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Dietary Whey Protein Hydrolysates Increase Skeletal Muscle Glycogen Levels via Activation of Glycogen Synthase in Mice

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ABSTRACT: Previously, we have shown that consuming carbohydrate plus whey protein hydrolysates (WPHs) replenished muscle glycogen after exercise more effectively than consuming intact whey protein or branched-chain amino acids (BCAAs). The mechanism leading to superior glycogen replenishment after consuming WPH is unclear. In this 5 week intervention, ddY mice were fed experimental diets containing WPH, a mixture of whey amino acids (WAAs), or casein (control). After the intervention, gastrocnemius muscle glycogen levels were significantly higher in the WPH group (4.35 mg/g) than in the WAA (3.15 mg/g) or control (2.51 mg/g) groups. In addition, total glycogen synthase (GS) protein levels were significantly higher in the WPH group (153%) than in the WAA (89.2%) or control groups, and phosphorylated GS levels were significantly decreased in the WPH group (51.4%). These results indicate that dietary WPH may increase the muscle glycogen content through increased GS activity.

KEYWORDS: *Whey protein hydrolysates, skeletal muscle glycogen, glycogen synthase, exercise*

INTRODUCTION

Glycogen is a carbohydrate that is stored in animal tissues; muscle glycogen is the main source of energy during moderate- to high-intensity exercise.[1] It has been reported that depletion of muscle glycogen stores is correlated with fatigue during endurance exercise.[2,3] In addition, the initial muscle glycogen content is correlated with endurance exercise performance.[4] Therefore, it is important for athletes to maintain adequate glycogen stores.

Dietary carbohydrate is an effective source of tissue glycogen. Several studies have reported that consuming a combination of carbohydrates and proteins after exercise more effectively replenishes muscle glycogen than consuming carbohydrates alone.[5,6] Ivy et al.[7] reported that muscle glycogen content was significantly greater after combined carbohydrate and protein intake than after consuming other types of foods with similar carbohydrate or caloric content.

Previously, we investigated the effects of consuming different types of dietary protein on muscle glycogen storage. We have shown that chronic feeding of whey protein to exercise-trained rats increased muscle glycogen content more than casein feeding.[8] Furthermore, we have shown that ingestion of carbohydrate plus whey protein hydrolysates (WPHs) increases skeletal muscle glycogen content in the 2 h after exercise in rats more than the ingestion of intact whey protein or branched-chain amino acids (BCAAs).[9] BCAAs, especially leucine, enhance muscle glycogen storage; our previous study indicated that not only BCAAs but also the bioactive peptides included in WPH might activate muscle glycogen synthesis. However, the mechanism whereby ingestion of WPH enhances glycogen synthesis has yet to be elucidated.

In skeletal muscle, glucose transport and glycogen synthase (GS) activity are considered the key regulatory factors for glycogen synthesis.[10] Furthermore, our previous study indicated that consumption of intact whey protein results in a greater increase in skeletal muscle glycogen content than casein consumption because of increased GS activity.[8] However, it remains to be determined which form of whey-based protein (intact protein, protein hydrolysates, or amino acids) is the most effective stimulator of GS in skeletal muscle. Thus, we hypothesized that WPH induces greater activation of glycoregulatory enzymes, especially GS, than amino acids.

The aim of the present study was to clarify the mechanisms of enhanced muscle glycogen synthesis with WPH consumption. We compared the effects of consuming WPH, whey amino acids (WAAs), or casein on mRNA expression, protein levels, and phosphorylation of glycoregulatory enzymes in skeletal muscle. We specifically focused on the effects of WPH on GS.

MATERIALS AND METHODS

Animals. This study used 5-week-old male ddY mice (Japan SLC, Shizuoka, Japan). Mice were maintained at 23 ± 2 °C, with lights on from 8:00 to 20:00. This study was approved by the Animal Committee of Food Science Research Laboratories, Meiji Co., Ltd.

