Blood analysis

Approximately 22–24 h after exercise sessions 1, 10, and 20, 0.7 ml of blood was collected from the tail of the rat into a 1.5 ml test tube containing 50 μl of EDTA (24 mg/ml, pH 7.4). The rats were gently restrained by wrapping them in a towel and placing them on a heating pad. The tip of the tail was then cut and the tail lightly massaged so that a sufficient amount of blood was easily and quickly obtained. All collected blood samples were centrifuged for 10 min at 3000 g at 4 °C with a FS-20 microtube rotor in a Sorvall RC-6 centrifuge (Thermo Fisher Scientific Inc. Waltham, MA). After centrifugation, the plasma samples were placed in capped microfuge tubes and stored at −80 °C for later analysis of corticosterone. The concentration of corticosterone was determined by an enzyme-linked immunosorbant assay kit (ELISA) with CV<10% (Enzo life sciences Inc. Ann Arbor, MI. Cat ADI-900-097).

2.6. Morphological analysis

Frozen OCT embedded muscle was transversely cut into 10 μm sections on a cryostat (Leica cm1900; Leica Microsystems Inc., Buffalo Grove, IL) set at −20 °C. Sections were mounted on glass slides. Hematoxylin and eosin (H&E) staining (Thermo Fisher scientific Inc.) was performed to identify muscle fiber CSA and the number of myonuclei. Slides were observed under a light microscope (Nikon Diaphot, Nikon Corp.; Tokyo, Japan) with a 20× objective lens for fiber CSA and with a 40× objective lens for quantification of nuclei and myonuclear domain. Three random histological fields were collected in each muscle for analysis. Images were then taken using a mounted digital camera, and measured and counted using Image J software (Hsieh et al., 2018).

2.7. Determination of muscle cross-sectional area

The following formula was used to calculate muscle CSA (Eng et al., 2008; Wang and Kernell, 2000):

Muscle CSA (mm^2) = muscle mass (mg) * cos θ (0.944) / [density (1.056 mg/mm^3) * muscle length (mm) * (fiber length/muscle length]

θ is the surface pennation angle between the fibers and the distal muscle tendon.

2.8. Immunoblot analysis

Immunoblot analysis was performed as previously described (Wang et al., 2015). The frozen muscle samples weighing 80–100 mg were homogenized in ice-cold homogenization buffer (20 mM HEPES, 2 mM EGTA, 50 Mm sodium fluoride (NaF), 100 mM potassium chloride (KCl), 0.2 mM EDTA, 50 mM glycerophosphate, 1 mM DL-dithiothreitol (DTT), 0.1 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM benzamidine, and 0.5 mM sodium orthovandate (Na_2VO_4) at a 1:8 dilution of wet weight muscle with a glass tissue grinder pestle (Corning Life Sciences, Lowell, MA; Caframo Stirrer Type RZR1, Wiarton, Ont. Canada). The crude muscle homogenates were centrifuged at 14,000×*g* for 10 min at 4 °C, and the supernatants were taken for measurements of protein concentration. Aliquots of muscle homogenates were stored at −80 °C until analyzed.

Muscle samples (60 μg) were combined with an equal amount of Laemmli sample buffer (125 mM Tris, 20% glycerol, 20% SDS, 0.25% bromophenol blue, and β-mercaptoethanol, pH 6.8) and boiled at 95 °C for 10 min in order to denature muscle proteins. Samples were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10–15% resolving gel at 100 V for 75 min (Bio-Rad Laboratories, Hercules, CA). The resolved proteins were then electrically transferred onto a nitrocellulose membrane (pore size: 0.45 μm; GE Healthcare Life Sciences, Pittsburgh, PA) using a wet transfer unit (Bio-Rad Laboratories) at 90 V for 60 min. The membranes were blocked in 7% nonfat milk in Tris-buffered saline with 0.06% Tween20 (TTBS) for 30 min at room temperature (RT). Then, the membranes were incubated with the appropriate primary antibody overnight at 4 °C. The targeted proteins were IGF-1 (sc-9013), myostatin (sc-134,345), pax 7 (sc-81975), myoD (sc-71629), and myogenin (sc-12732). The above antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc. Dallas, TX). Alpha-tubulin was used as an internal loading control (Cell Signaling Technology, Beverly, MA). Following overnight primary antibody probing, all membranes were washed 5 min with TTBS three times. Then, the membranes were incubated with HRP-conjugated secondary anti-rabbit IgG (Cell Signaling Technology) or anti-mouse IgG (EMD Millipore Corporation, Chicago, IL). After another three washes using TTBS, the membranes were visualized by enhanced chemi-luminescence (ECL) in accordance with the manufacturer's instructions (PerkinElmer, Boston, MA). All western blots were performed in duplicate for each muscle sample to ensure reproducibility (CV<10%). Images were then captured using a charge-coupled device camera in a ChemiDoc system (Bio-Rad). Intensity of each band was quantified with Quantity One analysis software (Bio-Rad) and expressed as a percentage of a standard.

