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PROTEÍNAS DO SORO DE LEITE E SUA SUPLEMENTAÇÃO COM L-LEUCINA: INFLUÊNCIA, NOS PARÂMETROS BIOQUÍMICOS, MOLECULARES E COMPOSIÇÃO CORPORAL, DE RATOS WISTAR EXERCITADOS

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RESUMO

Introdução: Em situações especiais é desejável estimular o anabolismo e reduzir o catabolismo. As proteínas do soro de leite apresentam excelente balanço de aminoácidos para o anabolismo. A suplementação com L-leucina vem sendo relacionada à inibição do catabolismo e ao estímulo do anabolismo, assim como o exercício físico. Na busca de estratégias para estimular o anabolismo e reduzir o catabolismo, optou-se por estudar o efeito combinado do consumo de proteínas do soro lácteo e a suplementação com L-leucina, aliados ao exercício físico. **Objetivo:** verificar o efeito dose-resposta da suplementação com L-leucina na composição corporal, ganho de massa corporal, parâmetros bioquímicos e ativação da proteína mTOR de ratos Wistar, consumindo proteínas do soro de leite (PSL), quando submetidos ao exercício. **Metodologia:** 96 ratos machos Wistar foram divididos em 16 grupos recebendo dieta AIN93 com PSL ou caseína (C) como fonte protéica, com 3, 4.5, ou 6% de suplementação de L-leucina, exercitados e sedentários, a saber: a) n=6, exercitados, dieta AIN93 com PSL (PSL E); b) n=6, exercitados dieta AIN93 com PSL + 3% de L-leucina; (PSL L3 E); c) n=6, exercitados, dieta AIN93 com PSL + 4,5% de L-leucina (PSL L4.5 E) e d) n=6, exercitados, dieta AIN93 com PSL + 6% de L-leucina (PSL L6 E). Para cada grupo exercitado houve um grupo controle sedentário e o mesmo desenho experimental foi repetido, com grupos sedentários e exercitados, alterando somente a fonte protéica da dieta para caseína que foi a proteína controle. Foram analisados: ingestão alimentar; evolução ponderal; perfil de aminoácidos

plasmáticos; composição corporal dos animais; atividades de CK, LDH, AST ALT e mTOR (muscular, cardíaca e diafragmática), ácido úrico, creatinina e massa relativizada do gastrocnêmio, coração e diafragma, tudo em função da dieta e da atividade física. Para análise estatística foi utilizado o software SPSS, versão 11.0 for Windows para análise de variância – ANOVA – utilizando o critério de significância de $p \leq 0,05$, com teste post-hoc de Tukey. **Resultados**: A ativação da via mTOR ocorreu com suplementação de L-L-leucina em ambas dietas por 30 dias no coração, diafragma e grastrocnêmio dos ratos sedentários e exercitados. Indicadores gerais de saúde, não pareceram alterados, exceto insulina e glicose, que demosntraram anormalidades nos animais sedentários com altas doses de L-leucina. O ganho de massa corporal foi significativamente menor nos animais submetidos à suplementação com 6% de L-L-leucina. As enzimas de prova hepática (TGO e TGP) mantiveram-se em níveis plasmáticos normais, assim como os indicadores de prova renal (ácido úrico e creatinina) não indicando dano renal com a suplementação descrita. **Conclusão:** A via mTOR foi ativada no coração, diafragma e músculo dos animais suplementados com L-L-leucina. A maior dose utilizada (6%) prejudicou o crescimento, níveis de glicose e insulina dos animais.

Palavras chave: L-leucina, Whey Protein, Caseína, exercício, suplementação dietética, composição corporal

ABSTRACT

Introduction: in both animals and humans L-L-leucine can activate protein synthesis in skeletal muscle by mTOR thus stimulating body growth. Currently, however, it is not clear if heart tissue is also subject to the same regulatory mechanism of protein synthesis. **Objective:** The purpose of this study was to assess heart mTOR activation, heart mass, growth and liver function in young Wistar rats fed standard AIN93-G diet supplemented with L-L-leucine at three levels. **Methods:** Ninety-six weanling male Wistar rats were divided into sixteen groups and fed one of the following diets for 30 days: a) Control (AIN 93-G); b) 3% (AIN93-G +3% L-L-leucine); c) 4,5% (AIN93-G +4,5% L-L-leucine); d) 6% (AIN93-G +6% L-L-leucine). Modified AIN 93-G diets containing whey protein instead of casein performed another 4 groups completing 8 sedentary groups, another set of 8 groups was trained, with the total of 16 groups. The supplemented diets, energy was adjusted at the expense of carbohydrate. mTOR pathway was quantified by Westernblot analysis. Serum insulin, uric acid, glucose, AST, ALT, and cardiac mass, total and body mass-adjusted protein were determined by standard methods. ANOVA and pos-hoc Duncan were applied to compare the means (significance $p<0.05$). **Results:** mTOR activation (phosphorylated mTOR/ total mTOR) was reached with L-L-leucine supplementation in heart, diaphragm and gastrocnemius. General health indicators did not show significant modifications except for glucose and insulin levels, which increased compared to control group. Supplementation did not adversely affect liver function as determined by AST, ALT,

but body mass in the 6% group was significantly lower than that of the 4.5% group, showing a negative effect of the highest dose on body mass accretion. Either absolute heart mass or adjusted heart mass showed no difference between any two groups. **Conclusion:** mTOR activities of heart, diaphragm and gastrocnemius of Wistar rats were increased by supplementing the AIN 93-G diet with L-L-leucine, loss of the body mass and abnormalities in insulin and glucose levels were shown in 6% of L-L-leucine supplementation. Even the 6% supplementation did not alter liver function, but this concentration adversely affected normal growth.

Keywords: L-leucine, whey protein, casein, exercise, dietary supplementation, body composition

Sumário

LISTA DE ABREVIATURAS

AIN93-G	*American Institute of Nutrition*
ALT	Enzima alanina aminotransferase
AST	Enzima aspartato aminotransferase
AT	Aminotransferase
ATP	Adenosina trifosfato
BCAAs	Sigla inglesa para aminoácidos de cadeia ramificada
CAS	Caseína
CK	Creatina quinase
LDH	Enzima lactato desidrogenase
PSL	Proteínas do soro do leite
PSLC	Proteína do soro do leite concentrada
PSLH	Proteína do soro do leite hidrolisada

Introdução geral

INTRODUÇÃO GERAL

Os seres humanos em situações específicas (crescimento, gestação, atividade física e convalescença), necessitam de uma quantidade maior de proteínas dietéticas para manter um balanço nitrogenado positivo (LEMON, 1997) e seria desejável uma resposta anabólica mais pronunciada em determinadas circunstâncias em que há desequilíbrio metabólico prevalecendo o catabolismo, tais como: I) Envelhecimento, quando há perda de massa muscular tanto em humanos como em roedores (HOLLOSZY et al. 1991, KLITGAARD et al., 1989); II) Patologias que estimulam estados catabólicos como, por exemplo, câncer, quando a maioria dos pacientes acaba morrendo por caquexia (BLUM et al., 2011); III) Pacientes acometidos por queimaduras ou em período pós-cirúrgico, quando há necessidade de reparação do tecido lesado (BIOLO et. al., 2002).

Desta forma, estudos sobre a estimulação do anabolismo ou inibição do catabolismo são importantes, e neles a leucina vem ganhando destaque (NICASTRO et. al., 2011).

Em indivíduos exercitados, a ingestão inadequada de proteína poderia induzir uma perda de proteína corporal, particularmente no músculo com a perda de desempenho, além disso, nos exercícios de longa duração o catabolismo protéico durante o exercício torna-se pronunciado quando as reservas de carboidratos são exauridas (KREIDER et. al., 2007).

As proteínas são reconhecidas como parte vital dos tecidos, apresentam uma incrível diversidade de funções, embora todas compartilhem a característica estrutural comum de serem polímeros lineares de resíduos de aminoácidos, os quais estão unidos em longas cadeias, em várias formas e combinações químicas para formar estruturas protéicas muito diversas (FARFAN et al., 2007).

Um adulto (70 kg) possui entre 10 e 12 kg de proteína, com a maior quantidade (6 a 8 kg) localizada dentro da massa dos músculos esqueléticos. Além disso, aproximadamente 210 g de aminoácidos existem na forma livre, principalmente como glutamina, um aminoácido chave com funções que incluem funcionar como combustível para as células do sistema imune. Recomenda-se ingestão de cerca de 10 a 15% (FARFAN et al., 2007) das calorias totais como proteína. Durante a digestão, a proteína é hidrolisada em seus aminoácidos constituintes e peptídeos a fim de ser absorvida pelo intestino delgado.

Durante a idade adulta, o conteúdo protéico da maioria dos adultos se mantém estável, e o fornecimento de aminoácidos em excesso, que não são utilizados para a síntese das proteínas, hormônios ou para o metabolismo energético são transformados em triacilgliceróis para armazenamento nos adipócitos (MCARDLE et al., 2001).

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Capítulo 1

INTRODUCTION AND RATIONALE

Human beings under specific situations (growth, pregnancy, physical activity and convalescence), require a higher amount of dietary protein to maintain a positive nitrogen balance (LEMON, 1997) and a more pronounced anabolic response would be desirable in certain circumstances where there is a metabolic imbalance prevailing over catabolism, such as: I)Aging, when there is loss of muscle mass in both humans and in rodents (HOLLOSZY et al. 1991, KLITGAARD et al., 1989). II)Pathologies that stimulate catabolic states such as cancer, when most patients eventually die due to cachexia (BACURAU, ROSA 1997); III)Patients suffering from burns or in postoperative period, when the repair of the damaged tissue is necessary (BIOLO et. al., 2002).

Thus, studies on the stimulation of anabolism or inhibition of catabolism are important, and in these studies L-leucine has been gaining attention (NICASTRO et al., 2011).

In subjects who practice exercises, inadequate intake of protein could induce a body protein loss, particularly in the muscle with the loss of performance; in addition, in the long-term exercises the protein catabolism during exercises becomes pronounced when the reserves of carbohydrates are exhausted (KREIDER et. al., 2007).

Proteins are recognized as a vital part of tissues, have an incredible diversity of functions, although all of them share the same structural feature of being linear

polymers of amino acid residues, which are bound in long chains, in several forms and chemical combinations to form very different protein structures (COLGAN, 1993; CHAMPE; HARVEY, 1996).

An adult (70 kg) has between 10-12 kg of protein, with the largest amount (6-8 kg) located within the mass of skeletal muscles. In addition, there are approximately 210 g of amino acids in the free form, mainly as glutamine, a key amino acid with functions that include working as fuel for the immune system cells. It is recommended the intake of approximately 10-15% (FARFAN et al., 2007) of total calories as protein. During digestion, the protein is hydrolyzed to its constituent amino acids and peptides, in order to be absorbed by the small intestine.

During adulthood, the protein content of most adults remains stable, and the excess amino acids, which are not used for the synthesis of proteins, hormones, or for the energy metabolism, are converted into triglycerides for storage in the adipocytes (MCARDLE et al., 2001).

Whey protein plays an important role in anabolism stimulation, due to its good digestibility, high nutritional value, since it has high concentration of branched chain amino acids (BCAA) and sulfurated amino acids (YVES et al., 1997; FRÜHBECK, 1998).

WHEY PROTEINS

Milk proteins are divided into 2 classes: insoluble (casein) and soluble (whey proteins - WPs). Casein makes up 80% of cow's milk proteins, and the other 20% are composed of whey proteins. WPs have high biological value, containing optimal concentrations of essential amino acids, according to the FAO recommendations (FARFAN et al., 2007).

Studies suggest that milk proteins, including whey proteins, in addition to their high biological value, have bioactive peptides, i.e., with biological activity and can work as antimicrobial agents, antihypertensive agents, immune function regulators, and even as growth factors (GROZIAK; MILLER, 2000; LÖNNERDAL, 2003).

Among the characteristics of the WPs the high rate of digestion and absorption, which can quickly increase plasma amino acid levels after their ingestion, and reduces gastric emptying time should be emphasized. The mixture of peptides quickly pass from the stomach to the small intestine, where the process of absorption begins (YVES et al., 1997; FRÜHBECK, 1998).

Another characteristic of the WPs is their ability to stimulate the release of insulin, anabolic hormone, which promotes the transport of amino acids to the intracellular medium, i.e., in addition to providing plenty of the basic units for the construction of proteins (amino acids), the intake of WPs stimulates the anabolism through this hormonal response produced (AKHAVAN et al., 2010).

Calbet & MacLean (2002) observed the insulin response after ingestion of a solution containing 25g/l of glucose (control) or the control solution with addition of 0.25 g/kg from different protein sources: a) WP; b) whole milk proteins; c) pea proteins. The highest insulin response was that of glucose solution with addition of WP, which was twice higher than the whole milk proteins and four times higher than the solution containing glucose only. In addition, the authors found that the glucose solution plus WP produced the most elevated concentrations of plasma essential amino acids, especially branched chain amino acids (BCAA - leucine, isoleucine and valine).

Because of the high supply of amino acids, favorable hormonal response and generation of bioactive peptides as growth factors, some researchers have tested the anabolic potential of WPs, Candow et al. (2006) in a double-blind experiment with 27 healthy sedentary men undergoing resistance training, divided into three groups, receiving daily supplementation of: a) WP; b) soy protein; or c) placebo (maltodextrin). They have observed a significant increase ($p<0.05$) in muscle mass in the groups receiving protein supplementation (regardless of protein source - WP or soy protein) compared to isocaloric placebo (maltodextrin).

Kerksick et al. (2006) in a human study, randomly divided 36 volunteers into 3 groups, who underwent resistance training (using weights) 4 times a week for 10 weeks, receiving daily supplementation with: a) 40 g of whey proteins plus 8 g of casein; b) 48 g of carbohydrate (placebo); or c) 40 g of WP plus 3 g of BCAA.

These authors observed significant increases in the muscle mass of the WP-supplemented subjects compared to the other groups.

Finally, we emphasize the high concentrations of BCAAs, including L-leucine in the WPs. The BCAAs comprise 21.2% of all amino acids contained in the WP, and the percentage of BCAAs in the essential amino acids of the WPs is 42.7%. This concentration of BCAA is above the average of other protein sources (ETZEL, 2004). This particular feature of the WPs provides them with an amino acid profile very similar to that of the skeletal muscle (HA; ZAMEL, 2003).

Since WPs have high digestibility and absorption rates, high concentrations of essential amino acids (mainly BCAA and L-leucine), and increased insulin response after ingestion, they are considered as an excellent protein source for stimulation of anabolism.

L-LEUCINE, AN ANABOLIC AND ANTI-CATABOLIC AGENT

In 1995, Louard et al. (1995) observed that the infusion of branched chain amino acids (BCAA) favored the maintenance of muscle mass. In 1996, Argilés et al. described the ability of BCAAs of inhibiting proteolysis in skeletal muscle, including under catabolic states (such as cachexia).

Since 2000, there were reports from different research groups claiming that protein synthesis depends on the availability of intracellular amino acids, increasing

when there is high concentration of amino acids in the cell (Proud, 2002). It was also observed that this stimulation in the protein synthesis occurs mainly in the presence of branched chain amino acids, being L-leucine the main responsible for this response (ANTHONY et al., 2002; KOOPMAN et al., 2005).

Therefore, among the branched chain amino acids (L-leucine, isoleucine and valine) L-leucine is the amino acid able to stimulate the acute protein synthesis in skeletal muscles. Some studies suggest L-leucine is a nutritional indicator of the availability of amino acids (ANTHONY at al., 2001; ANTHONY et al., 2002; GARLICK, 2005).

L-leucine is also recognized for its ability to stimulate the synthesis and release of insulin (BOLSTER, 2003). In animal models, L-leucine showed stimulating effect on protein synthesis through an insulin-independent mechanism – the anabolism-stimulating hormone (ANTHONY et al., 1999; ANTHONY et al., 2002). In 2002, Anthony et al showed that the infusion of L-leucine is able to stimulate the protein synthesis in rats, even when insulin concentrations were maintained low (by concomitant infusion of somatostatin, which inhibits insulin release). Data suggest that although L-leucine itself stimulates the insulin release, probably part of its anabolic effect does not depend on the presence of insulin.

In rats under fasting conditions for 18 hours, oral administration of L-leucine at 1.35 g/kg of body mass increased muscle protein synthesis when compared to the control group receiving saline solution (YOSHIZAWA et al., 2002).

However, in humans, Rieu et al. (2006) observed in 20 elderly volunteers (70 ± 1 years old), eating balanced meals and with supplementation of L-leucine, the plasma concentrations of this amino acid in the post-absorptive period were higher with concomitant increase in muscle protein synthesis, verified by the method of constant infusion of labeled phenylalanine and further muscle biopsy (vastus lateralis), allowing for the calculation of the muscle synthesis rate.

The anabolic response of L-leucine was also observed in young subjects. According to Koopman et al (2005), 8 healthy men (22.3 ± 0.9 years old) underwent an anaerobic workout session and were provided with: a) supplementation with L-leucine, carbohydrate and proteins (25g of glucose, 25g of maltodextrin, 16.6g of L-leucine and 33.3 g of whey protein); b) carbohydrate, protein (25g of glucose, 25g of maltodextrin and 33.3g of whey proteins); or c) carbohydrate (25g of glucose and 25g of maltodextrin). The volunteers who ate more supplementation with L-leucine plus carbohydrate and proteins showed higher anabolic response when compared to the other subjects; this was measured by the method of constant infusion of labeled phenylalanine and further muscle biopsy (vastus lateralis), allowing for the calculation of the muscle synthesis rate.

MECHANISMS OF ACTION OF L-LEUCINE

In addition to the ability of stimulating protein synthesis, "in vitro" studies suggest that high concentrations of L-leucine also act by inhibiting the degradation

of skeletal muscle tissue of rats (GARLICK, 2005), which may modulate the proteolysis rate, inhibiting the degradation of skeletal muscle proteins (ARGÍLES, 1996). Skeletal muscle proteolysis is regulated by several mechanisms, and the ubiquitin-proteasome system is the main non-lysosomal protein degradation pathway; this may be inhibited in the presence of L-leucine (NAKASHIMA et al., 2005).

Incubation of rat soleus in the presence of L-leucine reduced lysosomal and ATP-dependent protein degradation (ubiquitin-proteasome pathway) (BUSQUETS, 2000), and the regulation of muscle proteolysis is important for the energy homeostasis, control of muscle mass and body growth (KETTELHUT, 1988).

In the last decades, studies on the translation, function of ribosomes and translational control were performed and showed there is interrelationship between the translation system and the nutritional factors, such as amino acid supplementation. The control of translation in skeletal muscle is a multistep process that comprises the availability of eukaryotic initiation factors (eIFs) and the activation of ribosomal protein S6 kinase (S6K1) (SHAH, 2000). The process of protein synthesis is initiated through binding of initiation factor eIF2 to GTP and to met-tRNAi, forming the ternary complex eIF2-GTP-met-tRNAi. Part of this ternary complex binds to the ribosomal subunit 40S, establishing the 43S pre-initiator complex.

In order for this complex to recognize and bind to the mRNA, the activity of the eIF4F complex is necessary, formed by the following protein set: eIF4A, eIF4B, eIF4G, and eIF4E (ANTHONY et al.,2001). The eIF4F complex promotes the binding of 43S pre-initiator complex to the ribosomal subunit 60S, establishing the unit 80S. The eIF4F complex acts by recognizing, unfolding and guiding the mRNA during translation. The factor eIF4E associated with eIF4G identifies the 5'-terminal region of mRNA in the region m7GTP (SHAH, 2000). After recognition of the AUG codon, the translation begins, where the movement of the complex by the mRNA is driven by the eIF4A activity, stimulated by eIF4B.