Experimental Protocol. In preliminary training sessions, mice were fed an AIN-93G diet[11] for 3 weeks. All mice underwent initial measurements of exercise performance at 8 weeks of age and were

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then divided into three groups (n = 8) matched for exercise performance and body weight. All mice were allowed unlimited access to water and experimental diet for 5 weeks. At the end of 5 weeks, the mice were killed under ether anesthesia and the gastrocnemius muscle was quickly excised, washed, and then stored at −80 °C until further use. Liver and white adipose tissue (epididymal and retroperitoneal) were dissected and weighed.

Swimming Exercise Protocol. The swimming exercise protocol was modified from the protocol of Murase et al.[12] An improved adjustable-current water pool[13] was used to measure exercise performance. In preliminary training sessions, mice were acclimated to swimming for 30 min, 3 times a week, at a 13 L/min flow rate. After preliminary training, exercise performance was measured 3 times a week. To reduce the inherent variation in exercise performance, selection of mice was performed following the modified methods by Mizunoya et al.[14] We selected mice that had a standard deviation (SD) of less than 5 min for the three exercise performances. We excluded mice with a mean exercise performance longer than 35 min or shorter than 20 min; a total of 24 mice completed the experiment. During the experimental period, the mice exercised in the pool 5 times a week, at a 14 L/min flow rate; exercise performance was tested weekly for 4 weeks. All of the exercises were performed between 9:00 and 12:00. The last exercise occurred 2 days before dissection.

Exercise Performance Protocol. The exercise performance test was carried out following the modified methods by Ishihara et al.[13] Mice swam until they became fatigued, defined as a failure to rise to the surface of the water to breathe within a 7 s period. Changes in swimming posture accompanying fatigue have been described previously.[15] To evaluate exercise performance, we increased the flow rate of the pump by 1 L/min every 5 min from an initial flow rate of 13 L/min to a final rate of 18 L/min.

Experimental Diet. The design of the experimental diets followed the AIN-93 protocol,[11] and the composition of the experimental diets is shown in Table 1. Each diet contained an equal amount of protein.

Table 1. Composition of the Test Diets

ingredient	control (g/kg)	WAA (g/kg)	WPH (g/kg)
casein	245.900	200.000	200.000
L-cystine	3.000	3.000	3.000
cornstarch	351.586	347.486	347.486
dextrinized cornstarch	132.000	132.000	132.000
sucrose	100.000	100.000	100.000
soy oil	70.000	70.000	70.000
cellulose	50.000	50.000	50.000
mineral mixture	35.000	35.000	35.000
vitamin mixture	10.000	10.000	10.000
coline bitartate	2.500	2.500	2.500
tert-butylhydroquinone	0.014	0.014	0.014
WAA mixture		39.550	
WPH			50.000

The protein content of WPH (Meiji Co., Ltd., Japan) was measured by the Kjeldahl method.[16] The average chain length of the peptides was calculated as the ratio of total nitrogen/amino nitrogen in the protein samples. The average peptide length of WPH was 3.64. The WAA mixture was prepared by mixing pure crystalline L-amino acids (shown in Table 2).

Body Weight and Food Intake. Body weight was measured weekly throughout the study. Food intake was recorded for each cage throughout the study.

Muscle Glycogen Content. Perchloric acid extracts of gastrocnemius muscle were assayed for glycogen content according to the amyloglucosidase method.[17]

RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis. Total RNA was prepared from individual gastrocnemius muscle samples using a QIAGEN RNeasy Fibrous Tissue Kit (QIAGEN). The quality and quantity of RNA

Table 2. Composition of the WAA Mixture

amino acid	(g/100 g)
Ala	4.90
Arg	2.79
Asx	10.91
Cys	2.42
Glx	17.65
Gly	1.89
His	2.20
Ile	4.82
Leu	11.07
Lys	9.77
Met	2.18
Phe	3.39
Pro	5.45
Ser	5.02
Thr	5.30
Trp	1.77
Tyr	3.46
Val	5.01

samples was confirmed by measuring optical density at 260 and 280 nm and observing the gel electrophoresis pattern obtained using a BioAnalyzer (Agilent). A total of 1 μg of RNA was converted to single-strand cDNA using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD). Aliquots of the cDNA were assayed by quantitative RT-PCR using an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster, CA). Amplification was carried out for 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min using a TaqMan Universal PCR Master Mini Kit (Applied Biosystems) and Assays-on-Demand Gene Expression Probes (Applied Biosystems). The relative mRNA content for selected genes was calculated using the standard curve method. The quantity of mRNA was normalized to the amount of 18S rRNA in the cDNA sample.