2.9. Total muscle and myofibrillar protein measurements

Myofibrillar protein measurement was described previously (Hornberger and Farrar, 2004). In brief, 50 mg muscle was homogenized in ice-cold solution containing 8.5% sucrose, 5 mM EGTA, 50 mM KCl, and 100 mM $MgCl_2$ using a Polytron homogenizer (Virtishear, Virtis, Gardiner, NY, USA). An aliquot of the crude muscle homogenate (0.1 ml) was used to determine total protein concentration. The remaining homogenate was centrifuged at 2500×*g* for 15 min at 4 °C. The supernatant was discarded and the pellet was resuspended in a solution (pH 6.8) containing 5 mM EGTA, 100 mM KCl, 5 mM $MgCl_2$, and 0.05% Triton X-100. The suspension was then centrifuged at 2500×*g* for 10 min at 4 °C and the supernatant was discarded. This step was repeated two more times. The remaining pellet was washed in solution (pH 6.8) containing 5 mM EGTA and 100 mM KCl and centrifuged at 2500×*g* for 10 min at 4 °C. This step was then repeated a second time. Next, the myofibrillar pellet was resuspended in a solution (pH 7.4) containing 150 mM KCl and 5 mM tris-hydroxymethyl aminomethane. This suspension was used to determine myofibrillar protein concentration (mg/g total protein). Total muscle and myofibrillar protein concentrations were determined by a modified Lowry Protein Assay (Lowry et al., 1951).

2.10. Statistical approach

A standard formula provided by IACUC was used to calculate the minimum sample size required for this study. According to the findings of a previous study (Tarpenning et al., 2001), we used a group difference of 0.13, a standard deviation of 0.07, a power of 0.9, and an alpha level of 0.05. We determined that seven rats per group were required to achieve adequate power in this study. With consideration of 10% dropout rate, a total of 31 rats were included.

A repeated measure analysis of variance (ANOVA) was performed on a between-within mixed model design (treatment x time) for the measurements of body composition, maximal carrying capacity, and

corticosterone. When the interactive effect (treatment x time) was statistically significant, time effects within each group and group comparisons at each time point were examined using Fisher's LSD post hoc analysis. A one-way ANOVA was used for all the other data, and Fisher's LSD post hoc test was performed to compare mean differences among treatments. Differences with p-values ≤ 0.05 were considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics v19.0 software (IBM Corporation, Armonk), and all data was expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. Rat physical characteristics

Body mass, FHL muscle mass, epididymis adipose mass, and daily food and caloric consumptions are presented in Table 1. There were no differences in initial body mass, daily food and caloric consumptions across treatment groups. After 8 weeks of training, all rats had significant increases in body mass. The gains in body mass for the exercise groups, however, were significantly lower than occurred in the sedentary (SED) group. Body mass gains did not differ among the exercise groups. Likewise, resistance training significantly augmented FHL muscle mass, but the muscle mass did not differ among exercise groups. Both placebo (PLA) ($p = 0.05$) and CP ($p = 0.06$) groups showed a trend for lower epididymis adipose mass relative to the SED group. In spite of mass differences in the FHL muscle and epididymis adipose tissue after 8 weeks of training, total body composition measured by DEXA did not differ among groups. As shown in Table 2, percent of fat body mass was elevated whereas percent of lean body mass was reduced in all treatment groups after 4 weeks, and did not change during the subsequent 8 weeks of training. Additionally, there were no differences in percent fat or lean body mass across treatment groups. Changes in fat and lean mass showed a similar pattern as percent body composition. However, compared to week 4, SED and WP groups had a higher fat mass at week 8, and all groups had a greater increase in lean mass at week 8.

3.2. Maximal carrying capacity

After 8 weeks of resistance training, rats in the PLA group increased their maximal carrying capacity by 2.70-fold (from 475 ± 10 g to 1280 ± 37 g). Rats receiving WP immediately after each exercise session also increased their maximal carrying capacity by 2.73-fold (from 462 g ± 13 to 1260 ± 49 g). Rats receiving CP immediately after each exercise session increased their maximal carrying capacity by 3.02-fold (from 461 ± 14 g to 1390 ± 54 g) (Fig. 1). The percentage increase in maximal carrying capacity was significantly greater in the CP group compared with the PLA and the WP groups (Fig. 1).

Table 1
Food consumption, total calories, body mass, FHL muscle mass, and epididymis adipose tissue mass.

	SED	PLA	WP	CP
Daily food consumption (g)	29.0 ± 0.4	27.7 ± 0.3	27.6 ± 0.7	27.2 ± 0.8
Supplement			0.5 g/kg	0.5 + 1.2 g/kg
Total calories (kcal/d)	102.4 ± 1.4	97.9 ± 1.0	97.7 ± 2.6	97.2 ± 3
Initial body weight (g)	376.7 ± 14.2	394.9 ± 9.6	394.3 ± 10.4	373.5 ± 7.1
Final body weight (g)	552.6 ± 8.6 †	518.3 ± 14.5 * †	529.7 ± 16.9 * †	513.4 ± 12.2 * †
Gains in body weight (g)	190.6 ± 8.7	135.8 ± 9.0 *	148.0 ± 9.8 *	139.9 ± 14.7 *
FHL (mg)	743.1 ± 20.2	851.4 ± 19.9 *	829.8 ± 14.6 *	842.3 ± 18.6 *
Epididymis adipose tissue (g)	9.6 ± 0.6	7.7 ± 0.93 ($p = 0.05$)	8.9 ± 0.7	7.8 ± 0.2 ($p = 0.06$)

Data are presented as mean ± SEM (n = 7–8 per group). †, $p \leq 0.05$ vs. initial body weight in the same treatment. *, $p \leq 0.05$ vs. SED at the same time point.