The mechanism by which the activity of the eIF4F complex regulates the translation initiation involves the modulation and the availability of eIF4E. The factor eIF4E can bind to 4E-BP1 (translation repressor) and, while it is associated with the 4E-BP1 complex, the mRNA cannot bind to the ribosomal unit 80S. The dissociation of the 4E-BP1 complex is regulated by the activity of the protein mTOR (mammalian target of rapamycin), which phosphorylates 4E-BP1 and consequently causes dissociation of the complex, releasing eIF4E that binds to eIF4G (KIMBALL e JEFFSON, 2004).

mTOR regulates the activity of several molecules involved in the mechanism of translation (PROUD, 2002), primarily 4E-BP1 and S6K1 that are phosphorylated and activated by mTOR, increasing the cell’s ability to synthesize proteins (PROUD, 2002; KIMBALL and JEFFSON, 2004). The mTOR works as a

“bifurcation point” in the cascade of protein synthesis by the skeletal muscle cell, which may be signaled by insulin and L-leucine (ALFRED, 2010).

On the other hand, L-leucine can stimulate protein synthesis through its metabolites such as ketoisocaproic acid (KIC). In L-leucine catabolic process, it is transaminated to KIC, which in turn can be the controller of the L-leucine activities in the protein metabolism (NISSEN, ABUMRAD, 1997).

Therefore, the supplementation with L-leucine could be used to maximize the hypertrophic/hyperplastic effects of physical training, by the activation of mTOR cascade, stimulating protein synthesis (KYLE et al., 2004) associated with the consumption of whey proteins, which would act as suppliers of amino acids to the actual construction of proteins, in addition to maximizing the anabolic signaling through the insulin release.

Although the literature has indicated the effectiveness of L-leucine to stimulate the synthesis and inhibit protein degradation in the skeletal muscle, no consensus was reached on what would be the optimal dose to maximize the anabolic effects and on the repetition of this effect in non-skeletal muscle tissue (cardiac and smooth muscle).

FOOD PROTEIN AND EXERCISE

The protein intake-exercise ratio is frequently studied and discussed in scientific papers, most notably regarding anabolic effects on muscle mass (MM) (LEMON et al., 1997; TIPTON; WOLFE, 2003; SOCIEDADE BRASILEIRA DE MEDICINA NO ESPORTE, 2003; CANDOW et al., 2006; KERKSICK et al., 2006; COBURN et al., 2006).

Physical activity increases the need for protein intake, in order to maintain nitrogen balance or positive balance (TIPTON; WOLFE, 2003), but the optimal intake, as well as the time of this intake, taking the different sports into account, is still controversial (LEMON, 2000; TIPTON; WOLFE, 2003; SOCIEDADE BRASILEIRA DE MEDICINA NO ESPORTE, 2003). Two main reasons for the increase in protein needs during physical training are highlighted:

- Amino acids may be catabolized during exercise

- Protein synthesis for building muscles stimulated by the exercise or reconstruction of proteins damaged by the physical activity (by the physiological changes caused by the exercise) (CLOSE et al., 2005).

Therefore, in human beings protein intake for athletes exceeds the recommendation for sedentary subjects, currently 1 g.kg-1day-1. Athlete is defined as an individual who engages in regular organized physical

training within a particular sporting discipline and competes at county or national level (Sharma, 2003).

Table 1 – Protein requirements for athletes ($g.kg^{-1}.day^{-1}$)

Author	Strength Athletes	Endurance Athletes
Lemon (1997)	1.7 – 1.8	1.2 – 1.4
Weineck (1999)	3	-
SBME (2003)	1.4 – 1.8	1.2 – 1.6
Tipton (2004)	1.2 – 1.7	1.2 – 1.4

It is important that the protein provided to athletes under training has high quality and bioavailability, as the absence of one or more amino acids may impair the synthesis of proteins damaged or consumed during physical exercise.

ALANINE-GLUCOSE CYCLE

With the increase of the demand for energy during exercise, especially in long-term exercises, proteins can be used as energy source. Studies in humans have shown that there is an increased release of alanine by the muscles exercised:

as the exercise intensity increases a proportional increase in the production of alanine is observed (MCARDLE et al, 2001).

Alanine is synthesized in the skeletal muscle exercised through the reaction of transamination of BCAAs and from pyruvate. The newly formed alanine leaves the muscle and is transported to the liver where it is deaminated. In the liver, the carbon skeleton is used for gluconeogenesis, releasing the glucose produced in the blood, where it is transported to the muscles. During deamination, the amino acid loses its nitrogen group in the liver to form urea (H2NCONH2) for excretion purposes. After 4 hours of continuous low-intensity exercise, the liver production of glucose derived from alanine may be responsible for 45% of all glucose released by the liver. During the exercise, the production and elimination of alanine by the muscle is higher, which helps to maintain blood glucose, in order to meet the needs of the central nervous system and muscles. It is believed that 10 to 15% of total energy demand of the exercise may be generated by the alanine-glucose cycle, especially in high-intensity prolonged exercises. In well-fed subjects at rest, the protein fractionation contributes with 2-5% of total body energy demand (MCARDLE et al, 2001).

Thus, the hypothesis of this work is that the combination of L-leucine, whey protein and exercise may stimulate anabolism in heart, diaphragm and skeletal muscle.

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Objetivos

OBJETIVO GERAL

Verificar o efeito no longo prazo (30 dias) da suplementação com L-leucina em doses crescentes em ratos Wistar exercitados consumindo PSL.

OBJETIVOS ESPECÍFICOS

Em função das doses crescentes de L-leucina aplicadas por 30 dias avaliar:

Anabolismo muscular e ganho de peso corporal;

- ✓ Ativação da via mTOR no diafragma, coração e músculo esquelético;
- ✓ Composição corporal;
- ✓ Indicadores gerais de saúde;
- ✓ Massa dos órgãos relativizados pelo peso corpóreo;
- ✓ Concentrações de aminoácidos plasmáticos;

Capítulo 2

Preparado para *Medicine & Science in Sports & Exercise*

Medicine & Science in Sports & Exercise

Leucine-supplemented dietary whey protein. Dose-response effect on diaphragm mTOR of sedentary and trained rats

Short title: Dietary leucine on diaphragm anabolism

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ABSTRACT

Purpose. The milk-whey proteins are rich sources of L-leucine and affect mTOR-mediated skeletal muscle protein synthesis. Considering that the diaphragm has unique metabolic characteristics as a skeletal muscle, we wished to investigate the dose-response effect of chronic supplementation of the milk-whey proteins (WP) with L-leucine on this muscle's anabolic pathway proteins mTOR and p70S6K in the sedentary and exercised Wistar rats. **Methods**. Ninety-six weanling male Wistar rats were divided into eight groups and fed for 30 days diets containing either casein or WP, and increasing levels (0, 3, 4.5, 6% of diet) of L-leucine. A parallel set of eight groups was exercised for comparison. Serum uric acid, creatinin, glucose, AST, ALT, CK, LDH and cholesterol, were determined by standard methods, and mTOR and p70S6K, by Westernblot analysis. **Results**. The chronic leucine supplementation was capable of increasing both mTOR and p70S6K in a dose-dependent fashion in the diaphragm, independent of the type of dietary protein. Contrary to the normal reaction in other skeletal muscles, in the normal rat, these changes did not produce any significant increase in either diaphragm or protein mass. The plasma health biomarkers were not significantly altered even at the highest leucine supplementation. The higher doses of supplemental leucine reduced food intake and growth, followed by alterations in the plasma concentrations of branched-chain amino acids. **Conclusion**. Although

supplemental leucine showed the potential of increasing the anabolic pathway key proteins in the diaphragm, of casein- and whey-protein-fed normal rats, this did not result in muscle mass accretion, different from what could be expected for classical skeletal muscles, which was consistent with its unique metabolism and physiology.

Keywords: mTOR, leucine, exercise, diaphragm, amino acid supplementation, whey protein

INTRODUCTION

The diaphragm is a unique skeletal muscle that works uninterruptedly for the entire lifetime of lunged vertebrates, unlike other skeletal muscles (23). In spite of that, the effects of exercise on the diaphragm have been little explored in comparison with the voluntary skeletal muscles or even the myocardium. The presence of both slow and fast contracting fibers, two types of innervations, both voluntary and involuntary, and being responsible for its vital contracting-relaxing activity, are characteristics that fully justify the special histological and metabolic properties of this muscle. Functional insufficiency of the diaphragm can result from such serious conditions as amyotrophic lateral sclerosis (ALS), surgeries and the use of controlled mechanical ventilation (CMV), invariably related to the offset of the equilibrium between protein synthesis and degradation (27).

Leucine is a branched-chain amino acid which has been the subject of much investigation and is known to stimulate cellular protein synthesis (2, 10), which has been reported to fail only under such conditions as sepsis, alcohol intoxication and high infusions of glucocorticoids in old rats (4). The principal effects of protein synthesis activation by leucine, and leucine supplementation, have been described in skeletal muscle (2), but little attention has been given to the effects of this type of supplementation on the diaphragm.

It is well known that high quality dietary proteins can stimulate protein synthesis at higher rates than lower quality proteins (37). The milk-whey proteins (WP) possess high nutritive value and high speed of digestion and absorption, thus providing a fast and profuse supply of amino acids and small peptides with significant impact on the turnover of body proteins. For this reason, the milk whey proteins have been recognized to promote a positive metabolic balance, being classified by some researchers as proteins of rapid metabolism (7, 12), making them therefore attractive for situations of metabolic stress, including that caused by physical exercise (22), muscle mass loss (1), neurological disorders (28), and immune deficiencies, among others (20). It is pertinent to note that the concentrations of BCAAs (branched-chain amino acids) in the whey proteins, including leucine, are high. Altogether, BCAAs account for 21.2% of the amino acids that make up the milk whey proteins, or exactly 50% of the total of indispensable amino acids therein, a feature that is shared with few other proteins besides the skeletal muscle proteins (13).

Considering the unique characteristics of the diaphragm, the effect that exercise has on the mass (31) and on the improvement of this muscle's functionality (32, 34), besides the lack of studies on the possible effects of a key metabolic amino acid as leucine, or the milk whey proteins could have on the diaphragm, the objective of this work was therefore to determine the anabolic effects of three levels (3.0, 4.5, 6.0%) of long-term supplementation of the leucine-

rich milk whey proteins by monitoring the concentrations of mTOR and p70S6K, in both the phosphorylated and unphosphorylated forms in the diaphragm of trained and sedentary Wistar rats.

METHODS

Male Wistar (21-day old, specific-pathogen free; n = 96) rats, bred at the Multidisciplinary Center for Biological Research, University of Campinas, SP, Brazil, were housed (~22 °C, 55% RH, inverted 12-hour light cycle) in individual growth cages, with free access to commercial chow (Labina, Purina, Brazil) and water at all times, until they became 133,82 ± 5,6 g. The research methodology was approved by the Ethics Committee on Animal Experimentation (CEEA-UNICAMP, protocol 1835-1). Six animals were randomly assigned to each of sixteen groups according to the source of protein in the diet, leucine supplementation and regime of physical activity (Figure 1).

<Figure 1>

Training Protocol – The rats were selected in the treadmill beforehand considering as not fit those that stood for 30 seconds without departing from the baseline. Training began on the day the animals started to consume the experimental diets and lasted for 4 weeks following the progressive-resisted protocol adapted of Hohl et al (15).

Experimental diets – The experimental diets were isonitrogenous (approximately 17% protein, dry basis), isolipidic and isocaloric (approximately 360 kcal/100 g) and formulated following the recommendations of the American Institute of Nutrition, AIN93 (for growing rats) diet (26). The two diets differed among themselves with respect to the nature of the protein source, casein or milk-whey protein (Table 1).

<Table 1>

Protein extraction and immunoblotting – Total protein content of diaphragm muscle was determined by the Lowry method (19). For immunoblotting, tissue homogenates were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane, using a Wide Biocom Westernblot system (Bridge of Weir, UK). Blots were probed with the appropriate antibodies to mTOR total and phosphor-mTOR (Ser2448) (Cell signaling) and p70S6K total and phosphor-p70S6K (Thr 389) (Santa Cruz) to assess the levels of these proteins in diaphragm tissue, tubulin was the loading control (Santa Cruz). The appropriate secondary antibody conjugated to peroxidase and the BM chemiluminiscence blotting system were used for detection. The bands were visualized by chemiluminiscence (GE – ImageQuant LAS4000, Piscataway, NJ, USA). Band intensities were quantified by scanning and processing with the program ImageJ (v. 1.44 for Windows).

Biochemical parameters – Six hours after the training session, blood samples were collected in Vacutainers, kept at 4°C, and centrifuged at 3000xg (4°C, 12min) to obtain the serum. For the assessment of sera, uric acid, aspartate amino transferase (AST), alanine aminotransferase ALT, creatine kinase (CK) and lactate dehydrogenase (LDH), glucose and cholesterol standard enzymatic spectrophotometric determinations were performed employing Laborlab kits (São Paulo, Brazil).

Determination of plasma free amino acids – Serum free amino acids were extracted with methanol and derivatized with phenylisothiocyanate (PITC; White et al., 1986) and the PTH-derivatives chromatographed using a Luna C-18, 100 Á; 5 µ, 250 x 4.6 mm (00G-4252-EQ) column, at 50°C. Quantification was made by comparison with a standard mixture and DL-2-aminobutyric acid as an internal standard (Sigma-Aldrich Corp, St Louis, MO, USA). The free amino acids were extracted in 80% ethanol and 0.1M HCl, with 500 µL of 2-aminobutyric acid added as internal standard. The mixture was sonicated for 10 minutes and further homogenized for 1 hour, followed by centrifugation at 8500xg for 15 minutes. The supernatant was filtered through a 0.22 mm membrane and a 40 µL aliquot derivatized as described above for the injection of 20 µL into the liquid chromatograph.

Proximal composition – Moisture, total ash, protein, and lipids were determined according to Association of Official Analytical Chemists methods (5). The total carbohydrate content was inferred by difference. Protein efficiency ratio (PER) was calculated as the ratio of grams of protein feed per unit of wet weight gain protein fed.

Statistical analysis – The results were subjected to statistical analysis using the software SPSS (Statistical Package for the Social Sciences), version 17.0. The data were tested for normality (Kolmogorov-Smirnov test) and homogeneity using the tools available therein. For parametric data, the multivariate analysis of variance (ANOVA) was used and means were compared (Duncan test) adopting the value of $p \leq 0.05$ as a criterion for statistical significance.

RESULTS

The long-term, dose-dependent leucine supplementation showed no general trend on the diaphragm muscle mass with regard to the two dietary protein sources. It will be noted, however, that while supplementation resulted in slightly lower body mass gains, the mass of the diaphragms were maintained mostly unaltered, but showing a minor increase due to exercise only when the level of addition was 4.5% (Table 2).

<Table 2>

The long-term supplementation of normal rats with leucine produced in this experiment a general increase in mTOR and p70S6K proteins in the diaphragm, both phospohorylated and unphosphorylated (Figure 2). Comparing Figures 2A and 2B with 2C and 2D, it could be seen that the training also had a slight promoting effect in the production of the anabolic proteins, which was consistent with the increase in organ mass.

<Figure 2>

The consequences of supplementation on the activities of AST, ALT, CK and LDH enzymes, plus the serum levels of glucose, uric acid, creatinine and cholesterol were determined in order to further assess the impact of long term supplemental leucine (Table 3).

<Table 3>

The complete amino acid profiles (Table 4) of sera detected no clear changes, with the exception of alanine and the branched chain amino acids. With regard to leucine, commensurate increases were observed at every level of supplementation, except that the sedentary maintained levels of leucine 20% higher than the trained animals.

<Table 4>

DISCUSSION

No reports were found in the literature relating diaphragm and leucine, mTOR, or diaphragm and protein or muscle mass accretion. Considering that this skeletal muscle exhibits several distinct characteristics from the rest of skeletal muscles, it was remarkable to find that the dietary treatment resulted in concentration increases of both the unphosphorylated and phosphorylated biomarkers, but with no increase in muscle mass or protein content of the diaphragm, contrasting with the increases that have been observed in the gastrocnemius' protein content of and muscle mass (25).

The main mechanism by which leucine exerts its protein-synthesis stimulating function is by way of activating the mTOR, a key protein in the process of cell division (25). Once activated, the mTOR will in turn activate the 4E-BP1 and p70S6K proteins, thus potentiating the cell capacity to synthesize proteins (16,24). Besides the anabolic effect gained through the activation of mTOR, leucine also stimulates insulin secretion (9,11), thereby giving a further boost to anabolism. There is also evidence that supplementation with leucine for prolonged periods of time may have an anticatabolic effect in skeletal muscle (30).

The response of mTOR and p70S6K (Figure 2) was consistent with leucine supplementation studies in other skeletal muscles (8,38). Although no studies in diaphragm are available, our dose-dependent results suggest that the different

degrees of supplementation were well tolerated and that the animals responded by increasing the concentration of anabolic proteins. Since mechanically ventilated patients are potentially vulnerable to ventilator-induced diaphragmatic dysfunction (33), like diaphragmatic atrophy (36), it is likely that proper stimulation with leucine of the diaphragm anabolic pathways be useful in treating CMV-induced atrophy. Atrophy can develop rapidly, as early as 12 h after the initiation of CMV (21), and is particularly pronounced in the diaphragm, which commences the atrophy process ahead of peripheral skeletal muscles (36).

The stimulatory effect of leucine on protein synthesis does not appear to be a simple one. Anthony et al. (3) showed that oral administration of leucine to rats resulted in the stimulation of protein synthesis, even when the blood concentration of insulin was maintained sufficiently low by the concomitant infusion of somatostatin. For this reason, it was suggested by these authors that although leucine itself can stimulate the release of insulin, it is likely that a substantial part of its anabolic effect on protein synthesis may proceed directly via leucine, and not indirectly via insulin action.

In general, the supplementation of both casein and the leucine-rich whey proteins with leucine had some impact on the body mass gain, resulting in a greater mass accretion of the sedentary animals, compared to the trained cohorts, and reaching a maximum at the 4.5% level. This effect was reversed when the

leucine addition reached 6%, associated with a reduction in food intake (Table 2), and in accord with the observation made by Laviano et al. (18) and Blouet et al. (6) that high levels of dietary leucine may inhibit food intake.

No significant alterations of the general health parameters analyzed were observed with regard to chronic exercise. Neither were the muscle damage markers, CK and LDH, elevated by any of the levels of leucine supplementation tested, finding that was in agreement with those of other workers for peripheral skeletal muscles (17, 29).

The results of complete amino acid profiles (Table 4) were coherent with the results of Hambraeus et al. (14). The decreasing trend in concentration observed for both valine and isoleucine, as a function of leucine concentration in the diet, was expected according to the competitive absorption characteristics that exists among these amino acids. Finally, considering that the concentrations of BCAAs, including leucine, are higher in the whey proteins than in casein, it was a sign of consistency to encounter higher levels of the anabolic proteins when the dietary protein given was WP, as seen in most of the profiles shown in Figure 2.

Taken together, these data suggest that chronic leucine supplementation of normal Wistar rats will stimulate the protein anabolic pathways in the diaphragm, but in a unique fashion; different to what be expected from a typical skeletal muscle. At the present time, however, it is not possible to ascertain why the

increase in these biomarker proteins resulted in no net mass or protein content gain for the diaphragm, especially considering that the combination of training and leucine supplementation at the level of 4.5% did. The fact that supplemental leucine was translated in greater levels of mTOR and p70S6K, both in the phospohorylated and unphosphorylated forms, but not into the accretion of muscle protein or diaphragm mass, is an intriguing issue that should merit investigation.