Western Blotting. Muscle samples (50 mg) were weighed and homogenized in ice-cold RIPA buffer (Cell Signaling Technology, Beverly, MA) containing 1 mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 4000g for 30 min at 4 °C, and the supernatants were then aliquoted and stored at −80 °C for later analysis. The total protein content in the tissue homogenate supernatant was measured using bicinchoninic acid with bovine serum albumin as the standard; sample volumes were adjusted to give the same protein concentration in homogenates of muscles from different animals. Protein phosphorylation was determined by western blotting; 50 μg of muscle protein samples was separated by electrophoresis on a 5.0−10% gradient sodium dodecyl sulfate−polyacrylamide gel (Perfect NT Gel, DRC, Co., Ltd., Tokyo, Japan). The proteins were then transferred and blotted with gentle agitation using an iBlot Gel Transfer Device (Invitrogen, Carlsbad, CA) in freshly prepared TBS containing 0.5% nonfat dry milk and 0.1% polysorbate 20. The membranes were then incubated with gentle agitation at 4 °C overnight with a primary antibody. The primary antibodies used were rabbit anti-p-GS (Ser641; 1:1000 dilution; Cell Signaling Technology) for phosphorylated glycogen synthase (p-GS), rabbit anti-GS (1:1000 dilution; Cell Signaling Technology) for GS, rabbit anti-GLUT4 (1:1000 dilution; AbD Serotec) for GLUT4, and rabbit anti-β-actin (1:1000 dilution; Cell Signaling Technology) for β-actin, which was used as the internal control protein. After the membranes were washed in a 0.1% Tris-buffered saline−Tween 20 solution, they were incubated with agitation with a secondary reagent at room temperature for 1 h. Anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology) was used at a dilution of 1:1000 to detect p-GS, GS, GLUT4, and β-actin. The polyvinylidene difluoride membranes were then washed in 0.1% Tris-buffered saline−Tween 20, and the antibody-bound proteins were visualized using ECL Plus Western Blotting Detection Reagents (Amersham Pharmacia Biotech,

Amersham, U.K.) according to the protocol of the manufacturer. The images were visualized using a Chemi-doc Gel Quantification System (Bio-Rad, Hercules, CA).

Statistical Analysis. Data are expressed as means ± standard error of the mean (SEM). One-way analyses of variation (ANOVAs) were used to compare data between groups. Significant differences were analyzed using Tukey's post-hoc analysis. Associations between the variables were analyzed using Pearson's correlation coefficients. Differences between groups were considered statistically significant at $p < 0.05$.

RESULTS

Body Weights and Tissue Weights. Table 3 shows food intake, final body weight, and liver and adipose tissue weights. There were no significant differences in final body weight or tissue weights among the groups.

Table 3. Food Intake, Body Weight, Liver Weight, and Fat Weight of Mice Fed Casein, WAA, or WPH Diets for 5 Weeks[a]

	control	WAA	WPH
food intake (g mouse^{-1} day^{-1})	4.20	4.35	4.22
final body weight (g)	41.6 ± 1.0	41.6 ± 0.4	41.7 ± 1.2
liver weight (g/100 g of body weight)	4.29 ± 0.13	4.46 ± 0.15	4.44 ± 0.06
epididymal fat weight (g/100 g of body weight)	1.80 ± 0.22	2.03 ± 0.28	1.80 ± 0.28
retroperitoneal fat weight (g/100 g of body weight)	0.68 ± 0.09	0.54 ± 0.11	0.53 ± 0.11

[a]Values are means ± SEM ($n = 8$).

Gastrocnemius Muscle Glycogen Levels. Consistent with our previous study, the gastrocnemius muscle glycogen content was significantly greater in the WPH group than in the WAA or control groups (Figure 1). The WPH group had 70% higher glycogen levels than the control group and 40% higher levels than the WAA group; glycogen levels were significantly higher in the WAA group than in the control group.

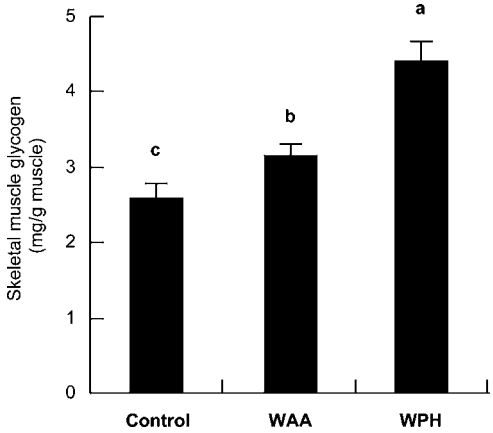

Figure 1. Skeletal muscle glycogen content of mice fed control, WAA, or WPH diets for 5 weeks. Values are means ± SEM ($n = 8$). Means without common letters differ; $p < 0.05$ (Tukey's post-hoc analysis).

Skeletal Muscle mRNA Levels. Dietary WPH significantly increased glycogen synthase I (GSI) mRNA more than dietary WAA or control; GSI mRNA expression was not different between the WAA and casein groups. GSI mRNA expression was 27% higher in the WPH group than in the control group; it was 18% higher than in the WAA group. In contrast, GLUT4 and hexokinase II mRNA levels did not differ among groups (Figure 2).

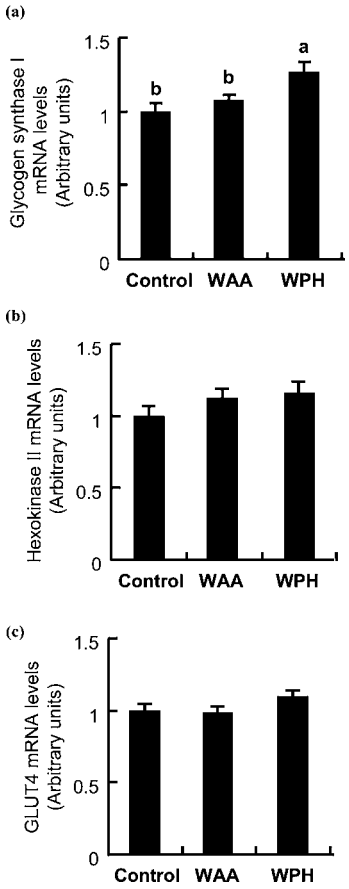

Figure 2. Skeletal muscle mRNA levels of (a) GSI, (b) hexokinase II, and (c) GLUT4 in mice fed control, WAA, or WPH diets for 5 weeks. Values are means ± SEM ($n = 8$). Means without common letters differ; $p < 0.05$ (Tukey's post-hoc analysis).

Western Blot Analysis of Gastrocnemius Muscle GS and GLUT4 Levels. GS is a highly regulated enzyme. GS activity is controlled allosterically and is activated by dephosphorylation.[18] Total GS levels were significantly higher in the WPH group than in the casein or WAA groups (Figure 3a). In contrast, phosphorylated GS levels were significantly lower in the WPH group than in the casein or WAA groups (Figure 3b). GLUT4 levels did not differ among groups (Figure 4).

Exercise Performance. There were no significant differences in exercise performance among groups. The WPH group exercised 49% longer than the control group at week 4 and 13% longer than the WAA group; however, these differences was not statistically significant (Table 4).

Correlation between Glycogen Content and Exercise Performance. We found a significant positive correlation between skeletal muscle glycogen content and exercise performance (Figure 5).

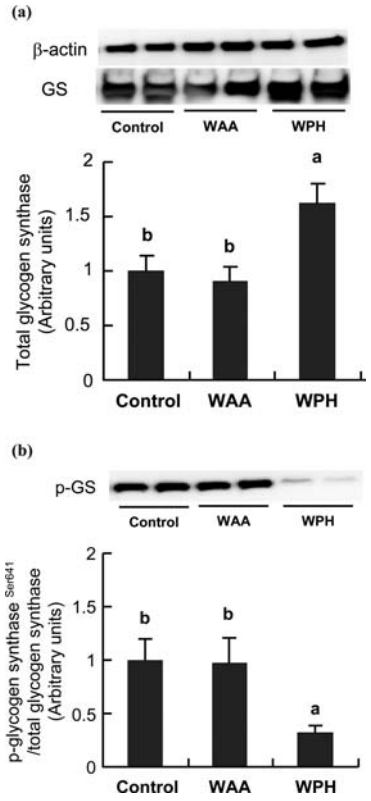

Figure 3. Skeletal muscle protein levels of (a) total GS and (b) phosphorylated GS in mice fed control, WAA, or WPH diets for 5 weeks. Phosphorylated GS was normalized to total GS. Values are means ± SEM (n = 8). Means without common letters differ; $p < 0.05$ (Tukey's post-hoc analysis).

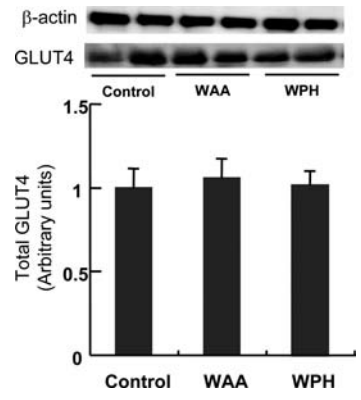

Figure 4. Skeletal muscle GLUT4 protein levels of mice fed control, WAA, or WPH diets for 5 weeks. Values are means ± SEM (n = 8).

DISCUSSION

We have previously shown that ingestion of carbohydrate plus WPH increases glycogen content significantly more after glycogen-depleting exercise than ingestion of whey protein, casein hydrolysates, or BCAAs.[9] In this study, we compared the effects of long-term feeding of a WAA mixture and WPH on muscle glycogen synthesis and glycoregulatory enzyme levels. This is the first study to demonstrate that WPH increases glycogen levels more than an amino acid mixture by activating GS.

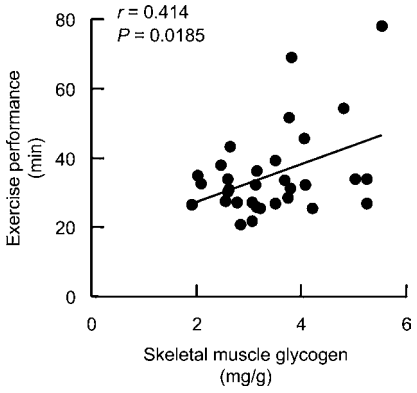

Figure 5. Correlation between skeletal muscle glycogen content and exercise performance.

Glucose uptake and GS activity are key regulatory factors for synthesizing skeletal muscle glycogen.[10a,b] In the present study, we found that dietary WPH significantly increased GSI mRNA levels; however, expression of the proteins that regulate glucose uptake (hexokinase II and GLUT4) was unchanged. Furthermore, we demonstrated that dietary WPH significantly increased GS protein levels. These findings strongly indicate that long-term feeding of WPH increases GS protein levels by activating GSI mRNA expression.

GS activity is allosterically regulated by phosphorylation; dephosphorylation activates GS.[19,20] Previous research shows that GS is activated after exercise.[21] As glycogen levels increase during recovery from exercise, GS activity is rapidly reduced. As in previous studies, the muscle glycogen supercompensation occurred in the face of muscle GS activities that were far lower than those found in immediately post-exercise.[22,23] In this study, although the WPH group had the highest levels of muscle glycogen, this group had significantly lower GS phosphorylation. These results indicate that long-term WPH feeding maintains the rate of GS activity, even if muscle glycogen levels are already high. Long-term feeding of WPH further increased muscle glycogen storage; however, long-term feeding of WAA or casein did not affect GS activity. This result may be explained by the relatively low WAA content (4.0%) in the diet (Table 1). In contrast, even small amounts of dietary WPH activated GS.

We have previously shown that WPH replenishes glycogen more efficiently than other protein sources in the 2 h after cessation of glycogen-depleting exercise.[9] In this study, we found that long-term feeding of WPH results in more skeletal muscle glycogen storage than feeding a WAA mixture or casein.