Table 2
Total body composition measured by DEXA.

	SED	PLA	WP	CP
Fat percentage (%)				
Week 0	35.7 ± 0.7	37.3 ± 1.3	36.1 ± 0.6	36.6 ± 0.5
Week 4	42.7 ± 1.2 †	43.6 ± 2.7 †	43.6 ± 0.8 †	44.0 ± 0.8 †
Week 8	43.1 ± 1.6 †	43.6 ± 2.3 †	44.5 ± 1.5 †	44.3 ± 0.7 †
Lean mass percentage (%)				
Week 0	64.3 ± 0.7	62.7 ± 1.3	63.9 ± 0.6	63.4 ± 0.5
Week 4	57.3 ± 1.2 †	56.4 ± 2.7 †	56.4 ± 0.8 †	56.0 ± 0.8 †
Week 8	56.9 ± 1.6 †	56.4 ± 2.3 †	55.5 ± 1.5 †	55.7 ± 0.7 †
Fat mass (g)				
Week 0	116.6 ± 3.9	127.0 ± 4.7	124.8 ± 3.8	123.6 ± 4.4
Week 4	198.6 ± 8.3 †	196.9 ± 12.8 †	199.4 ± 7.5 †	194.5 ± 6.1 †
Week 8	229.9 ± 10.1 † ‡	217.1 ± 14.5 †	226.4 ± 14.2 † ‡	218.6 ± 7.0 †
Lean mass (g)				
Week 0	210.0 ± 8.5	215.1 ± 11.1 †	220.8 ± 7.4	214.4 ± 8.6
Week 4	266.0 ± 7.4 †	255.5 ± 14.3 †	257.3 ± 7.2 †	247.5 ± 6.0 †
Week 8	302.9 ± 7.7 † ‡	279.5 ± 12.3 † ‡	279.8 ± 6.1 † ‡	273.4 ± 5.7 † ‡

Data are presented as mean ± SEM (n = 7–8 per group). †, $p \leq 0.05$ vs. week 0 in the same treatment. ‡, $p \leq 0.05$ vs. week 4 in the same treatment.

3.3. Muscle and fiber CSA

Eight weeks of resistance training produced a larger FHL muscle and fiber cross sectional area (CSA) relative to the SED group. Nevertheless, no significant differences were observed among the three exercise groups for either muscle mass or fiber CSA (Fig. 2A & B).

3.4. Total and myofibrillar proteins

To investigate the underlying mechanisms on increases in maximal carrying capacity, total and myofibrillar proteins were measured in the FHL. Exercise training did not affect total protein concentration (Fig. 3A). Total protein content was not significantly different between SED, PLA, and WP, but protein content for CP was significantly increased above SED (Fig. 3B). No difference occurred among the exercise trained groups. From the total protein extracted for each muscle, we determined the

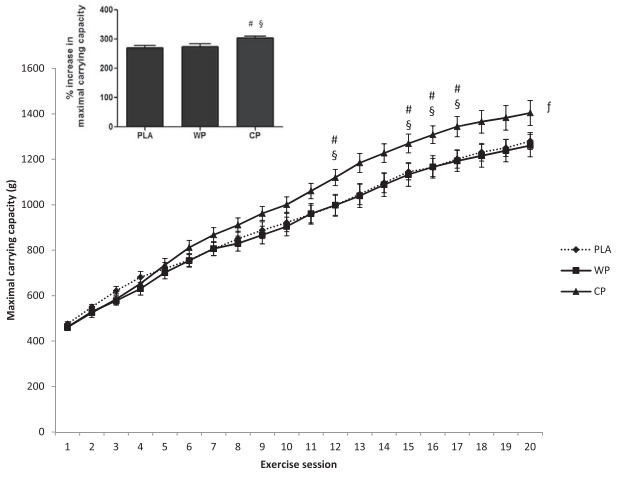

Fig. 1. Maximal carrying load per training session over 8 weeks. Percentage increase in maximal carrying capacity over 8 weeks of training is displayed on the upper left corner. Data are presented as mean ± SEM (n = 7–8 per group). ƒ, significant treatment effect in CP vs. PLA and WP. #, $p \leq 0.05$ vs. PLA. §, $p \leq 0.05$ vs. WP.