Although the highest doses of supplemental leucine elicited lower food intakes and lower body mass gains as compared to the controls, followed by some alterations in the plasma concentrations of BCAAs, the plasma health biomarkers AST, ALT, uric acid, CK, LDH, glucose and cholesterol, were not significantly altered.

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CONFLICT OF INTEREST

The authors declare not to exist any conflicts of interest of any type, regarding the conception, objectives or materials used in this investigation.

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FIGURES LEGENDS

Figure 1 (legend) – Distribution of groups. The AIN93-G WP diet was made by substituting the whey protein for the casein of the AIN93 (standard) diet. The Controls contained no added leucine.

Figure 2 (legend) – mTOR, phosphorylated mTOR(S2448), p70S6K and phosphorylated p70S6K(Y389) responses to the three degrees of supplementation with leucine (3, 4.5 and 6%) in both the sedentary (A,B, E and F) and trained (C, D, G and H) groups, in rats fed either casein (CAS ■) or milk-whey protein (WP □). Different letters denote significant differences between groups (columns) ($p \leq 0.05$).

Figure 1

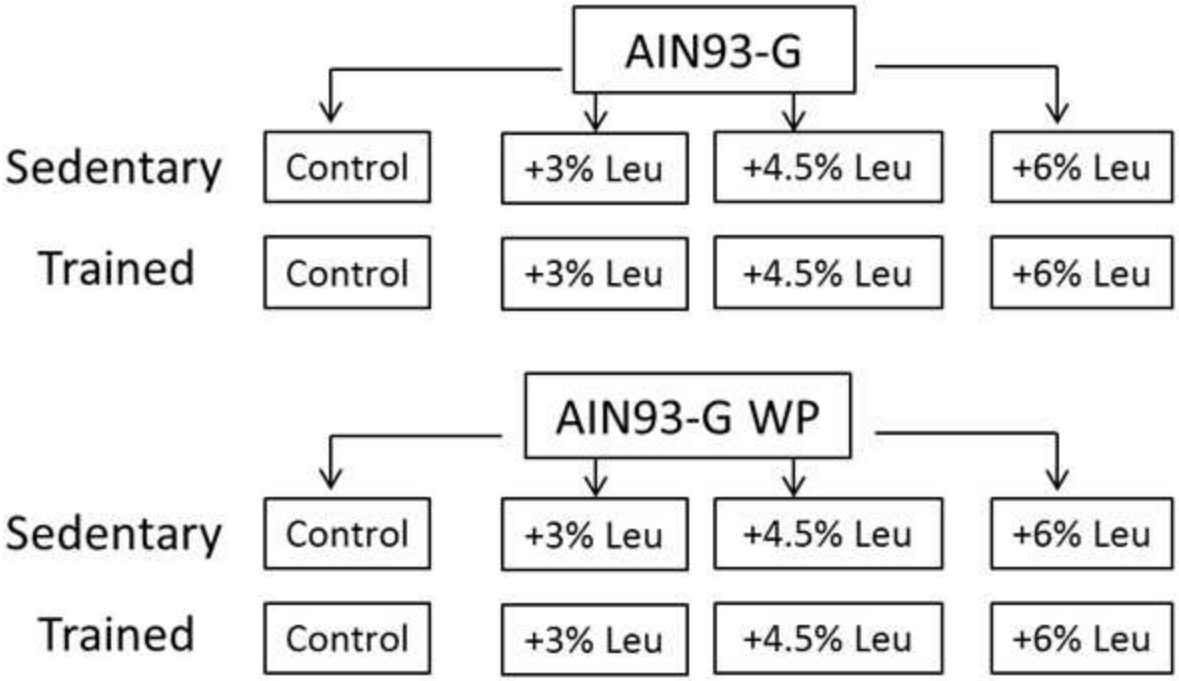

AIN93-G
Sedentary
Control
+3% Leu
+4.5% Leu
+6% Leu
Trained
Control
+3% Leu
+4.5% Leu
+6% Leu
AIN93-G WP
Sedentary
Control
+3% Leu
+4.5% Leu
+6% Leu
Trained
Control
+3% Leu
+4.5% Leu
+6% Leu

Figure 2

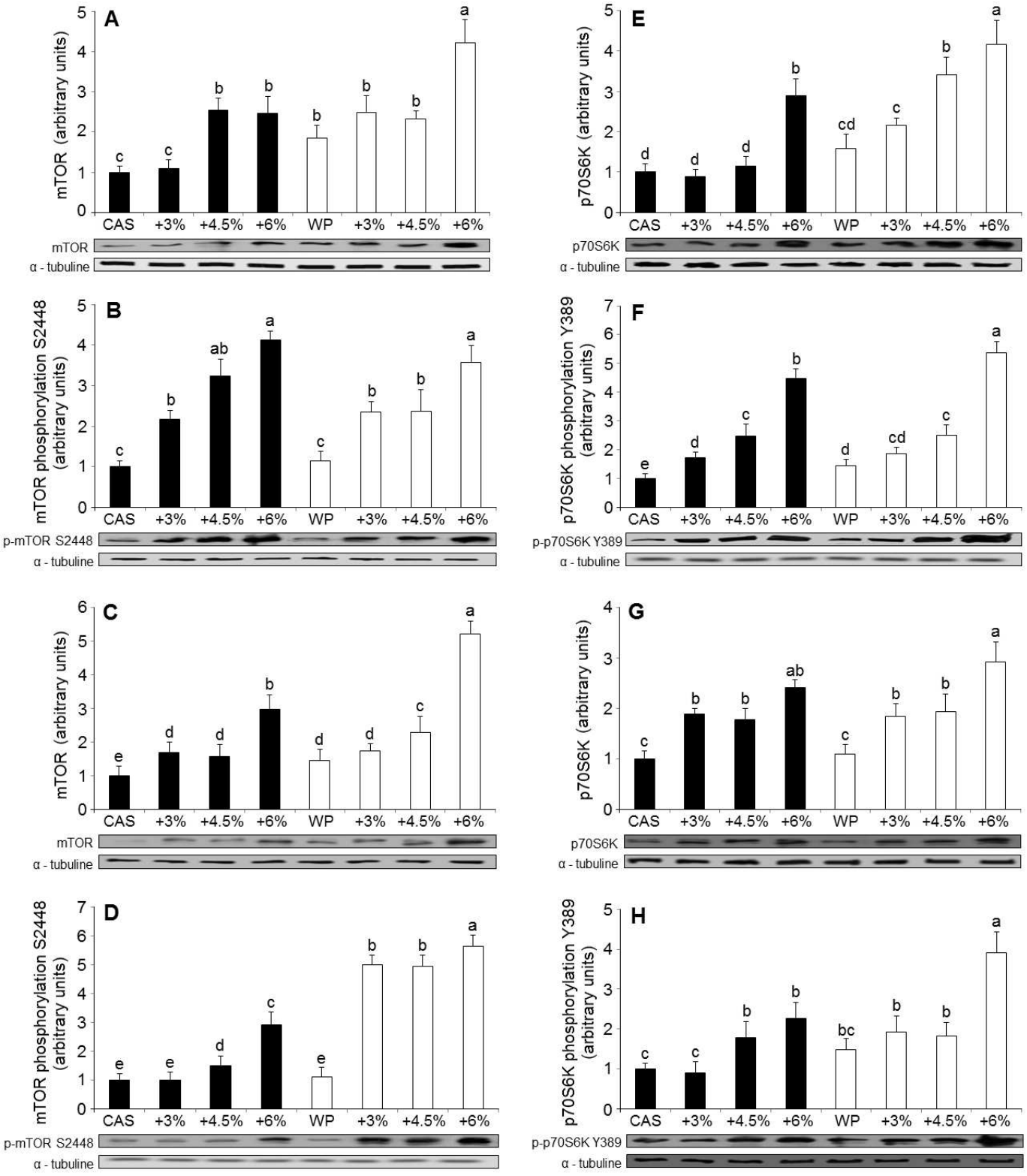

A
mTOR (arbitrary units)
CAS +3% +4.5% +6% WP +3% +4.5% +6%
mTOR
α - tubuline
B
mTOR phosphorylation S2448 (arbitrary units)
p-mTOR S2448
C
D
E
p70S6K (arbitrary units)
p70S6K
F
p70S6K phosphorylation Y389 (arbitrary units)
p-p70S6K Y389
G
H

Table 1 – Composition of the diets (g/kg diet)

Ingredient	AIN93-G (Casein) +				AIN93-G (WP) +			
	Control	3%L	4.5%L	6%L	Control	3%L	4.5%L	6%L
L-leucine added	-	30	45	60	-	30	45	60
Corn starch	403.4	373.4	358.4	343.4	407.3	377.3	362.3	347.3
Casein (87.6% protein)	194.1	194.1	194.1	194.1	-	-	-	-
WP (89.4% protein)	-	-	-	-	190.2	190.2	190.2	190.2
Dextrinized corn starch	132	132	132	132	132	132	132	132
Sucrose	100	100	100	100	100	100	100	100
Soybean oil	70	70	70	70	70	70	70	70
Fiber	50	50	50	50	50	50	50	50
Mineral mix	35	35	35	35	35	35	35	35
Vitamin mix	10	10	10	10	10	10	10	10
L-Cystine	3	3	3	3	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Tert-butylhydroquinone	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014

Table 2 – Dietary intake and anthropometric parameters for each diet and level of activity, as a function of L-leucine addition. Different letters denote significant differences between diets, within each activity group (columns) ($p \leq 0.05$).

		AIN93 CAS +								AIN93 WP +							
		Sedentary				Trained				Sedentary				Trained			
		0% L	3%L	4.5%L	6%L	0% L	3%L	4.5%L	6%L	0% L	3%L	4.5%L	6%L	0% L	3%L	4.5%L	6%L
Diet intake[1]	M	16.55^{a}	16.89^{a}	17.80^{a}	14.90^{b}	16.65^{a}	16.29^{a}	15.80^{ab}	15.40^{b}	16.00^{ab}	16.40^{a}	17.33^{a}	15.40^{b}	17.10^{a}	17.35^{a}	17.3^{a}	17.2^{a}
	SEM	0.3	0.48	0.35	0.3	0.23	0.32	0.25	0.17	0.11	0.16	0.11	0.17	0.14	0.17	0.13	0.34
Protein intake[1]	M	2.81	2.87	3.03	2.53	2.83	2.77	2.62	2.69	2.72	2.79	2.95	2.62	2.91	2.89	2.95	2.92
	SEM	1.42	2.27	1.67	1.41	1.09	1.5	1.18	0.81	0.52	0.78	0.55	0.83	0.65	0.81	0.62	1.61
PER[2]	M	1.89^{b}	1.99^{ab}	2.06^{ab}	2.08^{a}	1.74^{c}	1.77^{bc}	1.6^{d}	1.7^{cd}	1.73^{cd}	1.81^{bc}	2.03^{ab}	2.02^{ab}	1.66^{d}	1.75^{c}	1.78^{c}	1.81^{bc}
	SEM	0.08	0.13	0.06	0.06	0.06	0.12	0.1	0.11	0.17	0.09	0.14	0.07	0.1	0.04	0.09	0.12
Body mass gain[4]	M	207.5^{b}	222.2^{ab}	239.3^{a}	207.9^{b}	207.5^{b}	205.7^{b}	196.2^{b}	194.8^{b}	197.9^{b}	212.3^{ab}	233.2^{a}	209.9^{ab}	199.6^{b}	206.3^{b}	211.5^{ab}	219.3^{ab}
	SEM	8.6	11.9	7.5	7.5	6.9	11.5	10.9	9.9	12.9	9.9	9.7	6.2	11.8	7.6	5.1	8.5
L-leucine intake[1]	M	0.04^{f}	0.55^{e}	0.84^{c}	0.93^{b}	0.04^{f}	0.53^{e}	0.73^{d}	0.98^{b}	0.05^{f}	0.55^{e}	0.84^{c}	0.98^{b}	0.06^{f}	0.57^{e}	0.84^{c}	1.09^{a}
	SEM	0	0.02	0.02	0.02	0	0.01	0.01	0.01	0	0.01	0.01	0.01	0	0.01	0.01	0.03
Diaphragm mass[3]	M	0.54^{b}	0.58^{b}	0.54^{b}	0.54^{b}	0.65^{ab}	0.69^{ab}	0.75^{a}	0.67^{ab}	0.53^{b}	0.52^{b}	0.51^{b}	0.56^{b}	0.66^{ab}	0.69^{ab}	0.73^{a}	0.69^{ab}
	SEM	0.06	0.05	0.06	0.06	0.08	0.08	0.08	0.08	0.06	0.06	0.06	0.06	0.08	0.08	0.08	0.08
Diaphragm protein[4]	M	21.6	22.1	22.7	23.5	22.6	23.0	21.0	21.4	20.7	21.3	21.8	22.4	21.7	22.2	20.5	20.8
	SEM	0.71	0.69	0.54	0.60	0.27	0.33	0.42	0.44	0.36	0.26	0.63	0.67	0.60	0.64	0.50	0.47
Liver Mass[3]	M	9.15	9.14	9.23	9.02	9.27	9.31	9.08	9.23	9.19	9.27	9.21	9.26	9.28	9.02	9.19	9.27
	SEM	0.1	0.1	0.06	0.09	0.11	0.11	0.08	0.1	0.11	0.09	0.1	0.1	0.06	0.06	0.07	0.12

1 – grams / day
2 – Protein efficiency ratio
3 – in grams
4 – in percentage

Table 3 - Biochemical blood parameters. Different letters denote significant differences between diets, within each activity group (columns) ($p \leq 0.05$).

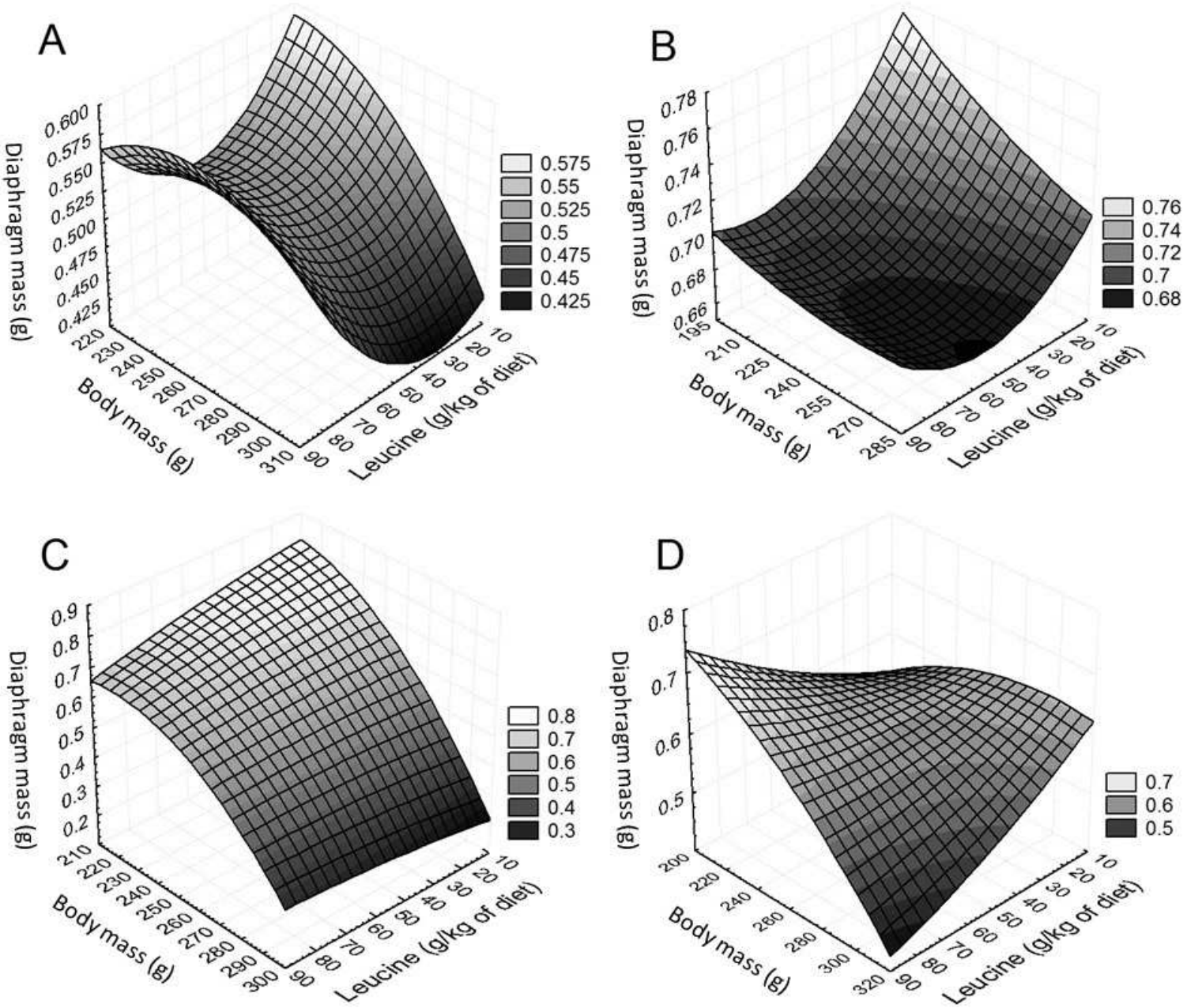
		AIN93 CAS +								AIN93 WP +							
		Sedentary				Training				Sedentary				Training			
		0% L	3%L	4.5%L	6%L	0% L	3%L	4.5%L	6%L	0% L	3%L	4.5%L	6%L	0% L	3%L	4.5%L	6%L
ALT[1]	Mean	17.3^{c}	27.5^{a}	21.9^{abc}	21.6^{bc}	25.2^{ab}	19.4^{bc}	22.2^{abc}	22.5^{abc}	22.3^{abc}	20.8^{bc}	20.8^{bc}	21.6^{bc}	24.6^{ab}	21.7^{bc}	22.0^{abc}	22.2^{abc}
	SEM	3.6	3.3	1.1	1.1	1.6	1.6	2.1	0.8	1.4	1.1	1.1	1.0	1.5	1.1	1.3	1.2
AST[1]	Mean	88.1^{abc}	97.1^{ab}	91.7^{abc}	92.2^{abc}	97.7^{ab}	94.9^{ab}	91.0^{abc}	99.4^{a}	86.2^{bc}	90.7^{abc}	80.9^{c}	91.9^{abc}	92.4^{abc}	90.0^{abc}	89.4^{abc}	96.9^{ab}
	SEM	2.1	2.9	3.9	3.3	3.5	4.0	5.9	3.4	2.4	2.0	4.7	2.3	3.9	5.0	2.4	6.0
Glucose[2]	Mean	141.2	142.0	151.3	158.8	134.0	132.5	142.5	138.7	124.8	142.3	144.0	149.7	141.7	146.2	150.2	143.8
	SEM	6.5	4.1	5.3	2.2	3.8	3.4	6.0	5.2	3.2	6.4	6.3	7.7	7.2	3.7	5.1	6.5
Uric acid[2]	Mean	1.0^{abc}	1.0^{abc}	1.2^{ab}	1.1^{ab}	0.6^{c}	1.2^{ab}	1.0^{abc}	0.8^{bc}	1.2^{ab}	1.1^{abc}	1.0^{abc}	1.0^{abc}	1.0^{abc}	1.1^{abc}	1.4^{a}	0.9^{abc}
	SEM	0.1	0.2	0.1	0.2	0.1	0.2	0.2	0.1	0.1	0.1	0.2	0.2	0.1	0.2	0.2	0.1
Creatinine[2]	Mean	0.6^{ab}	0.7^{ab}	0.5^{ab}	0.5^{ab}	0.4^{ab}	0.5^{ab}	0.3^{b}	0.5^{ab}	0.6^{ab}	0.5^{ab}	0.8^{a}	0.7^{ab}	0.5^{ab}	0.5^{ab}	0.6^{ab}	0.4^{ab}
	SEM	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Cholesterol[2]	Mean	59.3^{ab}	58.4^{ab}	65.6^{a}	62.4^{ab}	63.2^{ab}	59.9^{ab}	60.1^{ab}	58.5^{ab}	56.6^{ab}	63.0^{ab}	58.5^{ab}	61.8^{ab}	58.0^{ab}	59.1^{ab}	55.4^{b}	61.7^{ab}
	SEM	3.3	2.7	2.5	3.0	2.1	4.2	2.6	3.2	2.4	1.9	3.9	2.1	3.6	2.2	1.7	2.9

1 – U/L
2 – mg/DL
3 – ng/mL

Table 4 – Amino acids in plasma. Different letters denote significant differences between diets, within each activity group (columns) ($p \leq 0.05$).