Table 4. Exercise Performance of Mice Fed Casein, WAA, or WPH Diets through the Experimental Period[a]

	0 week (min)	1 week (min)	2 week (min)	3 week (min)	4 week (min)
control	25.5 ± 0.7	26.4 ± 1.1	27.5 ± 1.4	27.9 ± 1.5	28.5 ± 2.0
WAA	25.4 ± 1.2	25.4 ± 0.9	28.2 ± 2.7	35.3 ± 3.4	37.5 ± 5.0
WPH	26.1 ± 0.9	27.2 ± 1.0	34.8 ± 5.2	40.1 ± 6.2	42.4 ± 5.1

[a]Values are means ± SEM (n = 8).

Taken together, these results suggest that feeding WPH replenishes glycogen more efficiently during initial recovery from exercise and leads to increased glycogen storage over time. The WPH group exercised longer than the other groups, although this difference was not statistically significant. It is well-known that there is a positive correlation between muscle glycogen storage and exercise performance,[24] and this correlation was observed in the present study.

Previous research has investigated differences between the molecular forms of dietary protein. The results by Poullain et al.[25,26] suggested that, in rats, protein hydrolysates are used more effectively than free amino acids. However, Manninen et al.[27] suggested that the nutritional benefits of consuming protein hydrolysates instead of amino acids have not been clearly demonstrated. In this study, exercise-trained mice were fed experimental diets containing one of two whey-based protein sources: protein hydrolysates or amino acids. The two experimental diets differed only in the molecular forms of the whey-based proteins provided. The molecular form of protein consumed appears to affect long-term glycogen storage; long-term feeding of WPH leads to greater glycogen storage. These results suggest that consuming carbohydrates mixed with WPH may enhance sport performance by increasing glycogen storage.

BCAAs, especially leucine, play an important role in the activation of GS. Armstrong et al.[28] reported that amino acids stimulated p70S6 kinase and transiently inhibited glycogen synthase kinase 3, thereby increasing glycogen synthesis in cultured human muscle cells. Peyrollier et al.[29] also observed leucine-stimulated glycogen synthesis in L6 cells as a result of GSK-3 inactivation. The amino acid compositions of the two diets used in this study contained equal amounts of BCAA and leucine; however, muscle glycogen accumulation varied between diets. This result strongly suggests that not only the BCAA content but also the molecular form of BCAA found in the protein source might be important for muscle glycogen storage. Recently, we demonstrated that BCAA-containing peptides in WPH increased the rate of glucose uptake *in vitro*.[30] Furthermore, we have shown that the plasma levels of these peptides elevate markedly after ingestion of WPH.[31] WPH used in this study contained 21.6 mg/g of BCAA-containing bioactive peptides. These BCAA-containing peptides may be responsible for the GS activation-induced increase in muscle glycogen levels observed with long-term WPH feeding.

BCAAs increase glucose uptake in skeletal muscle tissue, mainly by enhancing GLUT4 translocation.[32] As an example of this, Doi et al.[33] reported that leucine supplementation significantly increased glucose incorporation into intracellular glycogen *in vivo*. Furthermore, we have previously shown that ingestion of glucose plus WPH activates key enzymes that regulate GLUT4 translocation.[9] Taken together, these results suggest that the molecular form of dietary protein may influence GLUT4 translocation as well. Thus, further studies are required to investigate the effect of WPH on GLUT4 translocation.

The findings of this study do not fully explain the effects of WPH on carbohydrate metabolism in mice. This may be due to the fact that intracellular glucose can undergo not only glycogenesis but also glycolysis and both of these pathways may be influenced by WPH. A previous study indicated that whey protein increases skeletal muscle glycogen content as a result of decreased 6-phosphofructokinase activity, a key regulatory enzyme in glycolysis.[8] Therefore, further studies are required to investigate the effects of WPH on glycolysis.

This is the first study to demonstrate that long-term feeding of WPH increases muscle glycogen storage more than a WAA mixture by stimulating GS. This increased rate of GS activation is maintained when muscle glycogen levels are already high. These results provide new insights into the use of different forms of dietary protein in sports nutrition; however, it remains unclear which components of the WPH are essential for enhanced muscle glycogen storage. Future studies are required to identify the bioactive peptide in WPHs and to determine the molecular mechanism of GS activation. These studies will help to establish the optimal formula for recovery after exercise.

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Notes

The authors declare no competing financial interest.

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