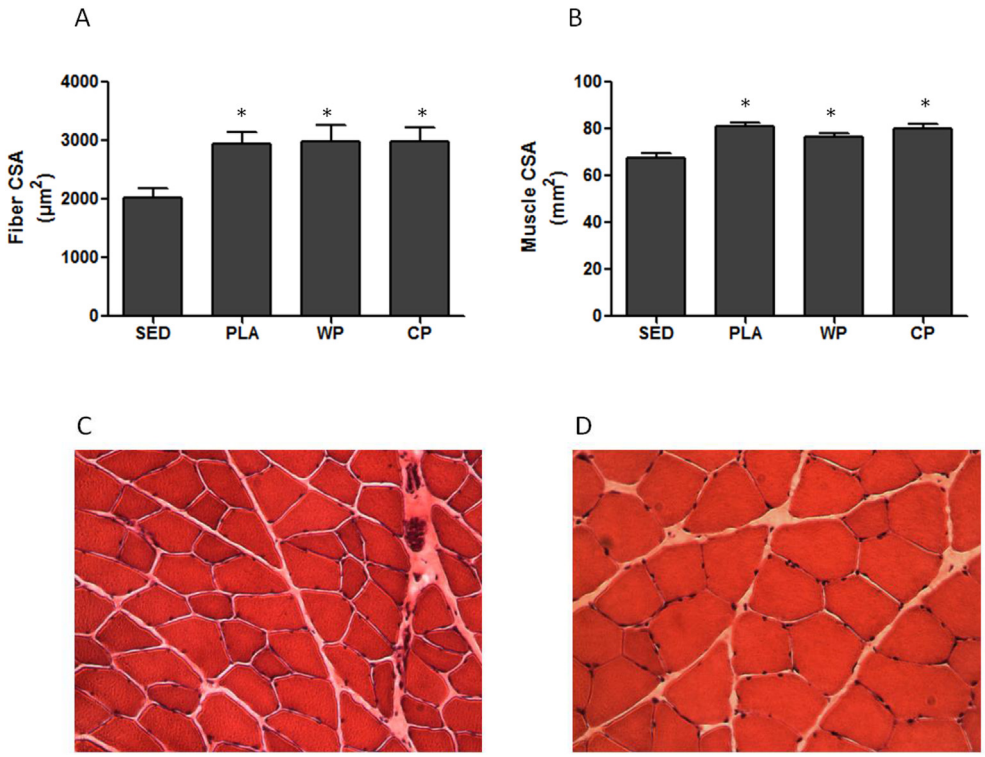

Fig. 2. Cross sectional area. A) Fiber cross sectional area. B) FHL muscle cross sectional area. C) Cross section of FHL muscle in untrained rats (20× objective lens). D) Cross section of FHL muscle in trained rats (20× objective lens). Data are presented as mean ± SEM (n = 7–8 per group). *, $p \leq 0.05$ vs. SED.