	AIN93 CAS +								AIN93 WP +							
	Sedentary				Trained				Sedentary				Trained			
	0% L	**3%L**	4.5%L	6%L	0% L	3%L	4.5%L	6%L	0% L	3%L	4.5%L	6%L	0% L	3%L	4.5%L	6%L
ASP	0.023	0.019	0.026	0.021	0.018	0.022	0.018	0.030*	0.021	0.022	0.023	0.019	0.020	0.023	0.020	0.031*
GLU	0.129^{d}	0.206^{ab}	0.259^{a}	0.286^{a}	0.204^{b}	0.254^{ab}	0.212^{b}	0.282^{a}	0.241^{ab}	0.227^{ab}	0.225^{ab}	0.270^{a}	0.186^{c}	0.252^{b}	0.253^{b}	0.258^{b}
H-Pro	0.032	0.038	0.041	0.041	0.037	0.037	0.040	0.050	0.041	0.040	0.039	0.040	0.038	0.034	0.033	0.040
ASN	0.105	0.096	0.129	0.117	0.107	0.104	0.090	0.119	0.145	0.136	0.114	0.089	0.109	0.131	0.106	0.134
SER	0.343	0.276	0.266	0.310	0.246	0.302	0.358	0.413*	0.346	0.332	0.323	0.282	0.266	0.362	0.274	0.291
GLN	0.518	0.517	0.596	0.600	0.584	0.665	0.693	0.79*	0.610	0.574	0.618	0.631	0.715	0.803*	0.792*	0.757*
GLY	0.154	0.105	0.131	0.106	0.158	0.106	0.179	0.124	0.133	0.125	0.113	0.090	0.107	0.098	0.105	0.095
HIS	0.139	0.081	0.097	0.102	0.106	0.099	0.109	0.082	0.085	0.101	0.088	0.103	0.085	0.062	0.076	0.089
ARG	0.227	0.208	0.288	0.226	0.245	0.272	0.269	0.301	0.242	0.351	0.249	0.206	0.199	0.242	0.347	0.272
THR	0.250^{d}	0.232^{d}	0.238^{d}	0.296^{d}	0.332^{d}	0.251^{d}	0.273^{d}	0.759^{a}	0.207^{d}	0.194^{d}	0.243^{d}	0.262^{d}	0.432^{c}	0.640^{b}	0.533^{bc}	0.556^{b}
ALA	0.437^{c}	0.432^{c}	0.467^{c}	0.643^{b}	0.365^{d}	0.503^{c}	0.527^{c}	0.720^{a}	0.463^{c}	0.492^{c}	0.569^{c}	0.796^{a}	0.512^{c}	0.575^{c}	0.521^{c}	0.856^{a}
PRO	0.290	0.218	0.389	0.188	0.202	0.337	0.265	0.248	0.265	0.287	0.317	0.355	0.219	0.275	0.191	0.256
TYR	0.202	0.128	0.179	0.119	0.138	0.137	0.120	0.083	0.091	0.158	0.132	0.130	0.085	0.083	0.069	0.103
MET	0.148	0.058	0.082	0.049	0.080	0.078	0.080	0.067	0.069	0.077	0.067	0.097	0.058	0.053	0.074	0.068
CIS	0.112	0.145	0.152	0.148	0.150	0.153	0.148	0.160	0.156	0.160	0.152	0.153	0.162	0.151	0.152	0.163
VAL	0.209^{a}	0.138^{b}	0.121^{c}	0.091^{c}	0.196^{a}	0.151^{b}	0.141^{b}	0.082^{c}	0.180^{a}	0.159^{b}	0.115^{c}	0.071^{c}	0.111^{b}	0.071^{c}	0.061^{c}	0.052^{c}
ILE	0.079^{a}	0.061^{b}	0.030^{c}	0.024^{c}	0.096^{a}	0.073^{b}	0.072^{b}	0.024^{c}	0.090^{a}	0.080^{a}	0.036^{c}	0.020^{c}	0.087^{a}	0.075^{b}	0.064^{b}	0.052^{b}
LEU	0.108^{e}	0.270^{d}	0.449^{b}	0.618^{a}	0.087^{e}	0.224^{d}	0.361^{c}	0.488^{b}	0.150^{e}	0.343^{d}	0.454^{b}	0.595^{a}	0.098^{e}	0.322^{d}	0.408^{c}	0.468^{b}
PHE	0.129	0.091	0.107	0.095	0.121	0.079	0.117	0.082	0.074	0.089	0.095	0.107	0.077	0.070	0.066	0.073
TRP	0.319	0.280	0.316	0.313	0.354	0.264	0.293	0.362	0.378	0.363	0.302	0.255	0.344	0.359	0.281	0.377
LYS	0.565	0.359	0.453	0.644	0.575	0.505	0.529	0.456	0.505	0.506	0.374	0.400	0.387	0.392	0.399	0.475
TOTAL	4.468	3.817	5.069	4.740	4.831	4.680	4.768	5.423	5.498	5.320	4.982	4.787	4.534	5.439	4.688	5.313

Supplementary data – Response surface of the diaphragm mass and body mass to total dose of L-leucine (g/kg) in diet. Dependent variables are over-all means. a)Sedentary; b)Trained; c) Casein; d) Whey Protein.



Capítulo 3

Preparado para *Laboratory Investigation*

L-leucine supplemented whey protein. Dose-response effect on heart mTOR activation of sedentary and trained rats

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ABSTRACT

Purpose. The milk-whey proteins are rich sources of L-L-leucine and affect mTOR-mediated skeletal muscle protein synthesis. We wished to investigate the dose-response effect of chronic supplementation of the milk-whey proteins (WP) with L-L-leucine on the anabolic pathway proteins mTOR and p70S6K in the heart of sedentary and exercised Wistar rats. **Methods**. Ninety-six weanling male Wistar rats were divided into eight groups and fed for 30 days diets containing either casein or WP, and increasing levels (0, 3, 4.5, 6% of diet) of L-L-leucine. A parallel set of eight groups was exercised for comparison. Serum uric acid, creatinin, glucose, AST, ALT, CK, LDH and cholesterol, were determined by standard methods, and mTOR and p70S6K, by Westernblot analysis. **Results**. The chronic L-leucine supplementation was capable of increasing both mTOR and p70S6K in a dose-dependent fashion in the heart, independent of the type of dietary protein. The plasma health biomarkers were not significantly altered even at the highest L-leucine supplementation. The higher doses of supplemental L-leucine reduced food intake and growth, followed by alterations in the plasma concentrations of branched-chain amino acids. **Conclusion**. Although supplemental L-leucine has the potential of increasing the anabolic pathway key proteins in the heart, of casein- and whey-protein-fed.

Keywords: mTOR, L-leucine, exercise, heart, amino acid supplementation, whey protein

INTRODUCTION

Cardiac hypertrophy involves increased heart size due to increased cardiomyocyte size. Initially, it is an adaptive response to increased workload or to defects in the efficiency of the contractile machinery. However, in the longer term it contributes to the development of heart failure and sudden death (Proud, 2004). Increased protein synthesis is a key feature of cardiac hypertrophy and likely underlies the increased cell and organ size observed under this condition (Hannan et al., 2003). Several other features are also seen, including changes in gene expression and reorganization of the contractile machinery, and it should be borne in mind that the protein accumulation that is characteristic of hypertrophy may reflect effects on protein breakdown as well as protein synthesis.

L-L-leucine is a branched-chain amino acid that has been the subject of much investigation and considered as capable of stimulating cellular protein synthesis (Anthony & Anthony 2001; Drummond & Rasmussen, 2008). Notwithstanding, its effectiveness has been reported to fail under such situations as sepsis, alcohol intoxication and high (excess) glucocorticoids in old rats (Anthony and Anthony, 2008). The attenuation of in-vivo skeletal muscle proteolysis by L-leucine has been also described but is, however, less documented than for protein synthesis (Zanchi et al., 2008). Anthony et al. (1999) that L-leucine increased skeletal muscle protein synthesis when given alone in rats following exercise.

The main mechanism by which L-leucine exerts its protein synthesis stimulating function is by way of activating the mTOR, a key protein in the process of cell proliferation (Proud, 2007). Once activated the mTOR phosphorylates and activates the 4E-BP1 and the S6K1, thus increasing the cell capacity to synthesize proteins (Proud, 2002; Kimball & Jeffson, 2004). Besides the anabolic effect gained through the activation of mTOR, L-leucine also stimulates insulin secretion (Bolster, 2003), thereby giving a further boost to anabolism. There is also evidence that supplementation with L-leucine for prolonged periods of time may have an anticatabolic effect in skeletal muscle (Sugawara et al., 2007).

The principal effects of protein synthesis activation by L-leucine have been described in skeletal muscle and its characteristics are maintained even in animals undergoing training (Anthony, 2001). In spite of this, little attention has been given to the effects of the supplementation on the cardiac muscle.

It is well known that high quality proteins can stimulate protein synthesis at higher rates than lower quality proteins can (Yoshizawa et al., 1999). The milk whey proteins possess high nutritive quality and high speed of digestion and absorption, thus providing a fast and profuse supply of amino acids and small peptides with significant impact on the turnover of body proteins. For this reason, the milk whey proteins have been recognized to promote a positive metabolic balance, being classified by some researchers as proteins of rapid metabolism

(Boire et al., 1997; Fruhbeck, 1998), making them attractive for situations of metabolic stress, including that caused by physical exercise (Nery-Diez, 2010), muscle mass loss (Agin et al., 2001) neurological disoreders (Silva et al., 2010) and immune deficiencies, among others (Marshall, 2004). It may be relevant to note that the concentrations of BCAA in the whey proteins, including L-leucine, are high. Altogether, BCAAs account for 21.2% of the amino acids that make up the milk whey proteins, or exactly 50% of the total of indispensable amino acids therein, a feature that is shared with few other proteins besides the skeletal muscle proteins (Etzel, 2004; Ha and Zamel, 2003). Hulmi et al. (2010) also reported that chronic (21 weeks) supplementation with whey proteins favored resistance training-induced muscle hypertrophy in young healthy individuals compared to a nonenergetic placebo. These data suggest that chronic 'high-quality protein' supplementation is able to improve exercise-induced muscle mass gain and performance.

Elucidating the signaling connect ions underlying cardiac hypertrophy is important for both our fundamental understanding of the process and developing potential therapeutic strategies for this condition, which is a major risk factor for cardiac failure. This is a vigorous research area and several signaling pathways have been implicated in hypertrophic responses in cardiomyocytes (Frey and Olson 2003; Molkentin and Dorn, 2001)

It is clear the effect of combination of L-leucine plus whey protein, in skeletal muscle, hypertrophy, but not in heart, the objective of this work therefore was to determine the metabolic effects of long-term supplementing the L-L-leucine-rich milk whey proteins with three steps of L-L-leucine (3.0, 4.5, 6.0%) on cell activation via phosphorylation of the m-TOR / S6K1 and 4EBP1 protein synthesis pathway.

METHODS

Male Wistar (21-day old, specific-pathogen free; n = 96) rats, bred at the Multidisciplinary Center for Biological Research, University of Campinas, SP, Brazil, were housed (~22 °C, 55% RH, inverted 12-hour light cycle) in individual growth cages, with free access to commercial chow (Labina, Purina, Brazil) and water at all times, until they became 133,82 ± 5,6 g. The research methodology was approved by the Ethics Committee on Animal Experimentation (CEEA-UNICAMP, protocol 1835-1). The animals were randomly assigned to either of sixteen groups according to the source of protein in the diet, L-leucine supplementation and whether they were exercise or sedentary (Figure 1).

<Figure 1>

Training Protocol – The rats were selected in the treadmill beforehand considering as not fit those that stood for 30 seconds without departing from the baseline. The animals began training on the day they started to consume the

experimental diets and lasted for 4 weeks following the progressive-resisted protocol adapted of Hohl et al (2009).

Experimental diets **–** The experimental diets were isonitrogenous (approximately 17% protein, dry basis), isolipidic and isocaloric (approximately 360 kcal/100 g) and formulated following the recommendations of the American Institute of Nutrition, AIN93 (for growing rats) diet (Reeves et al., 1993). The two diets differed among themselves with respect to the nature of the protein source, casein or milk-whey protein (Table 1).

<Table 1>

Protein extraction and immunoblotting **–** Total protein content of diaphragm muscle was determined by the Lowry method (1951). For immunoblotting, tissue homogenates were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane, using a Wide Biocom Westernblot system (Bridge of Weir, UK). Blots were probed with the appropriate antibodies to mTOR total and phosphor-mTOR (Ser2448) (Cell signaling) and p70S6K total and phosphor-p70S6K (Thr 389) (Santa Cruz) to assess the levels of these proteins in diaphragm tissue, tubulin was the loading control (Santa Cruz). The appropriate secondary antibody conjugated to peroxidase and the BM chemiluminiscence blotting system were used for detection. The bands were visualized by chemiluminiscence (GE – ImageQuant LAS4000, Piscataway, NJ, USA). Band

intensities were quantified by scanning and processing with the program ImageJ (v. 1.44 for Windows).

Biochemical parameters – Six hours after the training session, blood samples were collected in Vacutainers, kept at 4°C, and centrifuged at 3000xg (4°C, 12min) to obtain the serum. For the assessment of sera, uric acid, aspartate amino transferase (AST), alanine aminotransferase ALT, creatine kinase (CK) and lactate dehydrogenase (LDH), glucose and cholesterol standard enzymatic spectrophotometric determinations were performed employing Laborlab kits (São Paulo, Brazil).

Determination of plasma free amino acids – Serum free amino acids were extracted with methanol and derivatized with phenylisothiocyanate (White et al., 1986) and the PTH-derivatives chromatographed using a Luna C-18, 100 Å; 5 µ, 250 x 4.6 mm (00G-4252-EQ) column, at 50°C. Quantification was made by comparison with a standard mixture and DL-2-aminobutyric acid as an internal standard (Sigma-Aldrich Corp, St Louis, MO, USA). The free amino acids were extracted in 80% ethanol and 0.1M HCl, with 500 µL of 2-aminobutyric acid added as internal standard. The mixture was sonicated for 10 minutes and further homogenized for 1 hour, followed by centrifugation at 8500xg for 15 minutes. The supernatant was filtered through a 0.22 mm membrane and a 40 µL aliquot derivatized as described above for the injection of 20 µL into the liquid chromatograph.

Proximal composition – Moisture, total ash, protein, and lipids were determined according to AOAC methods (2002). The total carbohydrate content was inferred by difference. Protein efficiency ratio (PER) was calculated as the ratio of grams of protein feed per unit of wet weight gain protein fed.

Statistical analysis – The results were subjected to statistical analysis using the software SPSS (Statistical Package for the Social Sciences), version 17.0. The data were tested for normality (Kolmogorov-Smirnov test) and homogeneity using the tools available therein. For parametric data, the multivariate analysis of variance (ANOVA) was used and means were compared (Duncan test) adopting the value of $p \leq 0.05$ as a criterion for statistical significance.

RESULTS AND DISCUSSION

In general, supplementing both casein and the L-L-leucine-rich whey protein with L-L-leucine promoted the expression of mTOR in the heart muscle, at the three levels used in the experiment (Figure 2). Also, the degree of response was different depending on whether the protein was casein or the whey proteins, and whether the animal belonged to a sedentary or to a trained group.

Supplementation of the Casein-trained group (Figure 2B) with 3% L-L-leucine, for instance, resulted in a 42% increment in mTOR, whereas the 4.5% level resulted in the highest (four-fold) increment of expression. At the 6% level, however, this increment decreased to a level that was still above the basal level.

Compared to the trained, the Casein-sedentary mTOR followed a similar pattern, but of a lower magnitude than the former (Figure 2A), suggesting that training exerted an additional effect on supplementation. Similarly, it was also noted that the whey protein was less susceptible to the stimulus by L-L-leucine than was casein. This outcome was expected considering that the whey proteins have a higher content of L-L-leucine.

With regard to the stimulatory effect of supplementation on the expression of the phosphorylated form of mTOR (S2448), it was observed that regardless of the type of protein, the effect had a tendency to peak with the addition of 4.5% only in the sedentary groups, whereas in the trained groups, the effect increased with every successive addition (Figure 3). In the heart, mTOR is an important regulator of cardiac hypertrophy. Rapamycin, an inhibitor of mTOR, can attenuate load-induced cardiac hypertrophy in mice (Shioi et al., 2003). L-leucine is a very effective amino acid with insulinotropic effect (Anthony et al., 2008), the insulin can increase the activation of mTOR on serine 2448 that leads to increased protein synthesis via phosphorylation of downstream targets p70S6K and 4EBP1 (Proud, 2004). Overexpression of Akt/PKB by insulin, an upstream regulator of mTOR, results in cardiac hypertrophy (Shioi et al., 2003). The regulation of cell size by Akt is thought to be mediated by its phosphorylation and by the subsequent downstream phosphorylation of mTOR on the serine 2448.

In animal models, L-L-leucine showed to have a stimulatory effect on proteins synthesis, independent of insulin (Anthony et al., 1999; Anthony et al., 2002). Anthony et al. (2002) showed that infusion of L-L-leucine resulted in the stimulation of protein synthesis in rats, even if the concentration of insulin in the blood were maintained low by the concomitant infusion of somatostatin. The data presented, therefore, suggest that, although L-L-leucine itself can stimulate the release of insulin, it is likely that great part of the anabolic effect not be due to the action of insulin (Anthony et al., 2008).

Finally, it was noteworthy to observe the high concentrations of BCAA, including L-L-leucine, that are present in the whey proteins. Altogether, BCAAs account for 21.2% of the amino acids that make up the milk whey proteins, or exactly 50% of the total of indispensable amino acids contained therein. This signifies that BCAAs are predominantly contained in these proteins in contrast to most other proteins (Etzel, 2004). This particular feature of the whey proteins makes them approach the amino acid profile of the skeletal muscle proteins (Ha & Zamel, 2003).

<Figure 2>

<Figure 3>

< Table 2>

Another characteristic of the whey proteins is their capacity of stimulating the secretion of the anabolic hormone insulin, into the bloodstream, thereby increasing

the amino acid transport into the cell and setting up the necessary conditions for protein synthesis (Calbet, MacLean; 2002). L-L-leucine has been proved to reduce lyzosomal and ATP-dependent protein degradation (Busquets, 2000).

High total L-L-leucine in the diet lead to high body mass but normal heart mass in the rat (Figure 4), At the cellular level, cardiomyocyte hypertrophy is characterized by an increase in cell size, enhanced protein synthesis, and heightened organization of the sarcomere, hypertrophic growth accompanies many forms of heart disease, including ischemic disease, hypertension, heart failure, and valvular disease (Frey et al., 2004) and ventricular hypertrophy, that is associated with significantly increased risk of heart failure and malignant arrhythmia (Koren et al., 1991). Body-mass adjusted lean heart mass increased as body mass departed from a minimum of about 300 g, while body mass in turn, varied between the set extremes. This function also showed that the mass of the heart was more influenced by a high body mass than by the high content of L-L-leucine in the diet. Minimum relative heart mass was found around a body mass of 290g and a total L-L-leucine intake of about 20g/kg of diet. It can also be seen in Figure 3 that as the body mass approached 320 g and L-L-leucine increased beyond 70 g/100 of diet, the relative heart mass increased very rapidly. It is not yet known what the significance of this outcome can be, but one possibility is that as increases in total contents of L-L-leucine promote increases in body mass beyond a certain point, the corresponding increases in heart mass become disproportionately greater, with

no associated bearings on the general health of the animals. Liver mass in turn responded in a different fashion, yet consistent with the complementary data. As total L-L-leucine content of the diet increased, and body mass also increased.

<Figure 4>

The consequences of supplementation on the activities of AST and ALT enzymes, plus the serum levels of glucose, uric acid, creatinine, cholesterol and insulin were determined in order to further assess the impact of the supplemental L-L-leucine. Additionally, no alterations on indicators of heart cell damage, CK and LDH, (Dawie et al., 2011) (figure 5) suggest no damage in heart cells.

<Figure 5>

Serum insulin was the parameter which appeared to have been moderately raised, but only by the highest levels of total L-L-leucine. Meanwhile, the training exercise showed an expected reduction of the insulin levels, independent of the type of diet (Table 2), and the skeletal muscle appeared more sensitive to stimulatory effects of insulin than cardiac muscle (Forsyth and Vary 2008).

CONCLUSIONS

The supplementation of L-leucine plus whey protein activated cardiac mTOR pathway effectively, but the heart mass was not affected significantly by supplementation in any dose. The general health markers, AST, ALT, glucose, uric acid, creatinine was not affected, like the indicators of cardiac cell integrity, CK and

LDH. The higher doses of L-leucine supplementation led animals to smaller weight gain.

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Figure 1 – Distribution of groups. The AIN93-G WP diet was made by substituting the whey protein for the casein of the AIN93 (standard) diet. The Controls contained no added L-leucine.

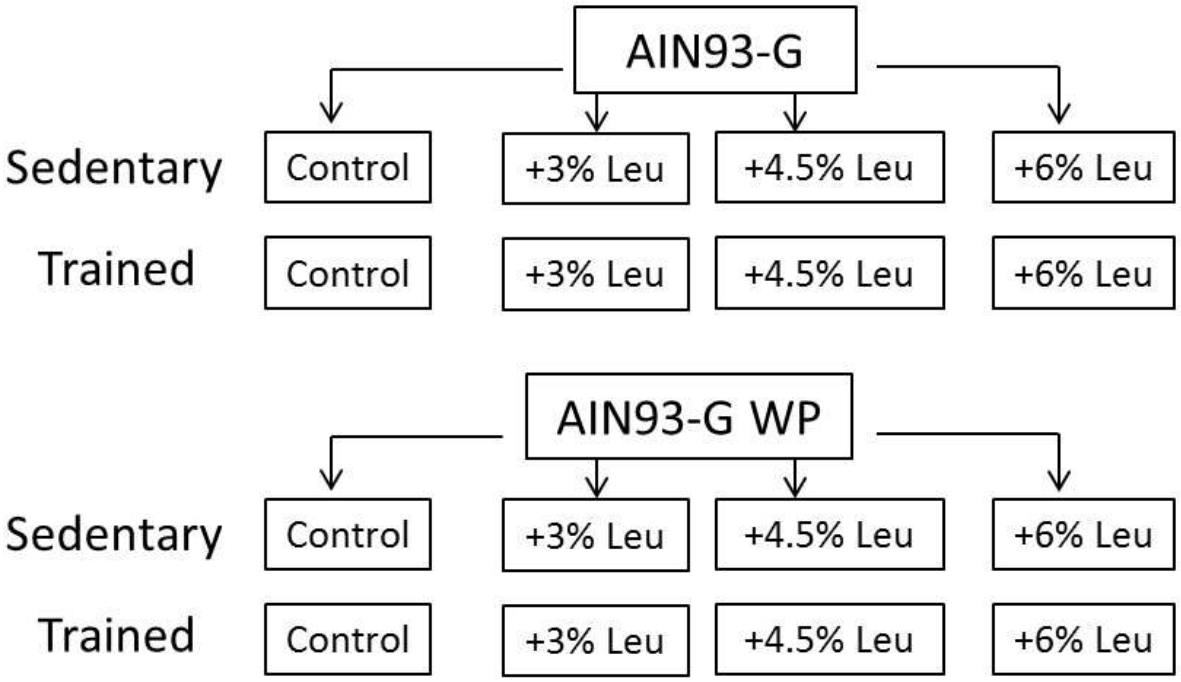


Table 1 – Composition of the diets (g/kg diet)

Ingredient	AIN93-G (Casein) +				AIN93-G (WP) +			
	Control	3%L	4.5%L	6%L	Control	3%L	4.5%L	6%L
L-leucine added	-	30	45	60	-	30	45	60
Corn starch	403.4	373.4	358.4	343.4	407.3	377.3	362.3	347.3
Casein (87.6% protein)	194.1	194.1	194.1	194.1	-	-	-	-
Whey Protein (89.4% protein)	-	-	-	-	190.2	190.2	190.2	190.2
Dextrinized corn starch	132	132	132	132	132	132	132	132
Sucrose	100	100	100	100	100	100	100	100
Soybean oil	70	70	70	70	70	70	70	70
Fiber	50	50	50	50	50	50	50	50
Mineral mix	35	35	35	35	35	35	35	35
Vitamin mix	10	10	10	10	10	10	10	10
L-Cystine	3	3	3	3	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Tert-butylhydroquinone	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014

Table 2 - Biochemical blood parameters. Different letters denote significant differences between diets, within each activity group (columns) ($p \leq 0.05$).

		AIN93 CAS +								AIN93 WP +							
		Sedentary				Training				Sedentary				Training			
		0% L	3%L	4.5%L	6%L	0% L	3%L	4.5%L	6%L	0% L	3%L	4.5%L	6%L	0% L	3%L	4.5%L	6%L
ALT[1]	Mean	17.3^{c}	27.5^{a}	21.9^{abc}	21.6^{bc}	25.2^{ab}	19.4^{bc}	22.2^{abc}	22.5^{abc}	22.3^{abc}	20.8^{bc}	20.8^{bc}	21.6^{bc}	24.6^{ab}	21.7^{bc}	22.0^{abc}	22.2^{abc}
	SEM	3.6	3.3	1.1	1.1	1.6	1.6	2.1	0.8	1.4	1.1	1.1	1.0	1.5	1.1	1.3	1.2
AST[1]	Mean	88.1^{abc}	97.1^{ab}	91.7^{abc}	92.2^{abc}	97.7^{ab}	94.9^{ab}	91.0^{abc}	99.4^{a}	86.2^{bc}	90.7^{abc}	80.9^{c}	91.9^{abc}	92.4^{abc}	90.0^{abc}	89.4^{abc}	96.9^{ab}
	SEM	2.1	2.9	3.9	3.3	3.5	4.0	5.9	3.4	2.4	2.0	4.7	2.3	3.9	5.0	2.4	6.0
Glucose[2]	Mean	141.2	142.0	151.3	158.8	134.0	132.5	142.5	138.7	124.8	142.3	144.0	149.7	141.7	146.2	150.2	143.8
	SEM	6.5	4.1	5.3	2.2	3.8	3.4	6.0	5.2	3.2	6.4	6.3	7.7	7.2	3.7	5.1	6.5
Uric acid[2]	Mean	1.0^{abc}	1.0^{abc}	1.2^{ab}	1.1^{ab}	0.6^{c}	1.2^{ab}	1.0^{abc}	0.8^{bc}	1.2^{ab}	1.1^{abc}	1.0^{abc}	1.0^{abc}	1.0^{abc}	1.1^{abc}	1.4^{a}	0.9^{abc}
	SEM	0.1	0.2	0.1	0.2	0.1	0.2	0.2	0.1	0.1	0.1	0.2	0.2	0.1	0.2	0.2	0.1
Creatinine[2]	Mean	0.6^{ab}	0.7^{ab}	0.5^{ab}	0.5^{ab}	0.4^{ab}	0.5^{ab}	0.3^{b}	0.5^{ab}	0.6^{ab}	0.5^{ab}	0.8^{a}	0.7^{ab}	0.5^{ab}	0.5^{ab}	0.6^{ab}	0.4^{ab}
	SEM	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Cholesterol[2]	Mean	59.3^{ab}	58.4^{ab}	65.6^{a}	62.4^{ab}	63.2^{ab}	59.9^{ab}	60.1^{ab}	58.5^{ab}	56.6^{ab}	63.0^{ab}	58.5^{ab}	61.8^{ab}	58.0^{ab}	59.1^{ab}	55.4^{b}	61.7^{ab}
	SEM	3.3	2.7	2.5	3.0	2.1	4.2	2.6	3.2	2.4	1.9	3.9	2.1	3.6	2.2	1.7	2.9
Insulin[3]	Mean	5.4	5.7	4.6	6.6	3.6	4.3	6.0	5.7	4.3	4.1	5.8	7.2	3.6	5.2	5.4	5.8
	SEM	1.4	1.5	0.2	0.3	0.6	1.4	0.7	1.3	0.4	0.5	0.3	1.4	1.1	0.2	1.5	0.4

1 – U/L
2 – mg/DL
3 – ng/mL

Table 3 – Dietary intake and anthropometric parameters. Different letters denote significant differences between diets, within each activity group (columns) ($p \leq 0.05$).

		AIN93 CAS +								AIN93 WP +							
		Sedentary				Trained				Sedentary				Trained			
		0% L	3%L	4.5%L	6%L	0% L	3%L	4.5%L	6%L	0% L	3%L	4.5%L	6%L	0% L	3%L	4.5%L	6%L
Diet intake[1]	M	16.55^{a}	16.89^{a}	17.80^{a}	14.90^{b}	16.65^{a}	16.29^{a}	15.80^{ab}	15.40^{b}	16.00^{ab}	16.40^{a}	17.33^{a}	15.40^{b}	17.10^{a}	17.35^{a}	17.3^{a}	17.2^{a}
	SEM	0.3	0.48	0.35	0.3	0.23	0.32	0.25	0.17	0.11	0.16	0.11	0.17	0.14	0.17	0.13	0.34
Protein intake[1]	M	2.81	2.87	3.03	2.53	2.83	2.77	2.62	2.69	2.72	2.79	2.95	2.62	2.91	2.89	2.95	2.92
	SEM	1.42	2.27	1.67	1.41	1.09	1.5	1.18	0.81	0.52	0.78	0.55	0.83	0.65	0.81	0.62	1.61
PER[2]	M	1.89^{b}	1.99^{ab}	2.06^{ab}	2.08^{a}	1.74^{c}	1.77^{bc}	1.6^{d}	1.7^{cd}	1.73^{cd}	1.81^{bc}	2.03^{ab}	2.02^{ab}	1.66^{d}	1.75^{c}	1.78^{c}	1.81^{bc}
	SEM	0.08	0.13	0.06	0.06	0.06	0.12	0.1	0.11	0.17	0.09	0.14	0.07	0.1	0.04	0.09	0.12
Body mass gain[4]	M	207.5^{b}	222.2^{ab}	239.3^{a}	207.9^{b}	207.5^{b}	205.7^{b}	196.2^{b}	194.8^{b}	197.9^{b}	212.3^{ab}	233.2^{a}	209.9^{ab}	199.6^{b}	206.3^{b}	211.5^{ab}	219.3^{ab}
	SEM	8.6	11.9	7.5	7.5	6.9	11.5	10.9	9.9	12.9	9.9	9.7	6.2	11.8	7.6	5.1	8.5
L-leucine intake[1]	M	0.04^{f}	0.55^{e}	0.84^{c}	0.93^{b}	0.04^{f}	0.53^{e}	0.73^{d}	0.98^{b}	0.05^{f}	0.55^{e}	0.84^{c}	0.98^{b}	0.06^{f}	0.57^{e}	0.84^{c}	1.09^{a}
	SEM	0	0.02	0.02	0.02	0	0.01	0.01	0.01	0	0.01	0.01	0.01	0	0.01	0.01	0.03
Liver Mass[3]	SEM	0.71	0.69	0.54	0.60	0.27	0.33	0.42	0.44	0.36	0.26	0.63	0.67	0.60	0.64	0.50	0.47
	M	9.15	9.14	9.23	9.02	9.27	9.31	9.08	9.23	9.19	9.27	9.21	9.26	9.28	9.02	9.19	9.27
	SEM	0.1	0.1	0.06	0.09	0.11	0.11	0.08	0.1	0.11	0.09	0.1	0.1	0.06	0.06	0.07	0.12

1 – grams / day
2 – Protein efficiency ratio
3 – in grams4 – in percentage

Figure 2 – mTOR and phosphorylated (S2448) mTOR response for Casein and WP to the three degrees of supplementation with L-L-leucine in both the Sedentary (A, C, E and G) and Trained (B, D, F and H) groups. Different letters denote significant differences between groups (columns) ($p \leq 0.05$).

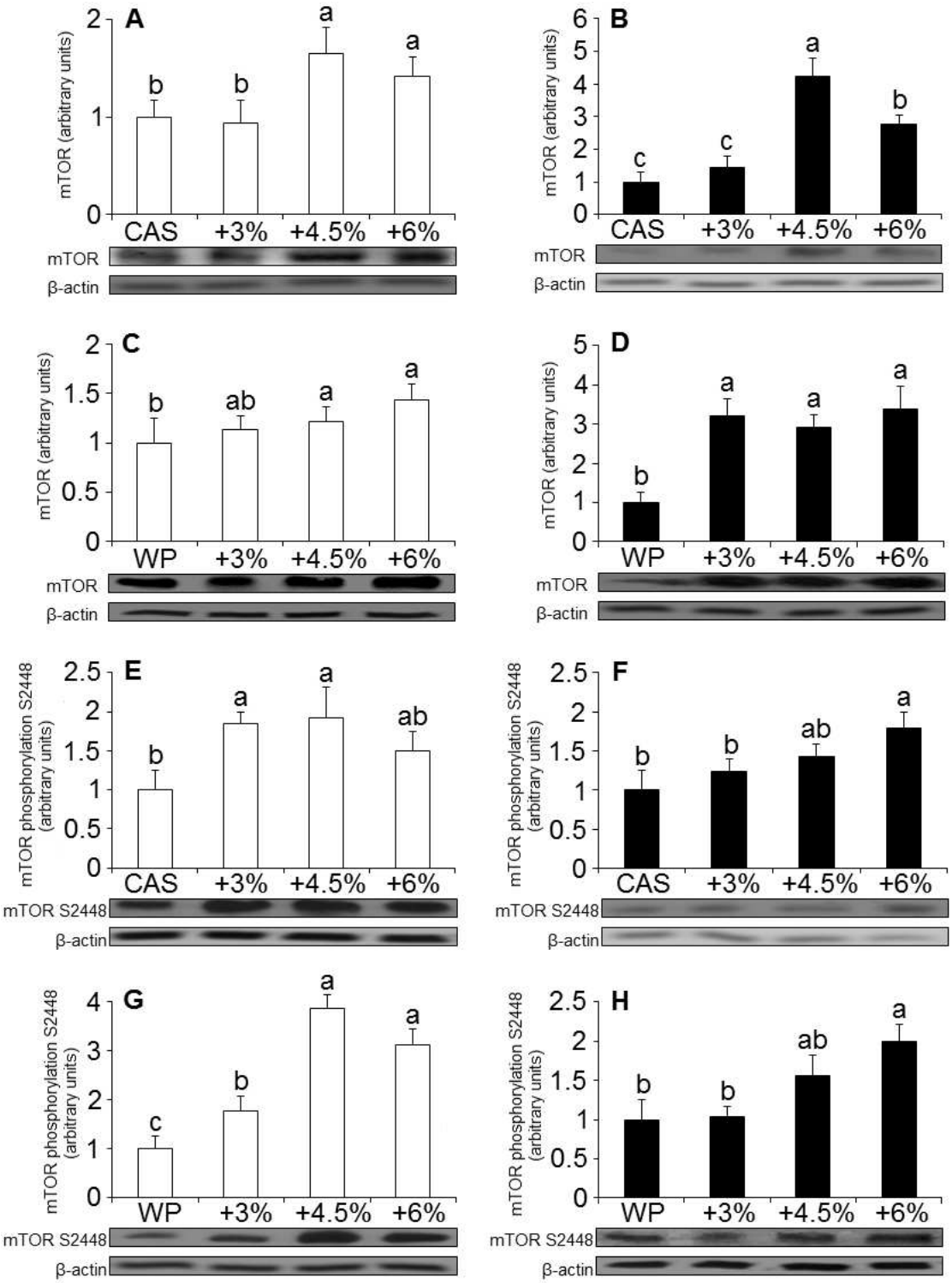


Figure 3 – p70S6K and p70S6K phosphorylated mTOR (Y389) response for Casein and WP to the three degrees of supplementation with L-L-leucine in both the Sedentary (A, C, E and G) and Trained (B, D, F and H) groups. Different letters denote significant differences between groups (columns) ($p \leq 0.05$).