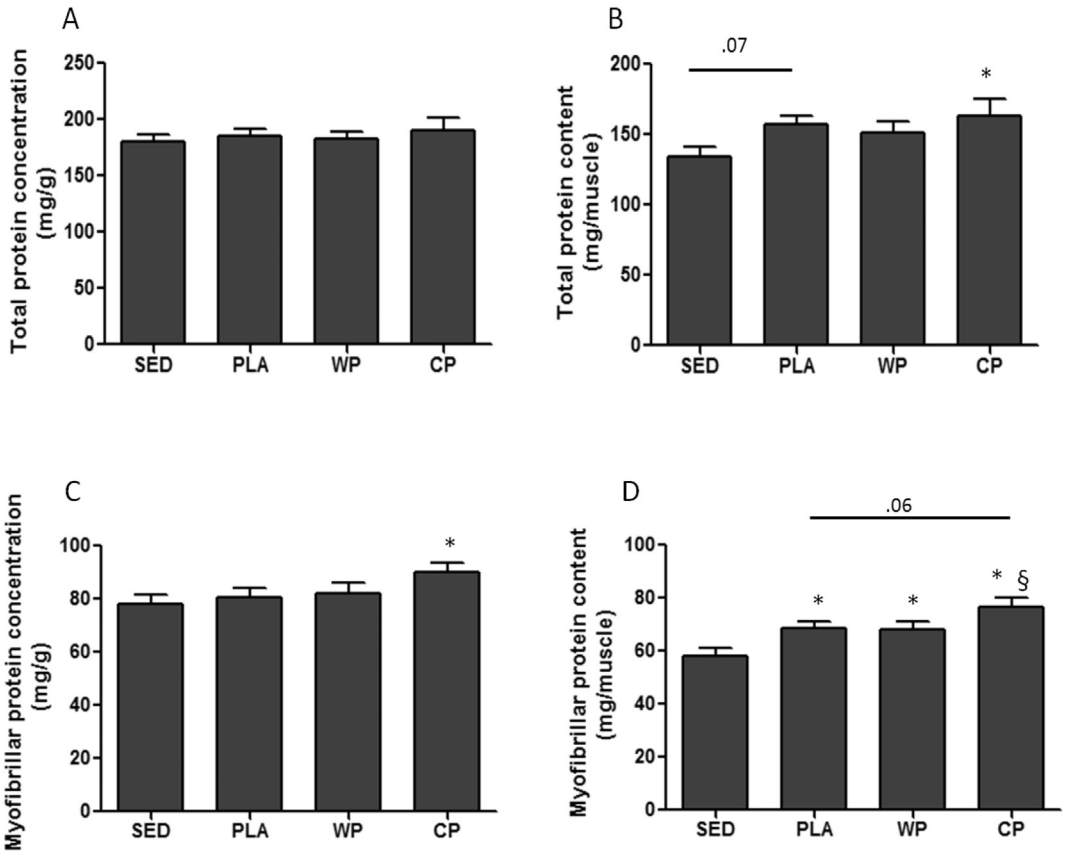

Fig. 3. Total and myofibrillar proteins. A) Total protein concentration. B) Total protein content per muscle. C) Myofibrillar protein concentration. D) Myofibrillar protein content per muscle. Data are presented as mean ± SEM (n = 7–8 per group). *, $p \leq 0.05$ vs. SED. §, $p \leq 0.05$ vs. WP.

myofibrillar protein component. Our results showed myofibrillar protein per g muscle from CP was significantly greater than SED (Fig. 3C). Myofibrillar protein of all 3 exercise groups was greater than SED when myofibrillar protein content was expressed as mg/muscle (Fig. 3D). Moreover, myofibrillar protein content of CP was significantly greater than WP, and approached significance relative to PLA ($p = 0.06$).

3.5. Signaling proteins that regulate the activation of SC

Expression of proteins involved in the activation of satellite cells was then investigated using Western blot. IGF-1 is a growth factor stimulating muscle protein synthesis and myogenesis. The results suggested that CP elicited a greater IGF-1 protein expression compared with SED (Fig. 4A). No other differences were noted for IGF-1 across treatments. Myostatin, another growth regulator, which plays a role in the down-regulation of muscle mass hypertrophy was investigated. However, no significant differences were found for myostatin protein expression across treatment groups (Fig. 4B). To investigate the possibility of satellite cell activation with training, protein expression of Pax 7, Myo D and Myogenin were examined (Fig. 4C–E). Neither exercise training nor nutrient supplementation appeared to have an effect on these proteins. To eliminate the possibility that the activation of satellite cells was not seen due to a missed time point, the number of nuclei per observed FHL muscle fiber was counted. However, the amount of nuclei per fiber did not differ across treatments (Fig. 5A), whereas nuclear domain per nucleus was significantly augmented by resistance training (Fig. 5B).

3.6. Corticosterone level

Plasma corticosterone level was measured 22–24 h after the 1st (week 0), 10th (week 4), and 20th (week 8) exercise sessions. Corticosterone level was significantly elevated by exercise at week 8 compared to week 0 for the PLA and WP groups (Fig. 6). Corticosterone level also showed a significant increase from week 4 to week 8 in the PLA group. There was no difference in corticosterone level in the CP group over the 8 weeks of training, and there were no differences between exercise groups at any time point.

4. Discussion

The primary finding of the present study was that maximum carrying capacity, indicative of muscle strength, was significantly greater in the

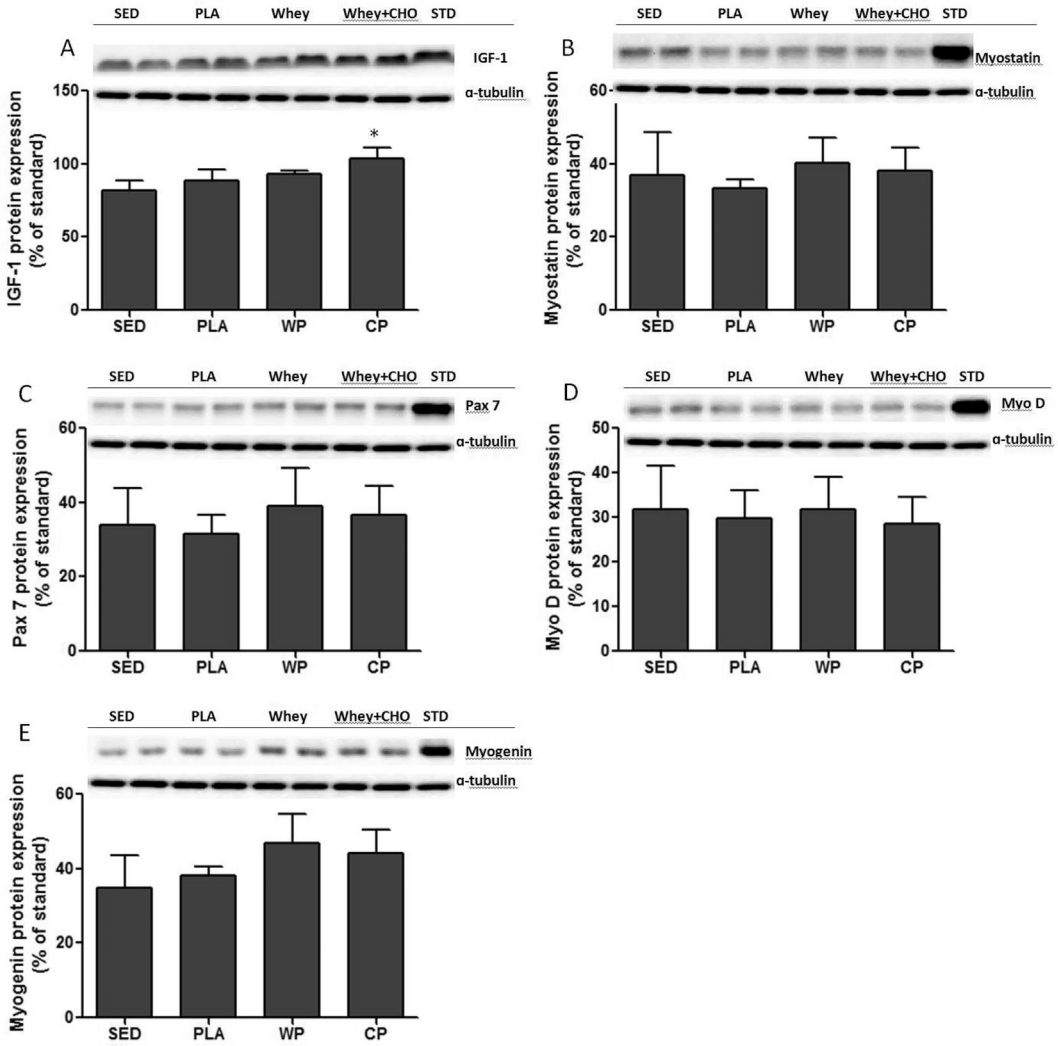

Fig. 4. Markers for new nuclei involvement. A) IGF-1 protein expression as a percentage of a standard sample obtained from an insulin-stimulated rat tissue. B) Myostatin protein expression as a percentage of a standard sample obtained from an insulin-stimulated rat tissue. C) Pax 7 protein expression as a percentage of a standard sample obtained from an insulin-stimulated rat tissue. D) Myo D protein expression as a percentage of a standard sample obtained from an insulin-stimulated rat tissue. E) Myogenin protein expression as a percentage of a standard sample obtained from an insulin-stimulated rat tissue. Data are presented as mean ± SEM ($n = 7–8$ per group). *, $p \leq 0.05$ vs. SED.

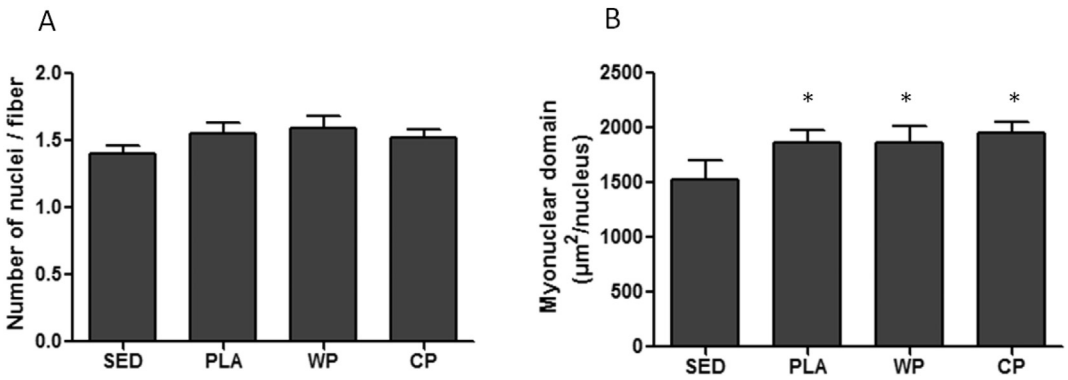

Fig. 5. Number of nuclei and myonuclear domain. A) Number of nuclei per FHL muscle fiber. B) Myonuclear domain per nucleus. Data are presented as mean ± SEM (n = 7–8 per group). *, $p \leq 0.05$ vs. SED.

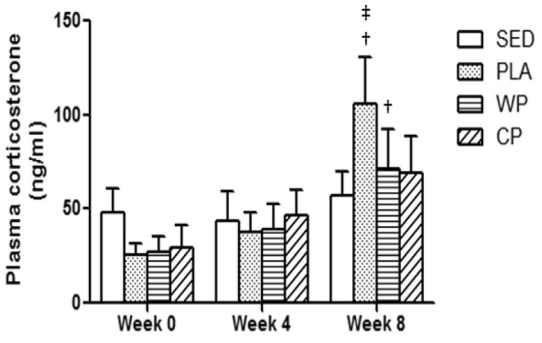

Fig. 6. Plasma corticosterone level 22–24 h after 1st (week 0), 10th (week 4), and 20th (week 8) exercise session. Data are presented as mean ± SEM (n = 7–8 per group). †, $p \leq 0.05$ vs. week 0 in the same treatment. ‡, $p \leq 0.05$ vs. week 4 in the same treatment.

CP group than the PLA or WP groups following 8 weeks of resistance training. Maximal carrying capacity during the first 4 weeks did not differ among exercise groups, and it may be explained by neural adaptation (Staron et al., 1994). The subsequent gains in carrying capacity were greater for CP than for the PLA and WP groups. CHO seemed to play a role in muscle protein accretion and strength. During the last week of training, however, the gain in the maximal carrying capacity became progressively more difficult. This phenomenon might be attributed to a "ceiling effect" as a result of the rat reaching their genetic potential (Schoenfeld et al., 2013; Peterson et al., 2005).

Previous studies have clearly demonstrated that the FHL muscle in the lower extremities is highly responsive to ladder climbing (Hornberger and Farrar, 2004; Lee et al., 2004). In agreement with this literature, our study showed that FHL muscle mass was significantly increased by exercise training, but did not differ among exercise groups. Also, while FHL muscle mass was enhanced by exercise training, the muscle hypertrophy to a small number of muscles in the lower extremities was not sufficient to impact changes of total body composition. Therefore, no significant treatment differences were detected in the percent of fat and lean mass. Interestingly, percent of fat mass was increased after 4 weeks in all groups while percent of lean mass was correspondingly reduced. To our knowledge, no animal studies exist to explain this phenomenon. Two-month old young adult healthy rats were used in our study. Over 8 weeks of training, rats were at puberty, with an age range comparable to 12-18 year-old humans (Sengupta, 2013; Andreollo et al., 2012). It has been well established that the puberty stage in humans results in rapid body growth, increased weight, and fat deposition (Rogol et al., 2000). Therefore, it was not surprising to observe an increased percent of fat mass in the present study.

Resistance training significantly increased muscle and fiber CSA leading to an increase in FHL muscle mass and indicative of muscle hypertrophy. Muscle hypertrophy is associated with protein accretion. In the present study, total protein concentration did not differ across groups. Total protein content for CP, however, was significantly elevated above SED. PLA and WP also showed a slightly greater increase in total protein content than SED, but significance was not reached. These results indicate that muscle hypertrophy was most likely due to an increase in protein content. Muscle protein pools consist mainly of myofibril, mitochondria, and sarcoplasmic proteins. Of the various proteins, myofibrillar protein contains myosin and actin contractile proteins, and their accumulation can add to muscular strength. Our results demonstrated that myofibrillar protein content was enhanced for CP over the SED and the WP group. There was also a trend ($p = 0.06$) for CP to have a higher myofibrillar protein content relative to PLA. Despite that such an increase in myofibrillar protein for CP did not result in a greater increase in muscle mass compared with PLA or WP, the CP induced rise in myofibrillar protein content seemed to bring about a greater strength relative to the other two exercise groups.

The net balance between muscle protein synthesis and protein degradation determines the accumulation of myofibrillar protein and muscle size. Hence, biomarkers related to muscle protein accretion were then investigated. Our result showed that muscle IGF-1 protein expression was upregulated in the CP group compared to the SED group. IGF-1 has been demonstrated to have autocrine/paracrine functions within muscle fibers (Adams, 1998; Goldspink, 1999). It is capable of stimulating protein synthesis and satellite cell (SC) activation (Hameed et al., 2003; Goldspink et al., 1995). Although not investigated directly in this study, an acute study from our laboratory clearly demonstrated that CP accelerated MPS via activating the mTOR signaling pathway compared to exercise alone (62). Moreover, Lee and colleagues found that protein synthesis was the source of muscle hypertrophy via increased IGF-1 expression in muscle (Lee et al., 2004). These results, along with the finding of the present study, support the hypothesis that CP may augment protein synthesis via increasing IGF-1 protein expression in skeletal muscle, as compared to the other two exercise groups.

Conversely, the more strenuous the resistance training, the greater the cortisol response, and the greater protein degradation occurs (Kraemer and Ratamess, 2005). The secretion of cortisol during high intensity resistance exercise can lead to negative net protein balance and muscle damage (Hickson et al., 1996). In the current study, we observed that plasma corticosterone levels were significantly elevated at the end of week 8 over week 0 in PLA and WP and over week 4 in PLA. As such, the elevation of circulating corticosterone may restrict or impede the anabolic effect of resistance training. In contrast, plasma corticosterone did not differ over 8 weeks of training in the CP group. This result is in agreement with the findings in human studies, which reported an attenuation of cortisol level by CHO intake after acute or chronic resistance exercise (Baty et al., 2007; Bird et al., 2006). Therefore, our results reveal that CHO may have attenuated MPB over the 8 weeks of resistance

training, and this could possibly explain, in part, the higher myofibrillar protein concentration and content in the CP group.

Once muscle protein is accumulated to a certain level, further muscle hypertrophy may be accompanied by an increase in the number of myonuclei in response to muscle loading (Allen et al., 1995). As mentioned above, muscle IGF-1 contributes to the activation of SC. SC represent muscle precursor cells. Their activation, proliferation and differentiation provide new myonuclei in the muscle fiber. Some myogenic regulatory factors (MRF) are responsible for the activation of SC. The increased expression of IGF-1 has been reported to mediate MRF expression and to enhance myoblast proliferation and differentiation (Allen and Boxhorn, 1989; Florini et al., 1991). On the other hand, myostatin, a member of the transforming growth factor-β superfamily, plays a role in the down-regulation of muscle mass change. In the current study, however, changes in Pax 7, MyoD, myogenin and myostatin levels were not observed among all groups.

To rule out the possibility that an activating window could be missed when these proteins were measured, and to further investigate whether SC were involved to promote muscle hypertrophy, the total number of nuclei per FHL muscle fiber CSA was measured. The amount of nuclei per fiber CSA did not differ across treatment groups, resulting in the myonuclear domain increasing in the exercise groups. These results indicate that resistance training can promote protein synthesis without additional nuclei involvement. Although some studies have reported an increase in the amount of nuclei per muscle fiber after resistance training (Olsen et al., 2006; Petrella et al., 2008), other studies have failed to observe the same change (Kadi et al., 2004; Mackey et al., 2011). This discrepancy may be due to the intensity of exercise training, the types of exercise training regimen, the magnitude of muscle hypertrophic response, muscle damage, amount of supplementation and timing, and genetic factors. These factors can affect the magnitude of muscle protein accretion and in turn muscle hypertrophy to varying degrees. Research suggests that myonuclei fusion by SC may be required to provide sufficient cytoplasmic space for muscle protein expansion only when the myonuclear domain reaches a threshold level, below which the stimulation of MPS is probably the sole factor necessary to induce hypertrophy (Petrella et al., 2006). Regardless, the lack of changes in the MRF and total number of observed nuclei in the current study suggest that our training load was not adequate to induce SC activation and myonuclei infusion. In agreement with our findings, McCarthy et al. observed the same increases in muscle mass induced by mechanical overload between normal mice and SC-depleted mice (McCarthy et al., 2011). Consequently, the myonuclear domain was expanded without an increase in myonuclei in hypertrophic muscle.

It was widely reported that consumption of additional protein supplementation after exercise results in an augmentation of protein synthesis and subsequent muscle hypertrophy (Dickinson et al., 2014; Pasiakos et al., 2015). Surprisingly, WP provided immediately after each exercise session did not stimulate a greater increase in muscle maximal carrying capacity, muscle mass, and myofibrillar protein compared to resistance training alone in the present study. There are several possible explanations for the discrepancy found in our study with others in the literature. First, the daily supply of dietary protein may have been adequate to promote muscle hypertrophy and no additional protein supplementation was needed. In the present study, WP was provided after each exercise session rather than every day. Therefore, average daily protein intake between the PLA and WP groups did not differ (4.54 ± 0.05 g/rat in the PLA group versus 4.68 ± 0.12 g/rat in the WP group). Second, the potential benefit of post-exercise protein supplementation may have been limited by the age of the rats. It is possible that because of the youth of the rats studied, normal rapid growth and developmental rates compromised the effects of the protein supplement. Why carbohydrate plus protein was more efficacious than protein alone is unclear, but could have been related to the different hormonal responses produced by the two supplements as addressed earlier. In addition, we previously observed that providing a carbohydrate and protein supplement to rats post exercise resulted in a greater insulin response than a protein supplement (Wang et al., 2017). Aside from having a positive effect on protein synthesis, this elevated insulin response could have resulted in a greater protein accretion by limiting protein degradation (Cermak et al., 2012; Borsheim et al., 2002).

This study evaluated the interaction of resistance exercise training and CP supplementation on muscle strength and mass development in young, male rats. Therefore, interpretation of our findings are limited to the age and gender of the rats tested. Several studies using the resistance exercise training program used in the current study observed similar increases in muscle mass and strength for young male and female rats (Hornberger and Farrar, 2004; Lee et al., 2004). Although there are significant hormonal differences between male and female rats, their response to resistance exercise prior to reaching maturity do not appear to differ, and therefore we suspect that whether we had used male or female rats we would have seen similar results. However, age can have a significant impact on training adaptation, and age may differentially affect the response of male and female rats. Further research, therefore, will be required to determine if the interactive effects of resistance exercise training and CP supplementation are affected by age and gender.

5. Conclusions

In summary, post exercise CP supplementation increased the magnitude of maximal carrying capacity following 8 weeks of resistance training. This greater increase in strength by CP, as compared to PLA or WP appeared to be due to a greater increase in myofibrillar protein content in the FHL muscle. However, muscle hypertrophy in response to resistance training was not sufficient to involve additional nuclei infusion into muscle fibers. We conclude that the addition of CHO to a protein supplement should be considered as part of the post exercise nutrition supplement to promote greater muscle strength during prolonged resistance training. The benefit of adding CHO to a protein supplement to enhance muscle strength development needs to be confirmed in human training studies.

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Declaration of Competing Interest

There is no conflict of interest for all authors.

CRediT authorship contribution statement

Wanyi Wang: Methodology, Investigation, Formal analysis, Writing - original draft. **Pei-ling Hsieh:** Investigation, Formal analysis. **Roger P. Farrar:** Methodology, Writing - review & editing. **John L. Ivy:** Methodology, Writing - original draft, Supervision, Writing - review & editing.

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