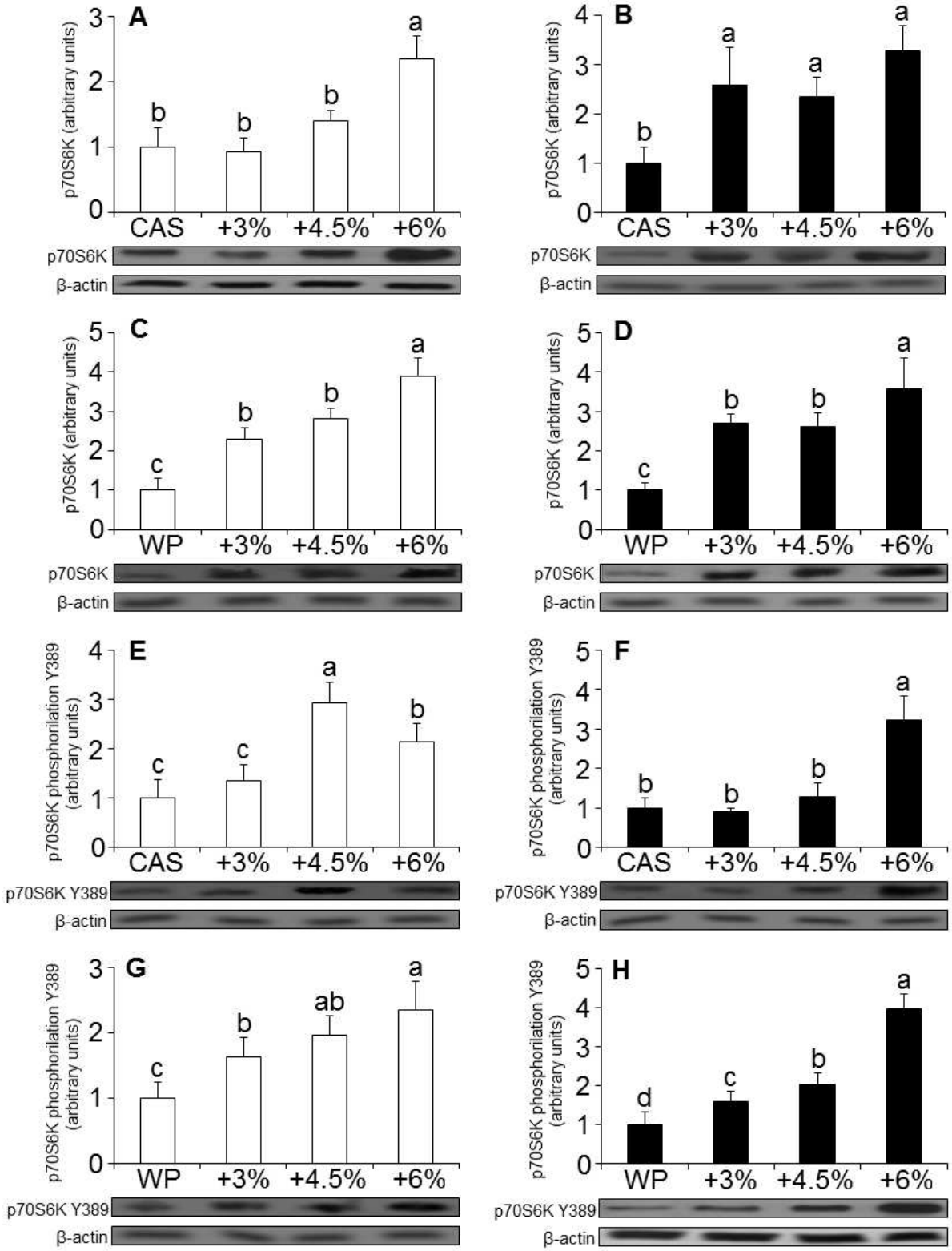


Figure 4 – Response of heart mass and protein contend, total and relativized for body mass, A) Sedentary; B) Trained; C) Casein; D) Whey Protein. Different letters denote significant differences between groups (columns) ($p \leq 0.05$).

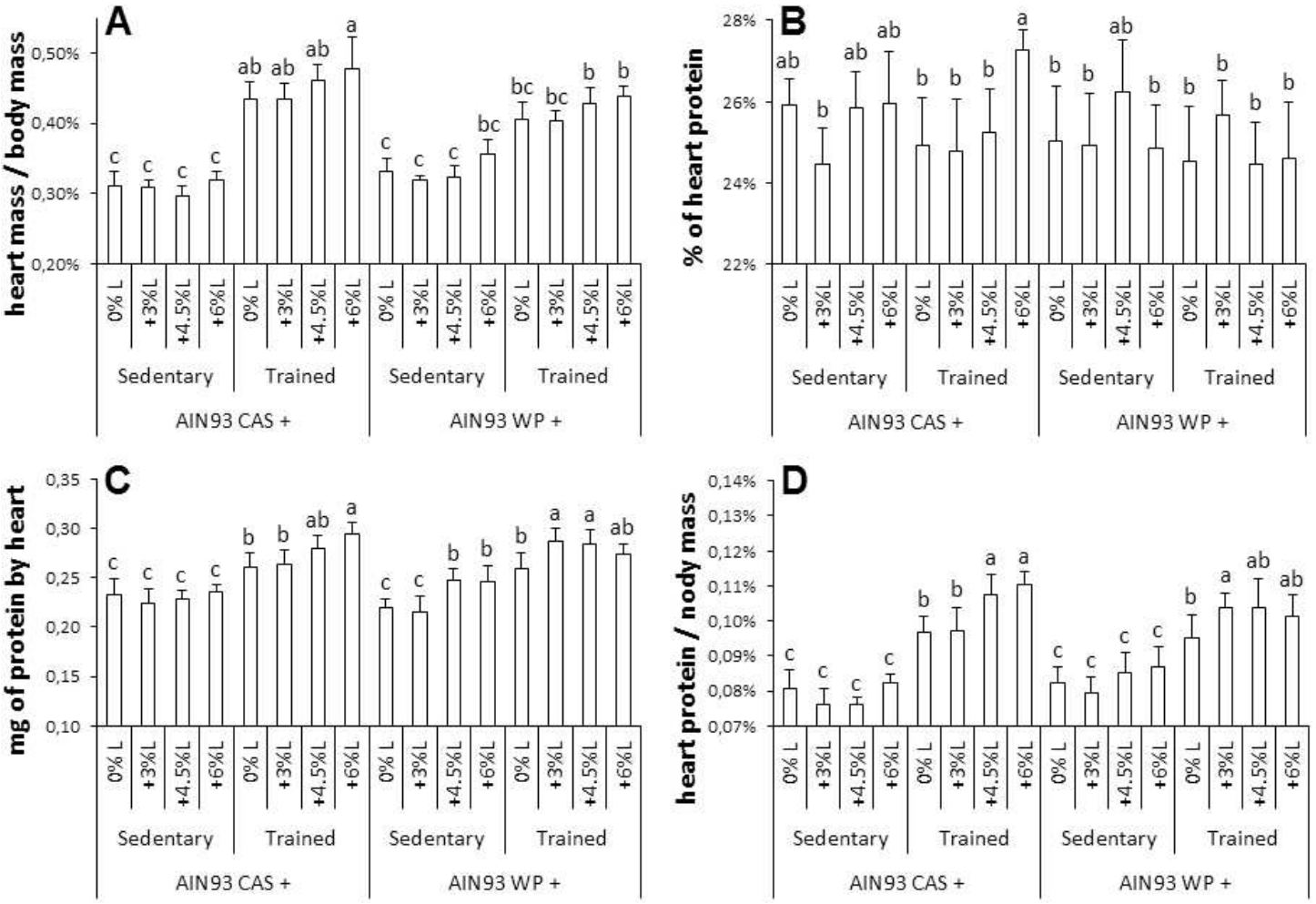
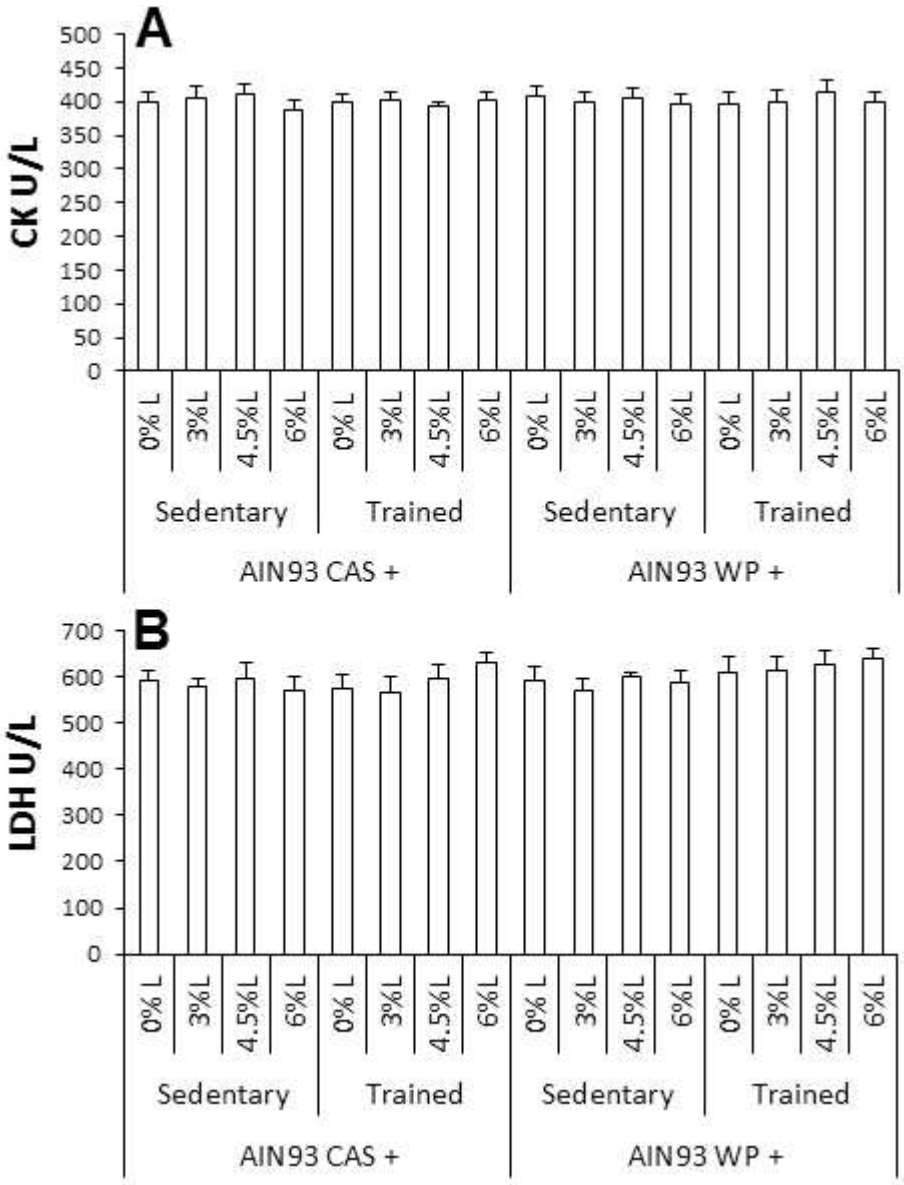
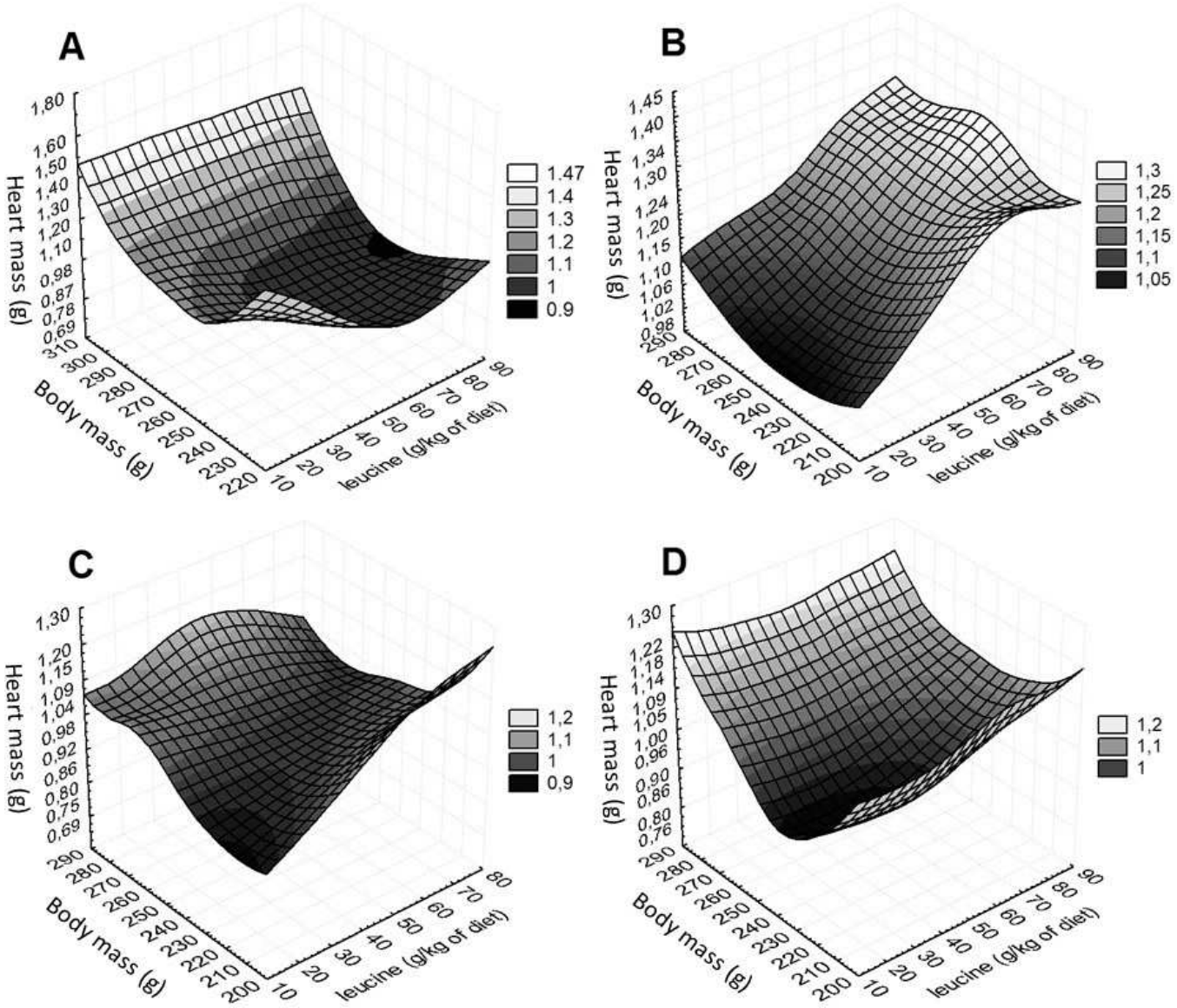


Figure 5 – Blood CK (A) and LDH (B).



Supplementary data – Response of heart mass and body mass to total dose of L-L-leucine (g/kg) in diet, A) Sedentary; B) Trained; C) Casein; D) Whey Protein.



Capítulo 4

Preparado para *International Journal of Sports Nutrition an Exercise Metabolism*

L-leucine supplemented whey protein. Effects on skeletal muscle mTOR pathway of sedentary and trained rats

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ABSTRACT

Purpose. The milk-whey proteins are rich sources of L-L-leucine and affect mTOR-mediated skeletal muscle protein synthesis. We wished to investigate the dose-response effect of chronic supplementation of the milk-whey proteins (WP) with L-L-leucine on the anabolic pathway proteins mTOR and p70S6K in the gastrocnemius of sedentary and exercised Wistar rats. **Methods**. Ninety-six weanling male Wistar rats were divided into eight groups and fed for 30 days diets containing either casein or WP, and increasing levels (0, 3, 4.5, 6% of diet) of L-L-leucine. A parallel set of eight groups was exercised for comparison. Serum uric acid, creatinin, glucose, AST, ALT, CK, LDH and cholesterol, were determined by standard methods, and mTOR and p70S6K, by Westernblot analysis. **Results**. The chronic L-leucine supplementation was capable of increasing both mTOR and p70S6K in a dose-dependent fashion in the gastrocnemius, independent of the type of dietary protein. The plasma health biomarkers were not significantly altered even at the highest L-leucine supplementation. The higher doses of supplemental L-leucine reduced food intake and growth, followed by alterations in the plasma concentrations of branched-chain amino acids. **Conclusion**. Although supplemental L-leucine has the potential of increasing the anabolic pathway key proteins in the gastrocnemius.

Keywords: mTOR, L-leucine, exercise, diaphragm, amino acid supplementation, milk whey protein

INTRODUCTION

A number of advantages have been attributed to the milk whey proteins when they are used as part of the diet of both animals and humans (Ha and Zemel, 2003; Nery-Diez et al., 2010). The milk whey proteins, or whey proteins (WP), have high concentrations of branched-chain amino acids, are known for having a good indispensable amino acid balance, high nutritional quality (Marshall, 2004) and digestibility (Boirie et al., 1997), and are therefore collectively marketed as a supplemental nurture for athletes. Tipton et. al. (2004) compared the acute anabolic response of the milk whey proteins (fast proteins) in humans with that of the caseins (slow proteins) during resistance exercise in short trials finding no difference between these two classes of proteins.

Protein or amino acid supply is closely associated with muscle mass gain or loss (Biolo et al., 1995; Roth, 2008) and oral solutions are a practical way of delivering supplements designed to promote muscle protein anabolism in exercising humans (Tipton et al., 1999). Between the aminoacids, the branched chain amino acids (BCAAs) and especially L-leucine have the potential to regulate muscle protein metabolism by stimulating protein synthesis and inhibiting protein degradation (Anthony et al., 2002; Proud 2007). It has been clearly confirmed in vivo that L-leucine was also able to stimulate muscle protein synthesis to the same extent as a complete meal (Anthony et al., 2000). The attenuation of in-vivo skeletal muscle proteolysis by L-leucine has been also described but is, however, less documented than for protein synthesis (Zanchi et al., 2008). L-leucine serves

not only as a substrate for protein synthesis but is also recognized as a potent nutrient signal that regulates protein turnover through activation of cell signaling pathways common to insulin, including the mammalian target of rapamycin complex (mTOR), which, in turn, activates two key regulatory proteins involved in the regulation of translation initiation such as S6K1 and 4EBP1 (Anthony et al., 1999).

There is some evidence that long-term L-leucine availability could improve muscle mass during exercise training. However, it needs to be associated with other amino acids to be efficient (Balage, Dardevet; 2010). At present, rare are the studies that investigated the effect of long-term L-leucine supplementation on whole body composition either in rodents. Considering that exercise, whey protein and L-leucine has the effect of increasing muscle mass the objective of this work was therefore to determine the anabolic effects of three levels (3.0, 4.5, 6.0%) of long-term supplementation of the L-leucine-rich milk-whey proteins combined with exercise on gastrocnemius mTOR pathway Wistar rats.

METHODS

Male Wistar (21-day old, specific-pathogen free; n = 96) rats, bred at the Multidisciplinary Center for Biological Research, University of Campinas, SP, Brazil, were housed (~22 °C, 55% RH, inverted 12-hour light cycle) in individual growth cages, with free access to commercial chow (Labina, Purina, Brazil) and water at all times, until they became 133,82 ± 5,6 g. The research methodology

was approved by the Ethics Committee on Animal Experimentation (CEEA-UNICAMP, protocol 1835-1). The animals were randomly assigned to either of sixteen groups according to the source of protein in the diet, L-leucine supplementation and whether they were exercise or sedentary (Figure 1).

<Figure 1>

Training Protocol – The rats were selected in the treadmill beforehand considering as not fit those that stood for 30 seconds without departing from the baseline. The animals began training on the day they started to consume the experimental diets and lasted for 4 weeks following the progressive-resisted protocol adapted of Hohl et al (2009).

Experimental diets – The experimental diets were isonitrogenous (approximately 17% protein, dry basis), isolipidic and isocaloric (approximately 360 kcal/100 g) and formulated following the recommendations of the American Institute of Nutrition, AIN93 (for growing rats) diet (Reeves et al., 1993). The two diets differed among themselves with respect to the nature of the protein source, casein or milk-whey protein (Table 1).

<Table 1>

<Table 2>

Protein extraction and immunoblotting – Total protein content of diaphragm muscle was determined by the Lowry method (1951). For immunoblotting, tissue homogenates were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane, using a Wide Biocom Westernblot

system (Bridge of Weir, UK). Blots were probed with the appropriate antibodies to mTOR total and phosphor-mTOR (Ser2448) (Cell signaling) and p70S6K total and phosphor-p70S6K (Thr 389) (Santa Cruz) to assess the levels of these proteins in diaphragm tissue, tubulin was the loading control (Santa Cruz). The appropriate secondary antibody conjugated to peroxidase and the BM chemiluminiscence blotting system were used for detection. The bands were visualized by chemiluminiscence (GE – ImageQuant LAS4000, Piscataway, NJ, USA). Band intensities were quantified by scanning and processing with the program ImageJ (v. 1.44 for Windows).

Biochemical parameters – Six hours after the training session, blood samples were collected in Vacutainers, kept at 4°C, and centrifuged at 3000xg (4°C, 12min) to obtain the serum. For the assessment of sera, uric acid, aspartate amino transferase (AST), alanine aminotransferase ALT, creatine kinase (CK) and lactate dehydrogenase (LDH), glucose and cholesterol standard enzymatic spectrophotometric determinations were performed employing Laborlab kits (São Paulo, Brazil).

Determination of plasma free amino acids – Serum free amino acids were extracted with methanol and derivatized with phenylisothiocyanate (White et al., 1986) and the PTH-derivatives chromatographed using a Luna C-18, 100 Á; 5 µ, 250 x 4.6 mm (00G-4252-EQ) column, at 50°C. Quantification was made by comparison with a standard mixture and DL-2-aminobutyric acid as an internal

standard (Sigma-Aldrich Corp, St Louis, MO, USA). The free amino acids were extracted in 80% ethanol and 0.1M HCl, with 500 μL of 2-aminobutyric acid added as internal standard. The mixture was sonicated for 10 minutes and further homogenized for 1 hour, followed by centrifugation at 8500xg for 15 minutes. The supernatant was filtered through a 0.22 mm membrane and a 40 μL aliquot derivatized as described above for the injection of 20 μL into the liquid chromatograph.

Proximal composition – Moisture, total ash, protein, and lipids were determined according to AOAC methods (2002). The total carbohydrate content was inferred by difference. Protein efficiency ratio (PER) was calculated as the ratio of grams of protein feed per unit of wet weight gain protein fed.

Statistical analysis – The results were subjected to statistical analysis using the software SPSS (Statistical Package for the Social Sciences), version 17.0. The data were tested for normality (Kolmogorov-Smirnov test) and homogeneity using the tools available therein. For parametric data, the multivariate analysis of variance (ANOVA) was used and means were compared (Duncan test) adopting the value of $p \leq 0.05$ as a criterion for statistical significance.

RESULTS AND DISCUSSION

The L-L-leucine supplementation in both, casein and the whey protein promoted the phosphorilation of mTOR in the gastrocnemius muscle in dose-

dependent manner (Figure 2), data of p70S6k phosphorylation confirm the activation of the mTOR pathway. Also, the degree of response was not different depending on whether the protein was casein or the whey proteins, and whether the animal belonged to a sedentary and trained group.

<Figure 2>

The mTOR, is a master regulator of mammalian cell growth (cell size) and proliferation (cell division) (Schmelzle and Hall, 2000; Hay and Sonenberg, 2004). mTOR integrates multiple inputs in a proliferating cell by sensing amino acid availability and cellular energy levels (Dennis et al., 2001; Inoki et al., 2003) , and at the same time mediating cytoplasmic relay of mitogenic signals (Chen and Fang, 2002). The catalytic activity of mTOR is capable of phosphorylating p70S6K in vitro, and is required for the regulatory phosphorylation in vivo (Hay and Sonenberg, 2004). The mTOR pathway has emerged as a major regulator of skeletal myogenesis (Park et al., 2005), and this control of translation initiation is an important mechanism, which tightly regulates the skeletal muscle protein accretion and growth, through multiple effector proteins. Coordinated regulation of these processes occurs through activation or inhibition of multiple signaling pathways allowing the skeletal muscle to integrate information regarding mitogenic signals and nutrient signals (Forsyth and Vary 2008).

<Figure 3>

<Figure 4>

This activation of mTOR pathway probably explain the increase of the muscle mass by L-L-leucine supplementation (Figure 3), but the higher doses of L-L-leucine in diet, shown the best activation of mTOR pathway, but poor enhance in muscle mass or in growth (Figure 4). This poor growth can be resulted by L-leucine has been shown to decrease food intake through mTOR activation in the hypothalamus (Cota et al., 2006) and the stimulation of the leptin secretion, the satiety hormone (Lynch et al., 2006). Additionally, L-leucine supplementation is known to induce a decrease in both valine and isoleucine concentrations (Dardevet et al., 2000; Hambraeus et al., 1976), this imbalance can lead to insufficient concentration of valine and isoleucine to build proteins in animals feeding higher doses of L-leucine. Our results supported another researches that shown the effect of L-leucine supplementation on accretion of muscle mass, despite demonstration by Anthony et al. (1999) that L-leucine increased skeletal muscle protein synthesis when given alone in rats following exercise, beyond, a ingestion of whey hydrolysate, which induced a rapid and large increase in plasma L-leucine concentration, was more efficient to stimulate muscle protein synthesis after exercise in men than ingestion of soy protein or casein, the authors suggested that L-leucine availability might be partly responsible for this beneficial effect (Tang et al., 2009).

The L-L-leucine supplementation promoted high concentration, of insulin (Table 3), it seems clear that L-leucine stimulates protein synthesis largely through insulin-independent mechanisms, the effects of insulin on mTOR signaling are

largely due to upstream control of mTOR through the tuberous sclerosis complex, and this upstream regulation of mTOR signaling through the TSC1-TSC2 complex is largely mediated by activation of protein kinase B (PKB or Akt) through a phosphatidylinositol (PI) 3-kinase-dependent pathway (Proud, 2004). Insulin activation of mTOR requires glucose hormones that regulate growth also have a substantial effect on metabolism (Stipanuk, 2007), and insulin not only enhances glucose uptake, but also regulates tissue growth via the mTOR signalling pathway (Yoshizawa et al., 1998).

<Table 3>

<Figure 5>

<Table 4>

The general health indicators (Table 3) seem indicate no abnormalities caused by L-leucine supplementation, with exception for increased in insulin levels. The body composition (Figure 4) showed no general trend with long-term supplementation with L-L-leucine mass with regard to dietary protein source, the growth and the diet intake (Table 4) was consistent with the observation of Laviano (2006), in which L-L-leucine was related like a suppressant of appetite.

CONCLUSION

There is some evidence that long-term L-leucine supplementation associated with high quality protein and fast protein, is sufficient to improve muscle

mass in sedentary or trained Wistar rats. This may be mediated by mTOR pathway activation and insulin increased levels. Excessive concentrations of dietary L-L-leucine, can suppress the dietary intake with concomitant loss of body weight, but apparently not affecting general health indicators o body composition consistently, the exercise seem no improve this effects

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Figure Legends

Figure 1 – Distribution of groups. The AIN93-G WP diet was made by substituting the whey protein for the casein of the AIN93 (standard) diet. The Controls contained no added L-leucine.

Figure 2 – Total mTOR, phosphorylated (S2448) mTOR, p70S6K and p70S6K phosphorylated response for Casein and WP to the three degrees of supplementation with L-L-leucine in both the Sedentary □ (A, C, E and G) and Trained ■ (B, D, F and H) groups. Different letters denote significant differences between groups (columns) ($p \leq 0.05$).

Figure 3 – Response of gastrocnemius mass to total dose of L-L-leucine (g/kg) in sedentary and trained rats, A, B and C are three different point of views.

Figure 4 – Growth of rats to the three degrees of supplementation with L-L-leucine in both the Casein (A) and Whey Protein group (B). Different letters denote significant differences between groups (columns) ($p \leq 0.05$).

Figure 5 – Proximal composition of of rats to the three degrees of supplementation with L-L-leucine in both the Casein and Whey Protein. Different letters denote significant differences between groups (columns) ($p \leq 0.05$).

Table 1 – Composition of the diets (g/kg diet).

Ingredient	AIN93-G (Casein) + Control	3%L	4.5%L	6%L	AIN93-G (WP) + Control	3%L	4.5%L	6%L
L-leucine added	-	30	45	60	-	30	45	60
Corn starch	403.4	373.4	358.4	343.4	407.3	377.3	362.3	347.3
Casein (87.6% protein)	194.1	194.1	194.1	194.1	-	-	-	-
Whey Protein (89.4% protein)	-	-	-	-	190.2	190.2	190.2	190.2
Dextrinized corn starch	132	132	132	132	132	132	132	132
Sucrose	100	100	100	100	100	100	100	100
Soybean oil	70	70	70	70	70	70	70	70
Fiber	50	50	50	50	50	50	50	50
Mineral mix	35	35	35	35	35	35	35	35
Vitamin mix	10	10	10	10	10	10	10	10
L-Cystine	3	3	3	3	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Tert-butylhydroquinone	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014

Table 2 – Aminogram of the diet protein sources (g/100 g)**.**

Amino Acid	CAS	WP	WPH
Asparagine	5.96	11.52	11.16
Glutamate	19	18.82	17.99
Serine	4.68	5.31	5.04
Glycine	1.39	1.74	1.75
Histidine	2.12	1.31	1.27
Arginine	3.03	2.66	2.31
Threonine	3.56	7.64	7.4
Alanine	2.3	5.11	4.89
Proline	8.85	5.89	5.68
Tyrosine	4.57	2.88	2.78
Valine	5.36	5.81	5.68
Methionine	2.32	2.51	2.52
Cystine	0.16	1.48	1.6
Isoleucine	4.51	6.97	6.88
L-leucine	7.62	10.15	10.14
Phenylalanine	3.89	2.86	2.78
Lysine	6.62	9.2	9.48

Table 3 - Biochemical blood parameters. Different letters denote significant differences between diets, within each activity group (columns) ($p \leq 0.05$).

		AIN93 CAS +								AIN93 WP +							
		Sedentary				Training				Sedentary				Training			
		0% L	3%L	4.5%L	6%L	0% L	3%L	4.5%L	6%L	0% L	3%L	4.5%L	6%L	0% L	3%L	4.5%L	6%L
ALT[1]	Mean	17.3^{c}	27.5^{a}	21.9^{abc}	21.6^{bc}	25.2^{ab}	19.4^{bc}	22.2^{abc}	22.5^{abc}	22.3^{abc}	20.8^{bc}	20.8^{bc}	21.6^{bc}	24.6^{ab}	21.7^{bc}	22.0^{abc}	22.2^{abc}
	SEM	3.6	3.3	1.1	1.1	1.6	1.6	2.1	0.8	1.4	1.1	1.1	1.0	1.5	1.1	1.3	1.2
AST[1]	Mean	88.1^{abc}	97.1^{ab}	91.7^{abc}	92.2^{abc}	97.7^{ab}	94.9^{ab}	91.0^{abc}	99.4^{a}	86.2^{bc}	90.7^{abc}	80.9^{c}	91.9^{abc}	92.4^{abc}	90.0^{abc}	89.4^{abc}	96.9^{ab}
	SEM	2.1	2.9	3.9	3.3	3.5	4.0	5.9	3.4	2.4	2.0	4.7	2.3	3.9	5.0	2.4	6.0
Glucose[2]	Mean	141.2	142.0	151.3	158.8	134.0	132.5	142.5	138.7	124.8	142.3	144.0	149.7	141.7	146.2	150.2	143.8
	SEM	6.5	4.1	5.3	2.2	3.8	3.4	6.0	5.2	3.2	6.4	6.3	7.7	7.2	3.7	5.1	6.5
Uric acid[2]	Mean	1.0^{abc}	1.0^{abc}	1.2^{ab}	1.1^{ab}	0.6^{c}	1.2^{ab}	1.0^{abc}	0.8^{bc}	1.2^{ab}	1.1^{abc}	1.0^{abc}	1.0^{abc}	1.0^{abc}	1.1^{abc}	1.4^{a}	0.9^{abc}
	SEM	0.1	0.2	0.1	0.2	0.1	0.2	0.2	0.1	0.1	0.1	0.2	0.2	0.1	0.2	0.2	0.1
Creatinine[2]	Mean	0.6^{ab}	0.7^{ab}	0.5^{ab}	0.5^{ab}	0.4^{ab}	0.5^{ab}	0.3^{b}	0.5^{ab}	0.6^{ab}	0.5^{ab}	0.8^{a}	0.7^{ab}	0.5^{ab}	0.5^{ab}	0.6^{ab}	0.4^{ab}
	SEM	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Cholesterol[2]	Mean	59.3^{ab}	58.4^{ab}	65.6^{a}	62.4^{ab}	63.2^{ab}	59.9^{ab}	60.1^{ab}	58.5^{ab}	56.6^{ab}	63.0^{ab}	58.5^{ab}	61.8^{ab}	58.0^{ab}	59.1^{ab}	55.4^{b}	61.7^{ab}
	SEM	3.3	2.7	2.5	3.0	2.1	4.2	2.6	3.2	2.4	1.9	3.9	2.1	3.6	2.2	1.7	2.9
Insulin[3]	Mean	5.4	5.7	4.6	6.6	3.6	4.3	6.0	5.7	4.3	4.1	5.8	7.2	3.6	5.2	5.4	5.8
	SEM	1.4	1.5	0.2	0.3	0.6	1.4	0.7	1.3	0.4	0.5	0.3	1.4	1.1	0.2	1.5	0.4

1 – U/L
2 – mg/DL
3 – ng/mL

Table 4 – Dietary intake and anthropometric. Different letters denote significant differences between diets, within each activity group (columns) ($p \leq 0.05$).

		AIN93 CAS +								AIN93 WP +							
		Sedentary				Trained				Sedentary				Trained			
		0% L	3%L	4.5%L	6%L	0% L	3%L	4.5%L	6%L	0% L	3%L	4.5%L	6%L	0% L	3%L	4.5%L	6%L
Diet intake[1]	M	16.55^{a}	16.89^{a}	17.80^{a}	14.90^{b}	16.65^{a}	16.29^{a}	15.80^{ab}	15.40^{b}	16.00^{ab}	16.40^{a}	17.33^{a}	15.40^{b}	17.10^{a}	17.35^{a}	17.3^{a}	17.2^{a}
	SEM	0.3	0.48	0.35	0.3	0.23	0.32	0.25	0.17	0.11	0.16	0.11	0.17	0.14	0.17	0.13	0.34
Protein intake[1]	M	2.81	2.87	3.03	2.53	2.83	2.77	2.62	2.69	2.72	2.79	2.95	2.62	2.91	2.89	2.95	2.92
	SEM	1.42	2.27	1.67	1.41	1.09	1.5	1.18	0.81	0.52	0.78	0.55	0.83	0.65	0.81	0.62	1.61
PER[2]	M	1.89^{b}	1.99^{ab}	2.06^{ab}	2.08^{a}	1.74^{c}	1.77^{bc}	1.6^{d}	1.7^{cd}	1.73^{cd}	1.81^{bc}	2.03^{ab}	2.02^{ab}	1.66^{d}	1.75^{c}	1.78^{c}	1.81^{bc}
	SEM	0.08	0.13	0.06	0.06	0.06	0.12	0.1	0.11	0.17	0.09	0.14	0.07	0.1	0.04	0.09	0.12
Body mass gain[4]	M	207.5^{b}	222.2^{ab}	239.3^{a}	207.9^{b}	207.5^{b}	205.7^{b}	196.2^{b}	194.8^{b}	197.9^{b}	212.3^{ab}	233.2^{a}	209.9^{ab}	199.6^{b}	206.3^{b}	211.5^{ab}	219.3^{ab}
	SEM	8.6	11.9	7.5	7.5	6.9	11.5	10.9	9.9	12.9	9.9	9.7	6.2	11.8	7.6	5.1	8.5
L-leucine intake[1]	M	0.04^{f}	0.55^{e}	0.84^{c}	0.93^{b}	0.04^{f}	0.53^{e}	0.73^{d}	0.98^{b}	0.05^{f}	0.55^{e}	0.84^{c}	0.98^{b}	0.06^{f}	0.57^{e}	0.84^{c}	1.09^{a}
	SEM	0	0.02	0.02	0.02	0	0.01	0.01	0.01	0	0.01	0.01	0.01	0	0.01	0.01	0.03
	SEM	0.1	0.1	0.06	0.09	0.11	0.11	0.08	0.1	0.11	0.09	0.1	0.1	0.06	0.06	0.07	0.12

1 – grams / day
2 – Protein efficiency ratio
3 – in grams
4 – in percentage

Figure 1

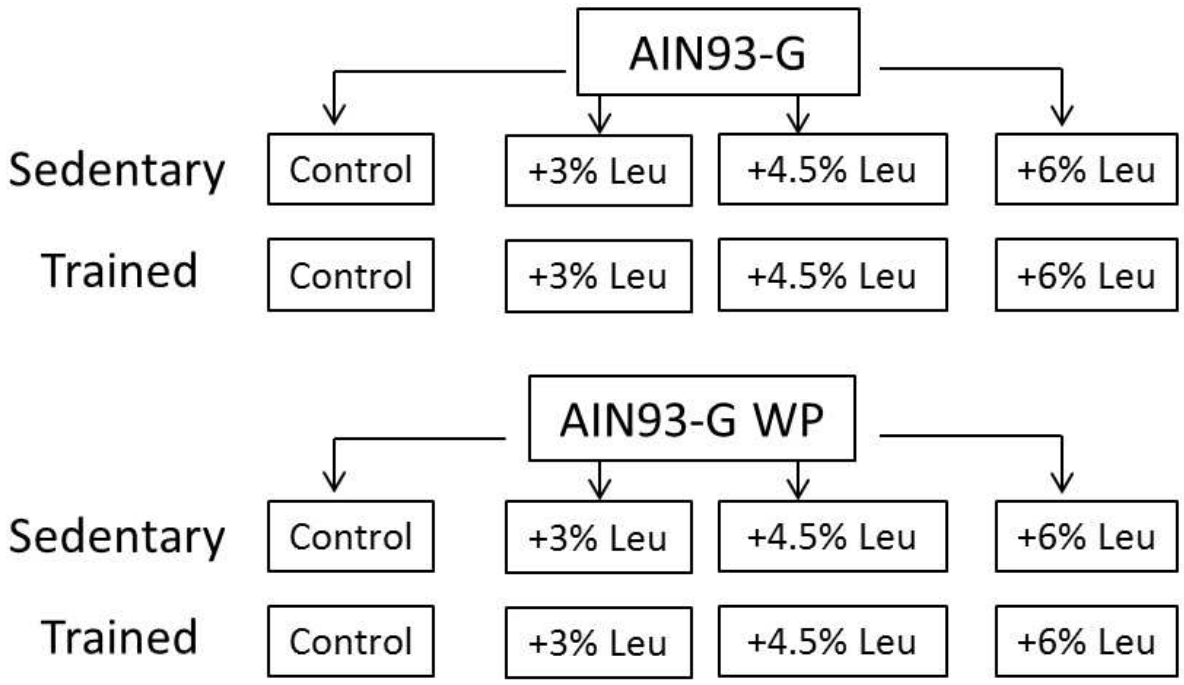

AIN93-G
Sedentary
Control
+3% Leu
+4.5% Leu
+6% Leu
Trained
Control
+3% Leu
+4.5% Leu
+6% Leu
AIN93-G WP
Sedentary
Control
+3% Leu
+4.5% Leu
+6% Leu
Trained
Control
+3% Leu
+4.5% Leu
+6% Leu

Figure 2

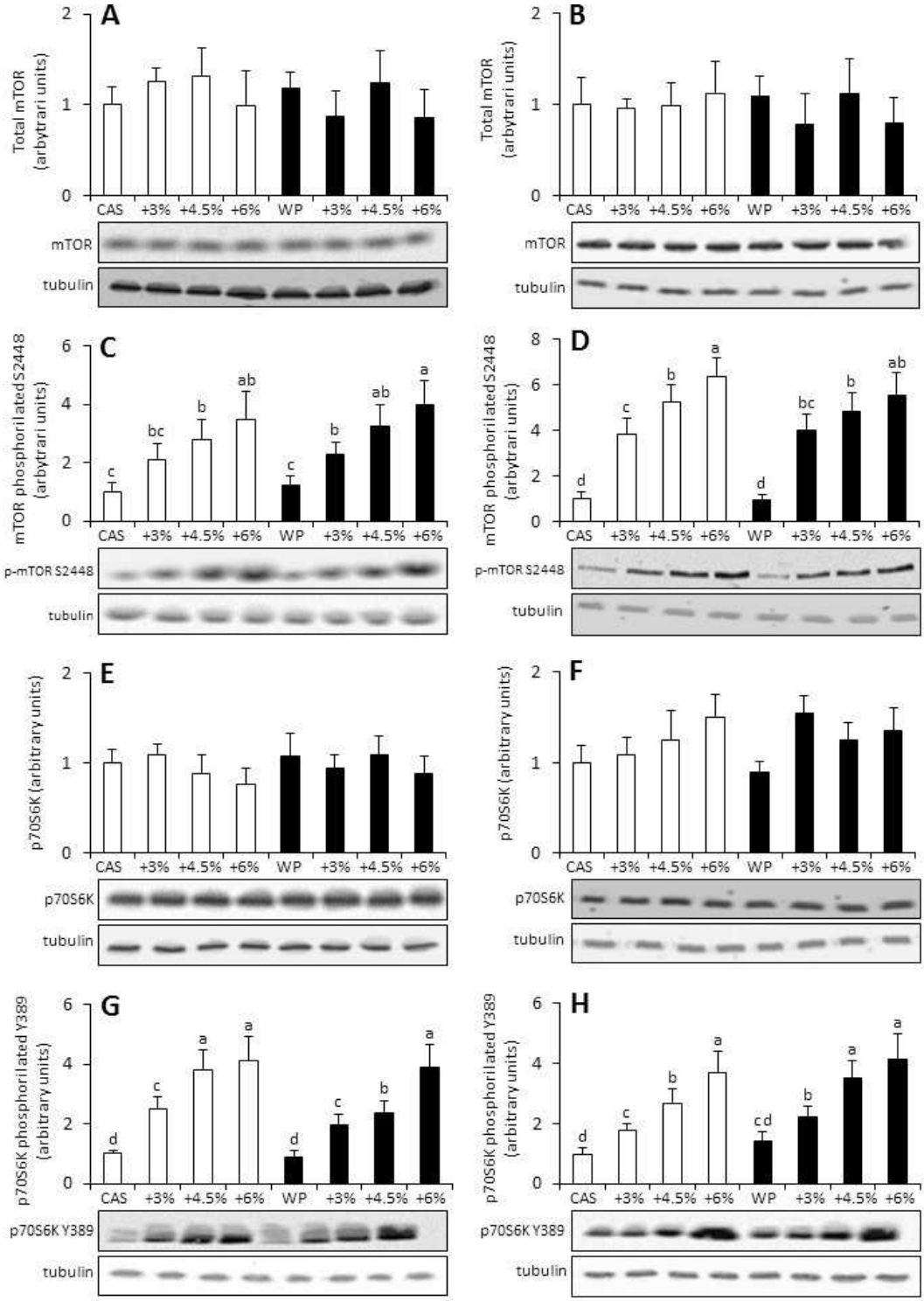

A
Total mTOR (arbytrari units)
CAS +3% +4.5% +6% WP +3% +4.5% +6%
mTOR
tubulin
B
Total mTOR (arbytrari units)
CAS +3% +4.5% +6% WP +3% +4.5% +6%
mTOR
tubulin
C
mTOR phosphorilated S2448 (arbytrari units)
CAS +3% +4.5% +6% WP +3% +4.5% +6%
p-mTOR S2448
tubulin
D
mTOR phosphorilated S2448 (arbytrari units)
CAS +3% +4.5% +6% WP +3% +4.5% +6%
p-mTOR S2448
tubulin
E
p70S6K (arbitrary units)
CAS +3% +4.5% +6% WP +3% +4.5% +6%
p70S6K
tubulin
F
p70S6K (arbitrary units)
CAS +3% +4.5% +6% WP +3% +4.5% +6%
p70S6K
tubulin
G
p70S6K phosphorilated Y389 (arbitrary units)
CAS +3% +4.5% +6% WP +3% +4.5% +6%
p70S6K Y389
tubulin
H
p70S6K phosphorilated Y389 (arbitrary units)
CAS +3% +4.5% +6% WP +3% +4.5% +6%
p70S6K Y389
tubulin

Figure 3

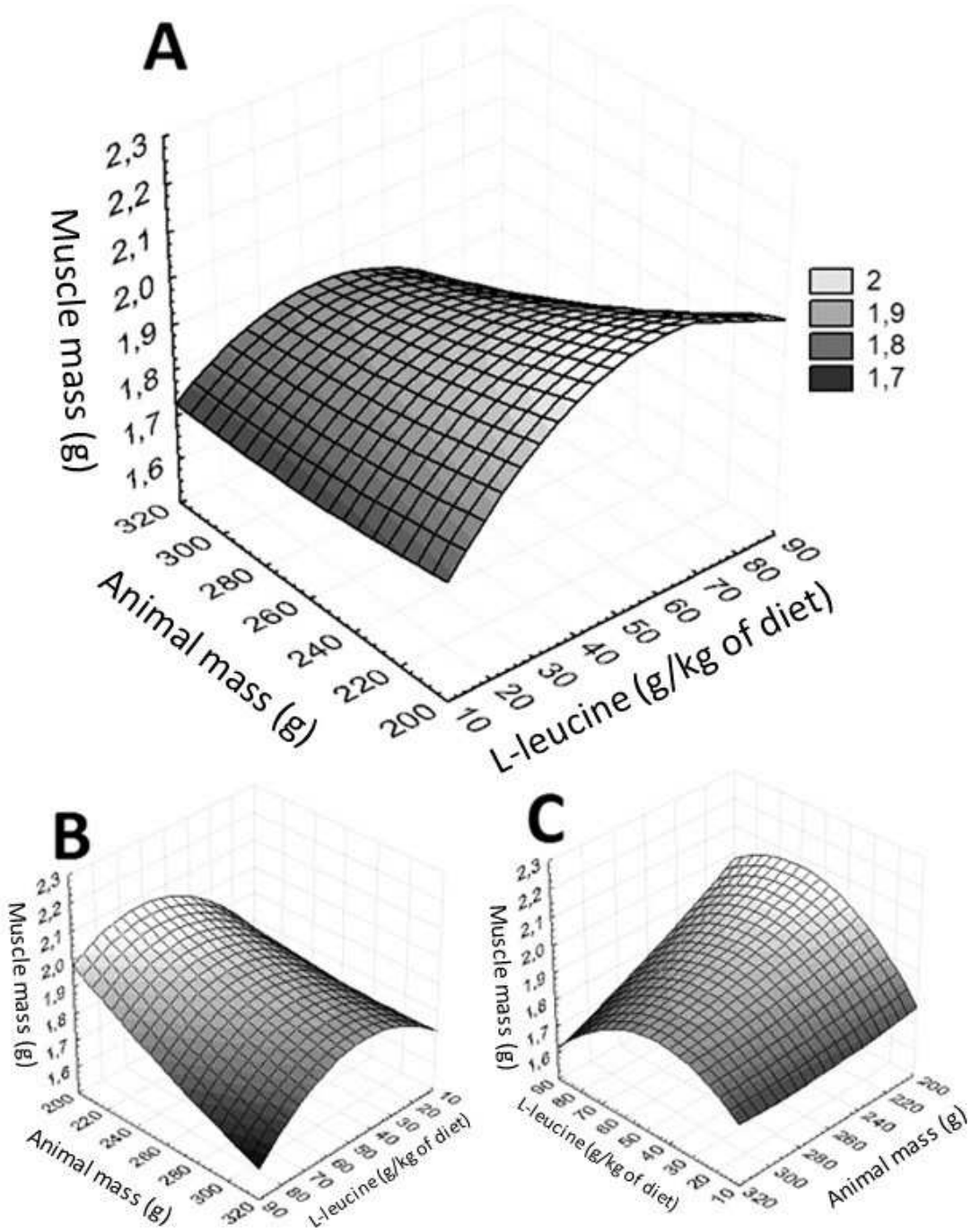

A
Muscle mass (g)
2,3
2,2
2,1
2,0
1,9
1,8
1,7
1,6
2
1,9
1,8
1,7
Animal mass (g)
320
300
280
260
240
220
200
L-leucine (g/kg of diet)
10
20
30
40
50
60
70
80
90
B
Muscle mass (g)
Animal mass (g)
L-leucine (g/kg of diet)
C
Muscle mass (g)
L-leucine (g/kg of diet)
Animal mass (g)

Figure 4

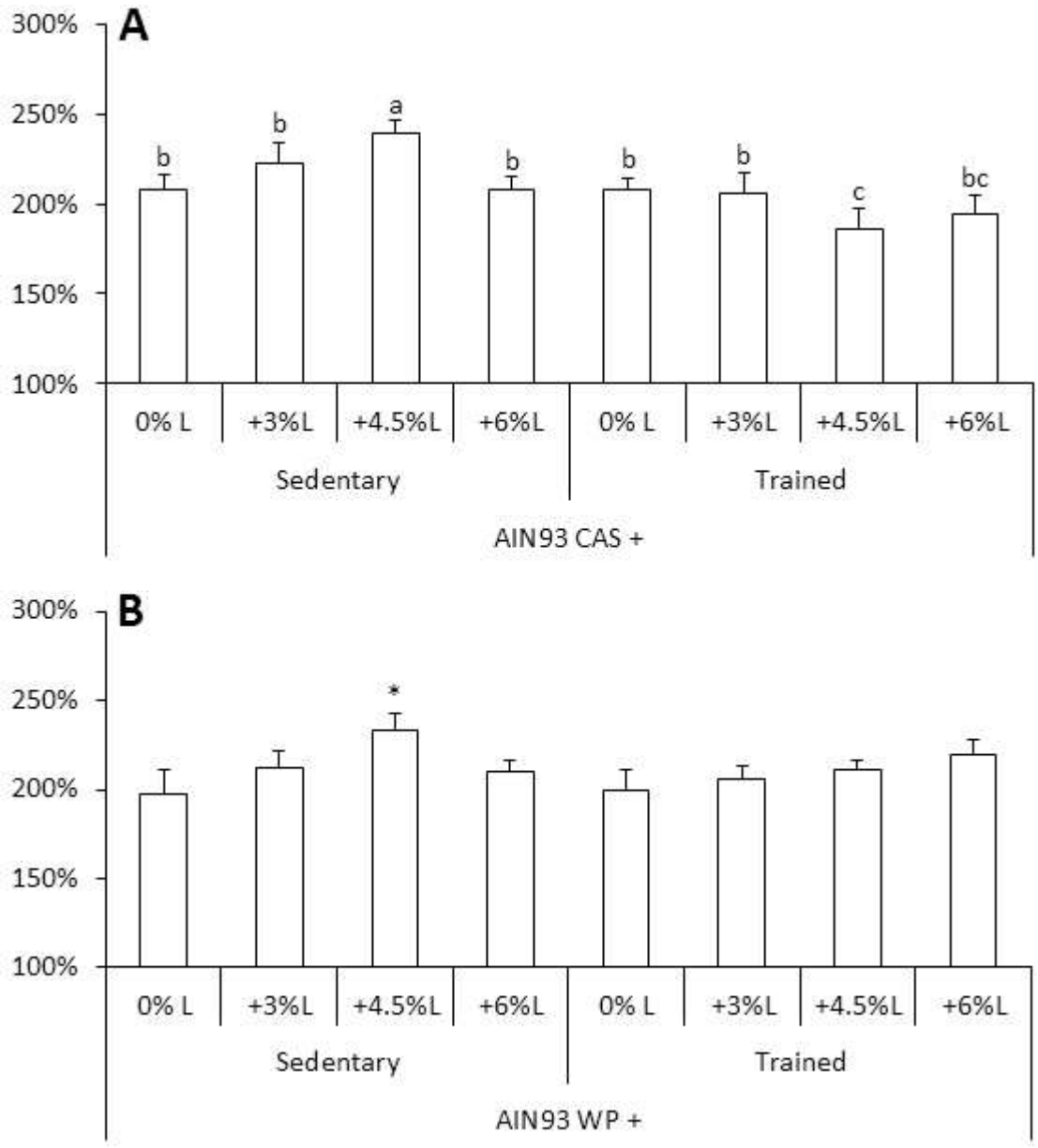
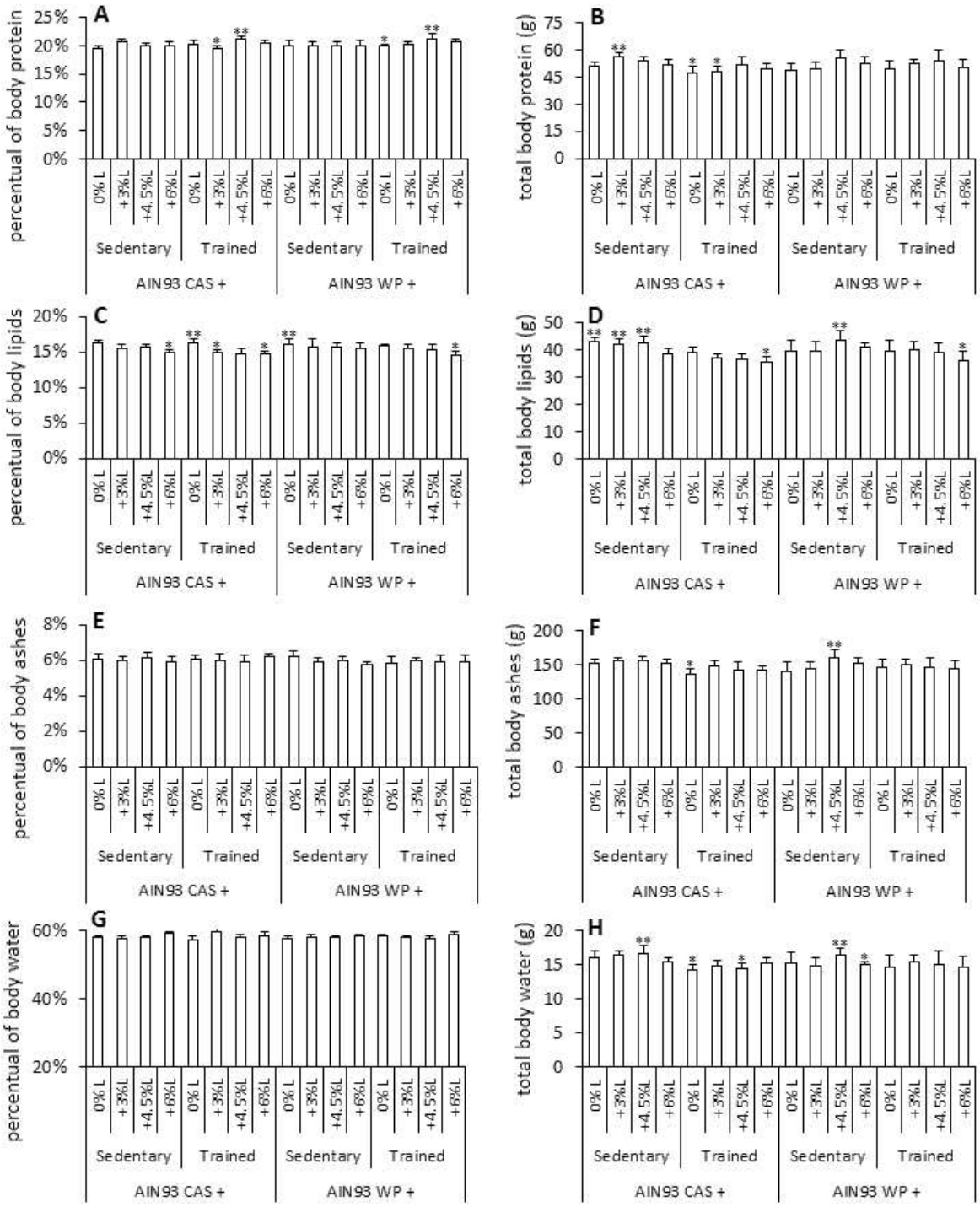

A
300%
250%
200%
150%
100%
b
b
a
b
b
b
c
bc
0% L
+3%L
+4.5%L
+6%L
0% L
+3%L
+4.5%L
+6%L
Sedentary
Trained
AIN93 CAS +
B
300%
250%
200%
150%
100%
*
0% L
+3%L
+4.5%L
+6%L
0% L
+3%L
+4.5%L
+6%L
Sedentary
Trained
AIN93 WP +

Figure 5


A
percentual of body protein
25%
20%
15%
10%
5%
0%
B
total body protein (g)
75
60
45
30
15
0
C
percentual of body lipids
20%
15%
10%
5%
0%
D
total body lipids (g)
50
40
30
20
10
0
E
percentual of body ashes
8%
6%
4%
2%
0%
F
total body ashes (g)
200
150
100
50
0
G
percentual of body water
60%
40%
20%
H
total body water (g)
20
15
10
5
0
0% L
+3%L
+4.5%L
+6%L
Sedentary
Trained
AIN93 CAS +
AIN93 WP +

CONCLUSÃO GERAL

O consumo de isolado proteico de soro do leite suplementado com L-leucina nas concentrações de 3, 4,5 e 6% da massa total da dieta (AIN93-G) foi efetivo na ativação da via mTOR, no miocárdio, diafragma e gastrocnêmio. A ativação foi dada pela fosforilação da mTOR no resíduo de serina 2448 e confirmada pela fosforilação da p70S6K no resíduo Y 389. A via mTOR é uma das principais vias anabólica em tecidos musculares, e as duas proteínas testadas, caseína e PSL, apresentaram comportamento semelhante.

A suplementação com L-leucina no nível de 6% do conteúdo total da dieta diminuiu o ganho de peso normal dos animais quando comparado ao grupo controle. Verificou-se também desbalanço no perfil aminoacídico plasmático, principalmente dos aminoácidos de cadeia ramificada (isoleucina e valina), com quedas significativas nas concentrações destes.

Os demais parâmetros indicadores gerais de saúde dos animais não apresentaram variações significativas fora dos limites de normalidade (grupo controle), sem alteração também em enzimas indicadoras da integridade celular hepática e cardíaca.

A suplementação com L-leucina, mostrou-se ainda efetiva no aumento da massa do gastrocnêmio de animais suplementados com 3 e 4,5%, tanto exercitado quanto sedentários e em ambas proteínas dietéticas testadas, sem distinção significativa entre elas. Novas pesquisas devem ser realizados sobre o

papel da L-leucina nas vias catabólicas e também sobre os efeitos colaterais, principalmente no pâncreas e ilhotas de Langherans

Anexo I

Comissão de Ética na Experimentação Animal
CEEA/Unicamp

CERTIFICADO

Certificamos que o Protocolo nº **1835-1**, sobre **"Proteínas do soro de leite e sua suplementação com leucina: influência nos parâmetros bioquímicos e composição corporal e no desempenho físico de ratos Wistar exercitados"**, sob a responsabilidade de **Prof. Dr. Jaime Amaya Farfám / Pablo Christiano B. Lollo**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal – CEEA/Unicamp em **04 de maio de 2009**.

CERTIFICATE

We certify that the protocol nº **1835-1**, entitled **"Milk whey proteins and leucine supplementation: influence on biochemical and body compositional parameters and on the physical performance of exercising Wistar rats"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **May 4, 2009**.

Campinas, 04 de maio de 2009.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva