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Proteína do soro do leite hidrolisado na síntese proteica muscular

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.

Enquanto houver esperança de lutar,
haverá esperança de vencer!
(SANTO AGOSTINHO)

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LISTA DE ABREVIATURAS E SÍMBOLOS

AAs	Amino acids
BCAA	Aminoácidos de cadeira ramificada
CAS	Casein
EAA	Essential amino acids
eIF4B	Eukaryotic translation initiation factor 4B
eIF4E	Eukaryotic translation initiation factor 4E
eIF4F	Eukaryotic translation initiation factor 4F
FSR	Fractional rates of protein synthesis
FOXO	Forkhead transcription factor
GSK3	Glycogen synthase kinase 3
IGF-1	Insulin Like Growth Factor 1
LEU	Leucin
MPS	Muscle protein synthesis
mTOR	Mammalian target of rapamicin
p70S6K	Ribosomal protein S6 kinase beta-1
PI3K	Fosfoinositol 3 kinase
PKB	Protein kinase B
p-4EBP1	Phosphorylation Eukaryotic translation initiation factor 4E-binding protein 1
SP	Soy protein
TSC2	tuberin
VAL	Valine
WP	Whey protein
WPI	Whey protein isolated
WPH	Whey protein hydrolyzed
WPC	Whey protein concentred
4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1

Proteína do soro do leitehidrolisado na síntese proteica muscular

RESUMO

Suplementação com proteína do soro do leite hidrolisado (WPH) é utilizado para estimular a síntese proteica muscular (MPS) via fosforilação da 4EBP1 e consequentemente, o ganho de massa muscular. Contudo, precisa ser esclarecido se o consumo de WPH é superior no estímulo da MPSapós o exercício físico, comparada a outras proteínas.**Objetivo:** Verificar a ativação da 4EBP1 em ratos Wistar alimentados com dieta AIN93-G modificada, tendo proteína do soro do leite hidrolisado (WPH), proteína do soro do leite concentrado (WPC) e Caseína (CAS) comofonte proteica. **Métodos:**Oitenta e quatro ratos Wistar foram divididos em três grupos: WPH, WPC e CAS; e subdivididos em: sedentários, treinados, sedentários exaustos e treinados exaustos. Técnica de Western Blot (WB) foi utilizada para quantificar a ativação da 4EBP1 no músculo gastrocnêmico e foi determinada a concentração de aminoácidos livres no músculo por métodos padrões. Para o tratamento estatístico ANOVA com post- hoc de Ducan foi utilizado. **Resultados:**A fosforilação da 4EBP1 foi superiorpara grupo alimentado com WPH nos grupos levados até a exaustão, quando comparado ao consumo de WPC e CAS. O consumo de WPH e WPC apresentou fosforilação significativamente superior da 4EBP1, quando comparado ao consumo de CAS, no grupo sedentário. Os animais dos grupos treinados não apresentaram diferença significativa na fosforilação da 4EBP1, nos grupos alimentados com WPH, WPC e CAS. O BCAA, especialmente a leucina (LEU), apresentou maiores concentrações livre no músculo após o consumo de WPH, nos ratos sedentários, treinados e exaustos, quando comparado aos grupos alimentados com WPC e CAS. **Conclusão:**A fosforilação da 4EBP1 foi significativamente superior, no músculo gastrocnêmio, em ratos levados até a exaustão e alimentados com WPH, sem diferença significativa nos ratos do grupo treinado. Os dados em conjunto apontam que a regeneração muscular depende do estímulo adequado durante o exercício, como o aumento no volume e intensidade eo consumo de WPH com fonte proteica apresenta resultados superiores na MPS.

Palavras-chave: Proteína do soro do leite; suplementação; caseína; exercício físico.

ABSTRACT

Supplementation with WPH is used to activate muscle protein synthesis by p-4EBP1 thus increasing body growth. However, it is not clear if WPH is higherin regulation mechanism of protein synthesis.**Objective:** The purpose of this study was to assess gastrocnemius 4EBP1 activation in Wistar rats fed standart AIN93-G diet supplemented with WPH, WPC and CAS. **Methods:** Eighty-four Wistar rats were divided into twelve groups and fed one of the following diets for three weeks: a) CAS; b) WPC; c) WPH; Modified AIN93-G diets containing casein, whey protein hydrolyzed and whey protein concentrate as a protein source. The diets were subdivided into 4 groups: sedentary; exercised; sedentary-exhausted and exercised-exhausted. P-4EBP1 phosphorylation was quantified by Western Blot analysis. Free amino acid concentration in muscle was determined by standard methods. ANOVA and post-hoc Duncan were applied to compare the means (significance $p<0.05$). **Results: P**hosphorylated 4EBP1 ofWistar rats were increased by feeding the AIN93-G diet with WPH compared to WPC and CAS to sedentary-exhaustive and exercise-exhaustive groups. Feeding WPC and WPH showing increase phosphorylation of 4EBP1 when compared to CAS in the sedentary condition. P-4EBP1showed no difference between any three groups to trained group. BCAA (specifically, Leucine) were significant increaseto WPH group in free amino acid concentration in muscle, compared to WPC and CAS groups. **Conclusion:**The phosphorylation of 4EBP1 was significantly higher in the gastrocnemius muscle in rats fed to exhaustion and fed WPH, with no significant difference in the rats of the trained group. The data together show that muscle regeneration depends on the appropriate stimulus during exercise, such as the increase in volume and intensity and the consumption of WPH with protein source presents superior results in MPS.

Keywords: whey protein; supplementation; casein; exercise.

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1. INTRODUÇÃO

O exercício exaustivo gera micro-lesões nas células musculares. As micro-lesões musculares ocorrem em resposta à sobrecarga mecânica e metabólica ao treinamento (SOUSA et al., 2014). Após o período de adaptação ao treinamento o organismo produz uma assimilação compensatória na musculatura esquelética exercitada, tornando o músculo apto a repetir contrações sucessivas. Para o aumento da resistência e performance muscular, após o período de adaptação, o estimulo devem apresentar uma sobrecarga mecânica (aumento de intensidade e volume) para continuar estimulando as vias de tradução de sinais e estimular a recuperação muscular (SCHOENFELD, 2010).

O exercício físico aumenta a necessidade de proteína para a reposição de proteínas perdidas e reconstrução da musculatura lesionada. A reorganização da estrutura celular depende do balanço positivo entre a taxa de síntese e a taxa de degradação proteica, portanto, o treino ativa vias de tradução de sinais gerando um aumento da síntese proteica muscular (MPS) (KIMBALL et al., 2006). A regulação das vias de tradução proteica aliada a uma dieta que contenha os aminoácidos essências (EAA), gera um balanço nitrogenado positivo(SANDRI, 2008). Para que o balanço nitrogenado seja positivo é necessária a biodisponibilidade de aminoácidos na circulação sanguínea favorecendo a regeneração muscular. Assim, para que ocorra uma melhora nas respostas agudas e adaptações crônicas do musculo esquelético, o exercício físico deve apresentar o estimulo adequado que desencadeia a ativação ou repressão superior de vias moleculares de sinalização de MPS, necessitando de uma proteína de boa digestão e absorção, que resultariam no aumento da transcrição e a tradução de proteínas (SOUSA et al., 2014).

O reparo do tecido muscular depende do consumo de proteínas de alto valor biológico. Para a recuperação muscular a proteína ingerida deve conter os EAA, responsáveis pela regeneração tecidual, como a proteína do soro do leite (WP), proteina do ovo, caseína (CAS) e proteina da soja (SP) (GORISSEN et al., 2015). Os suplementos a base de proteinas tem ação rapída aumentando os aminoacidos disponíveis na circulação sistemica facilitando o anabolismo de proteína no músculo (KERKSICK et al., 2006). Portanto, a ingestão de proteínas é essencial para a recuperação muscular.Porém o consumo de suplementação proteica acima da necessidade diária, não gera aumento de desempenho e recuperação muscular, pelo contrario, essa proteína adicional é armazenado na forma de gordura corporal.

O aumento no consumo de suplementos proteicos por praticantes de atividade físicagera a necessidade e o interesse, por meio dos pesquisadores, de estudos sobre o efeito e a utilização desses suplementos na recuperação muscular após o exercício. Dentre os suplementos a base de proteína, um suplemento muito utilizado por praticantes de atividade física é WP.O WP é utilizado por atletas para recuperação muscular e/ou hipertofia (TIPTON et al., 2007). Visto que, WP são proteínas de rápida digestão e absorção, que elevam os níveis de aminoácidos plasmáticos e musculares após sua ingestão, auxiliando na recuperação do musculo esquelético. A ingestão de WP após o exercício induziu a hiperaminoacidemia, sinalização de mTOR e MPS (BURKE et al., 2001), Yang et al. (2012) suplementou 30 idosos com 20 e 40g de WP e SP, logo após o exercício de resistido. A população idosa são menos sensíveis aos efeitos anabólicos da dieta e exercício, contudo, foram observados aumentos significativos na capacidade de estimular a MPS em idosos suplementados com WP, do que com SP, 1h após o exercício, nas doses de 20g e 40g.

O estudo com hidrolisados proteicos surge a partir da descoberta de transportadores para di e tri peptídeos intestinais. As proteínas hidrolisadas são fabricadas a partir da proteína intacta, por processo de hidrolise (ácida ou enzimática), são fontes de peptídiosde diversos tamanhos e apresentam rápida taxa de absorção no trato gastrointestinal em comparação às proteínas intactas (MEREDITH et al., 1990). Abecia-Soria et al. (2003) verificou que ratos alimentados com WPH e submetidos a exercício físico até a exaustão em esteira, atingiram a exaustão em maior tempo, quando comparada ao WPI. Entretanto, Ramos (2001) alimentou ratos com WPH submetidos à natação, os resultados não apresentaram diferença significativa na glicose sérica, insulina plasmática, proteínas totais, albumina, triacilgliceróis plasmáticos e colesterol total, quando comparados aos ratos alimentados com WPI ou CAS.Em hipótese, a utilização de WPH apresentaria uma disponibilidade de aminoácidos facilitada na circulação sanguínea, o que resultaria na recuperação muscular melhorada, quando comparada a proteínas intactas, após o exercício.

No desenvolvimento deste trabalho foi realizada uma revisão sistemática na literatura cientifica com a finalidade de verificar a utilização do WPH na MPS. Posteriormente, foi realizado um estudo experimental que mostrou o efeito do consumo do WPH na MPS. Assim, no presente estudo procurou-se de analisar se o consumo de hidrolisados proteicos (WPH) apresenta efeito superior na MPS após o exercício. Além deinvestigar as proteínas do leite, CAS e WPC, e comparar o efeito dessas proteínas na MPS.

2.Revisão de literatura

2.1. Artigo 1: Whey protein hydrolyzed supplementation in muscle protein synthesisduring exercise: a systematic review

Summary: The interest of the supplementation market for the consumption of whey protein to optimize physical and metabolic performance after exercise is increasing. Evidence suggests that the ingestion of the whey protein hydrolyzed (WPH) have higher anabolic properties when compared with other proteins, presenting high biological value and high absorption rate due to the hydrolysis process. This review was performed using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA). Articles were searched for in the Pubmed database and included studies verifying the effects of whey protein hydrolyzed consumption on protein synthesis in response to exercise.Thirteen trials were included in this review. The results showed that the consumption of WPH is superior to that of soy protein (SP), casein (CAS) and only exercise with respect to MPS. Future research comparing WPH with other proteins is needed to define protein source to be used in nutritional interventions to MPS in different populations after exercise.

Keywords: Whey Protein; Protein synthesis; Supplementation; Hydrolysis.

Introduction

Regular exercise promotes progressive muscular physiological adaptation. Each exercise session stimulates specific signaling pathways that regulate transcriptional and translational activity in the cell (SPIERING et al., 2008). Potentiating muscle recovery after exercise is essential to maximize muscle adaptations. The contractile muscle stimulates the secretion of insulin-like growth factor 1 (IGF-1)signaling pathway, responsible for the regulation of skeletal muscle atrophy and hypertrophy.

The IGF-1, connected toreceptor, initiates the muscle signaling cascade (Figure 1). The IGF-1, activates the protein *fosfoinositol 3 kinase* (PI3K), leading to the activation of *protein kinase B* (PKB) ou AKT, the PKB phosphoryl enzymes *glycogen synthase kinase* 3 (GSK3), *forkhead transcription factor* (FOXO) e *tuberin* (TSC2) and inactivating them. The phosphorylation of TSC2 prevents *mammalian target of rapamicin* (mTOR) inhibition.mTOR regulates muscle protein synthesis (MPS) by controlling the phosphorylation of 4EBP1 and p70S6K, key proteins in the process of the protein synthesis.Since phosphorylation of p70S6K activates the ribosomal protein S6, which is responsible for increased translation of messenger RNA and the phosphorylation 4E-BP disconnecting this protein to the initiating factor eIF4B, initiating the translation (COFFEY; HAWLEY, 2007).The literature indicates the key role of protein intake in the balance of metabolic functions after exercise (WILLIAMSON et al., 2006).

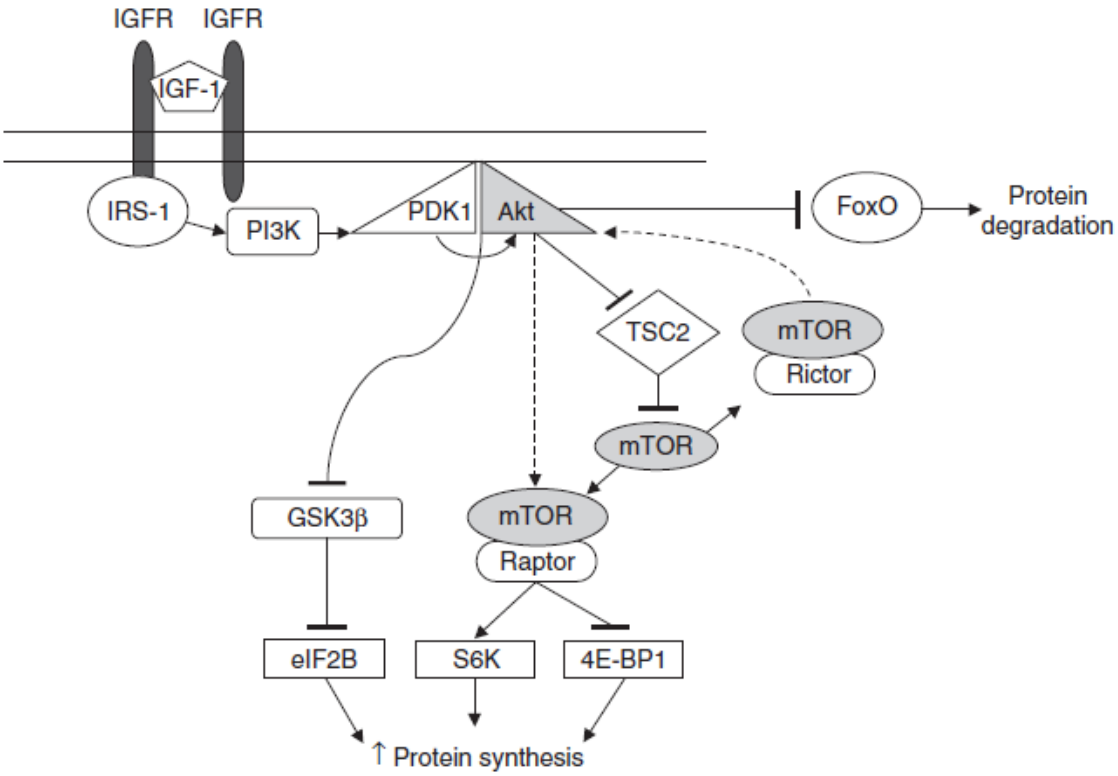

Figure 1. Simplified (IGF)-1 signalling pathway from receptor binding to MPS.Source: (Coffey; Hawley, 2007).

Whey protein (WP) is largely used by athletes for muscle recovery and hypertrophy (TIPTON et al., 2007).The WP supplementation stimulates and maintains muscle growth and strength,and the intake of WP after exercise was shown to induce hyperaminoacidemia, Mammalian target of rapamycin(mTOR) signaling and MPS(BURKE et al., 2001). Anthony et al. (2007) used supplementation of ~0.9g/kg of WP or soy protein (SP) in a high-carbohydrate diet for rats that took part in a 2h endurance exercise session. The results were higher for the induction of MPS 1h after consumption of the WP and SP, but mTOR phosphorylation was in the group that consumed WP than in that which consumed SP.

The WP can be classified according to its degree of purification, being whey protein isolate (WPI) containing content of protein> 90%, whey protein concentred (WPC) with protein content between 35-80%. Whey protein hydrolyzed (WPH) produced from the WPC after the protein is enzymatically hydrolyzed (MARSHALL, 2004).After the discovery

of intestinal transporters for di and tri peptides arises the interest, by the researchers, of the benefit of using pre-broken proteins in muscle recovery after physical exercise.We hypothesized that WPH would raise the stimulation of MPS by the fact that its digestion is facilitated.The purpose of this literature reviewwas to verified the effects of WPH on the MPS after exercise.

Methodology

This systematic review was carried out using explicit methods that allowedfor the selection of articles that analyzed the use of WPHsupplementation in MPS.This systematic review was performed using The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).The articles research was based on PubMed and Web of Sciencedatabase until January of 2019. In this search for articles the terms used for whey protein were: “whey”, “whey protein”, “milk protein” and “milk serum protein”, and the search was again carried out using the “OR” operator between the terms. The terms used for exercise: “physical fitness”, “physical performance”, “physical exercise”, “exercise”, “exercise training”, “physical training”, “exercise program”, “exercise performance”, “resistance training” and “resistance exercise”. The search was carried out using the “OR” operator, between the terms. The terms used for muscle protein synthesis were: “protein synthesis” and “anabolic signaling”, and the search was carried out using the "OR" operator between the terms. The combination of the terms related to soy protein, whey protein and exercise was carried out using the "AND" operator. Studies included were English language,randomized, double-blinded, placebo-controlled trials investigating the effects of WPHsupplementation on measuresofMPSfollowing exercise. In the initial search for the selected terms, 293 potential articles were identified for inclusion in the review. 131 were excluded because they did not contain the combination of terms in the title and abstract. Of

the remaining 64 studies for further evaluation with the use of the inclusion and exclusion criteria. The exclusion criteria were applied and the following studies were excluded: a) reviews; b)studies do not use WPH. Thirteen trials were included in this review. The study selection diagram and the selection steps are shown in Figure 2.

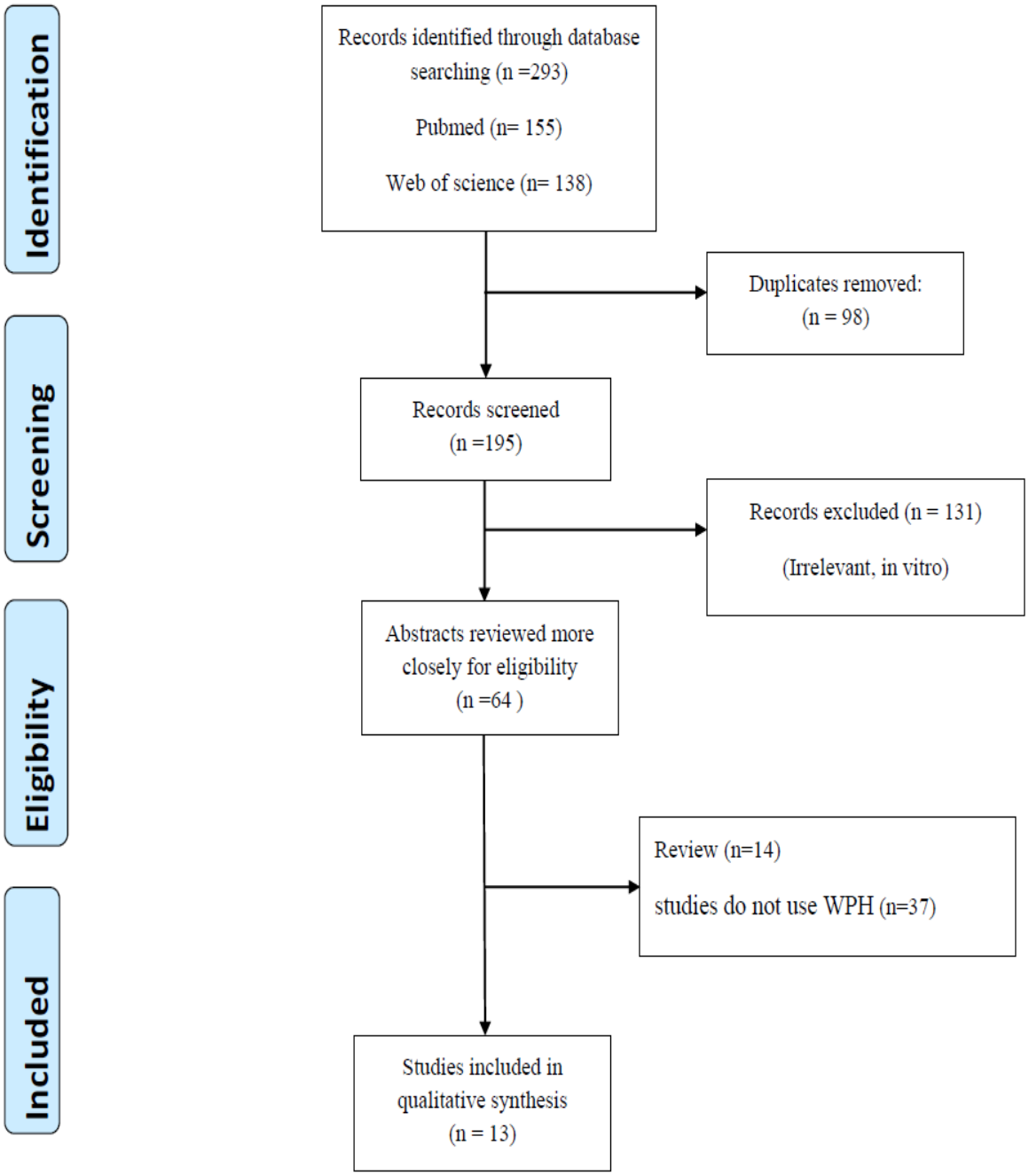

Figure 2.Diagram of selected studies.

Findings

Thirteen studies investigated the effects of WPH supplementation on MPS after resistance and endurance exercise. These studies are compiled with details in Table 1and discussed in this review.

Whey protein in muscle protein synthesis

Supplementation with whey protein is superior to that exercise only for MPS. Four experiments compared the effect of WP intake and exercise on MPS in young subjects, after resistance exercise. The myofibrillar proteins were calculated in the Western blot (WB) test.It was verified that WPH supplementation stimulates a significant increase in p70S6K1 (CAMERA et al., 2015; KAKIGI et al.,2013;MOORE et al., 2011), 4E-BP1 (KAKIGI et al., 2013; COOKE et al., 2011; MOORE et al., 2011) and mTOR phosphorylation (CAMERA et al., 2015; KAKIGI et al., 2013), but Cooke et al. (2011)showedsimilar results in mTORand p70S6K1 phosphorylation.Three experiments compared the effect of WPH intake and exercise on MPS in rats, after resistance exercise. The myofibrillar proteins were calculated in the WB.It was showed that WPH stimulated a significant increase in mTOR and p70S6K1 expression(WANG et al., 2015). However, Wang et al. (2017)showed similar results in mTOR, p70S6K1 and 4E-BP1 phosphorylation. Haraguchi et al. (2014) fed rats with WPH or were fed the AIN-93M standard diet, exercised rats were submitted to a resistance exercise program. It was observed that WPH stimulated muscle protein synthesis via mTOR phosphorylation to ahigher degree than in those only practiced the exercise.

Authors	**Sample**	**Exercise**	**Supplementation / grupos**	**Methods**	**MainFindings**
Tang et al. 2009	Young (19-25 years)	resistance exercise	~0.3g/kg/ WPH or SP	FSR	↑# FSR =# insulin concentration ↑#leucinemia ↑# EAA
Cooke et al. (2011)	Young (20-30 years)	resistance exercise	~0,15g/kg WPH or exercise	WB	↑* phosphorylation of 4E-BP1 =* phosphorylation of 4E-BP1 =* phosphorylation of mTOR ↑* insulin concentration
Moore et al. (2011)	Young (23-30 years)	resistance exercise	~0,30g/kg WPH or exercise	WB	↑* phosphorylation of P70S6K ↑* phosphorylation of 4E-BP1
Kakigi et al. (2013)	Young (20-30 years)	resistance exercise	~0,30g/kg WPH or exercise	WB	↑* phosphorylation of P70S6K ↑* phosphorylation of 4E-BP1 ↑* phosphorylation of mTOR
Haraguchi et al. (2014)	rats	resistance exercise	~0,25g/kg/ WPH or exercise	WB	↑* phosphorylationofmTOR
Wang et al. (2015)	rats	resistance exercise	0,4g/kg/ WPH orexercise	WB	↑* phosphorylation of 4E-BP1 ↑* phosphorylation of mTOR ↑* EAA
Camera et al. (2015)	Young (20-23 years)	resistance exercise	0,3g/kg/ WPH or exercise	WB	↑* phosphorylation of P70S6K ↑* phosphorylation of mTOR ↑* insulin concentration
Mobley et al. (2015)	rats	resistance exercise	~0,25g/kg/ WPH or BCAA (Leu)	WB	=¨ phosphorylation of P70S6K =¨ phosphorylation of 4E-BP1 = phosphorylation of mTOR
Mitchell et al. 2015	13 men (60–75 years)	resistance exercise	~0.5g/kg WPH or SP	WB	↑# phosphorylation of P70S6K
Francaux et al. (2016)	Elderly (68-72 years)	resistance exercise	0,35g/ kg/ WPH or exercise	WB	↑* phosphorylation of mTOR ↑*insulin concentration ↑* leucinemia
Reitelseder et al. (2017)	Elderly (68-70 years)	resistance exercise	0,45g/kg/WPH or CAS	WB	=! phosphorylation of P70S6K ↑! insulin concentration ↑!leucinemia
Wang et al. (2017)	rats	resistance exercise	0,37g/kg/ WPH or exercise	WB	=* phosphorylation of P70S6K =* phosphorylation of 4E-BP1 =* phosphorylation of mTOR
Moura et al. (2017)	rats	running	0,75g/kg/ WPH ou BCAA (LEU-VAL)	WB	↓¨ phosphorylation of 4E-BP1 ↓¨ phosphorylation of mTOR

Table 1. Comparison the effects of the intake of WP in muscle protein synthesis during exercise.**Subtitle:** WP: Whey protein; SP: Soy protein; FSR: fractional rates of protein synthesis; WB: Western Blotting; BCAA: branched- chainamino acids; EAA: essential amino acids; TAA: total of all amino acids; =: no significant differences; ↑: significantly greater; ↓: significantly lower;[#]: WPH vs. SP; *: WPH vs. exercise; ¨: WPH vs. BCAA; [!]: WPH vs. CAS;

Oneexperiment compared the effect of WPH intake and exercise on MPS in elderly individuals, after resistance exercise. The myofibrillar proteins were calculated in the WB.They observed more significant increases in the ability to stimulate MPS in elderly individuals supplemented with WPH than in those only practiced the exercise, indicate that WPH induced a significant increase in mTOR expression (FRANCAUX et al.,2016).

Supplementation with WPH is superior to that with SP for MPS. Two experiments compared the effect of WP and SP intake on MPS, after resistance exercise in humans. The fractional synthetic rates (FSR) of myofibrillar proteins were calculated. Mitchell et al. (2015) supplemented elderly individuals with ~0.5g/kg of WPH and SP. It was observed that WP and SP stimulated muscle protein synthesis via p70S6K phosphorylation, however, they observed more significant increases in the ability to stimulate MPS in elderly individuals supplemented with WPH than in those supplemented with SP, 4h after exercise.Tang et al. (2009) supplemented 18 young subjects with ~0.3g/kg of WPH and SP. It was showed that WPH stimulated MPS to a greater degree than SP after resistance exercise.

Supplementation with WPHand CAS stimulate MPS. One experimentcompared the effect of WPH and CAS intake on MPS, after resistance exercise in elderly individuals. Reitelseder et al. (2017) used supplementation of ~0,45g / kg of WPH and CAS. FSR of myofibrillar proteins were calculated. The results were similar for the induction of MPS via p70S6K phosphorylation, 3h after consumption of the WPH and CAS.

Moura et al. (2017)compared the effect of four branched-chain amino acid-(BCAA)containingdipeptides and WPH intake on MPS, after at the running for 60 min. They observed more significant increases in the ability to stimulate MPSincreased p-4EBP1 and p-mTOR expression, in rats supplemented with BCAA (Leu-Val)than in those supplemented with WPH.Mobley et al. (2015) compared the effect of WPH and LEU intake on MPS3 h following resistance exercise in rats. This study observed that was similar for the induction of MPS via mTOR, p70S6K and 4E-BP1 phosphorylation.

Plasma insulin concentration was higher following WPH as compared to CAS (REITELSEDER et al., 2017) and only exercise (FRANCAUX et al., 2016; CAMERA et al.,2015;COOKE et al., 2011)but one study observed that plasma insulin concentrationwas similar for WPH and SP (TANG et al., 2009).Higher amplitudes in blood leucinemia were achieved following WPH as compared to SP (TANG et al., 2009), CAS (REITELSEDER et al., 2017) and only exercise (FRANCAUX et al., 2016). WPH produced significantly higher blood amino acids levels compared with SP(TANG et al., 2009) and only exercise (WANG et al., 2015).

Discussion

Supplementation with WPH, SP and CAS may accentuate these adaptations and promote an increased cross-section of muscle, known as muscle hypertrophy. However, the ingestion ofWPH results in higher postprandial MPS rates than does the ingestion of SP or only exercise, results in a higher skeletal muscle hypertrophy when performed chronically.Regular resistance exercise promotes progressive physiological muscle adaptation. Each exercise session stimulates specific signaling pathways that regulate transcriptional and translational activities in the cell (SPIERINGet al., 2008). To achieve lean mass gain, MPS must be greater than muscle protein degradation, resulting in a positive

protein balance (ATHERTON; SMITH,2012). Chronic adaptations come from the accumulation of acute effects and are sustained by the regulation of MPS(REYNOLDS;BODINE; LAWRENCE, 2002).

Postprandial MPS measurements are used to verify the maintenance or increase of skeletal muscle mass. Changes in MPS are not a quantitative estimate of skeletal muscle remodeling, but indicate hypertrophy when performed chronically (PHILLIPSet al., 2005).What is known is that mTORC1 activation to enhance MPS. It was verified that increase in MPS activates the protein kinases such as the ribosomal protein of 70-kDa S6 kinase 1 (p70S6K1) and 4E-binding protein-1 (4EBP1) promoting ribosomal binding to mRNA to initiate protein synthesis (MCGLORY; DEVRIES; PHILLIPS, 2016). In this review, the articles showed that WPH, SP, CAS increased MPS, mTOR, p70S6K and 4E-BP1 phosphorylation, but that supplementation with WPH obtained better results than does the ingestion of SP (MITCHELL et al., 2015; TANG et al., 2009) and the articles showed that supplementation with WPH obtained better resultscompared only exercise(WANG et al., 2015; CAMERA et al., 2015; HARAGUCHI et al., 2014; KAKIGI et al.,2013;MOORE et al., 2011; COOKE et al., 2011).However, Reitelseder et al. (2017) showed no significant difference of MPS via p70S6K phosphorylation among CAS and WPH.

WP has high nutritional valuepresenting concentration elevated and the rapid delivery of amino acids into the systemic circulation(KERKSICK et al., 2006). Protein supplementation with WPH, SP and CAS increases the circulating amino acids, making the protein balance positive. Tang e Phillips (2009) found that WP and SP supplementation may increase MPS, but that WP was superior to SP, since the amino acids contained in this protein were available in larger quantities in the bloodstream, facilitating absorption by the muscle cell.The protein digestion rate and absorption kinetics of the ingested protein source after the exercise are important for modulating postprandial MPS. Rapidly digested proteins

support increased rates of MPS (BOS et al., 2003). In theory, WPH would be more readily absorbed than the isolated protein, because they are a source of bioactive peptids, elevating the MPS.This review found a significantly higher increase in MPS after the consumption of WPH than after the consumption of SP and after exercise. However, exhibit no significant difference when compared to CAS.

Insulin supportsin the uptake of amino acids by muscle tissue, facilitating MPS.The increases insulin secretion after exercise influence in increases mTOR activity in muscle (BUTTEIGER et al., 2013).WPHinduced increase in insulin levels compared to CAS(REITELSEDER et al., 2017) and only exercise (FRANCAUX et al., 2016; CAMERA et al.,2015;COOKE et al., 2011). However, one study showed no significant difference in plasma insulin levels among SP and WP (TANG et al., 2009).

Leucine (LEU) is an important essential amino acid responsible for the increase in MPS. It is known that BCAAs (branched chain amino acids), particularly LEU, have an important role as metabolic regulators of MPS (ANTHONY et al., 1999), activating mRNA translation from the rapamycin target (mTOR) (LANEet al., 2017).Lollo et al. (2012) stated that leucine was an important amino acid in the activation of the mTOR and p70S6K pathways. The leucine content of whey protein is higher than that of SPand CAS(12% in WP, 9% in CAS and 8% in SP) (MORIFUJI et al.,2005)(Figure 3).

	Casein	Whey	Soy
Ala	2.79	4.91	4.28
Arg	3.53	2.21	7.74
Asp+Asn	6.51	10.73	11.46
Cys	0.37	1.91	1.31
Glu+Gln	20.63	17.55	18.54
Gly	1.95	1.91	4.12
His	2.97	2.11	2.53
Ile	4.74	5.12	4.54
Leu	8.74	12.04	8.09
Lys	7.16	9.23	6.26
Met	2.60	2.51	1.32
Phe	4.55	3.51	5.41
Pro	11.90	6.22	5.10
Ser	5.58	3.71	5.42
Thr	3.81	4.91	3.93
Trp	1.12	1.91	1.38
Tyr	5.11	3.81	3.86
Val	5.95	5.72	4.70

Figure 3. Amino acid profile of protein sources. Source: Morifuji et al.(2005)

Supplementation with BCAA (Leu-Val)was higher than in those supplemented with WP on MPS. And supplementation with LEUwas similar to WP for the induction of muscle protein synthesis. The results indicated that WPH elevated the plasma leucine levels significantly more than SP (TANG et al.,2009). However, supplementation with BCAA (Leu-Val)was higher than in those supplemented with WPH on MPS. And supplementation with LEUwas similar to WPHfor the induction of MPS. The high amount of these amino acids in WPH partially explains its superior effect on MPS.

Conclusion

The review of the articles indicated that supplementation with WPH stimulated the initiation of MPS and translation to a greater degree when compared to supplementation with SP and exercise. Physical activity and protein ingestion has been shown to sensitize skeletal muscle tissue to the anabolic properties. Only a few studies have compared the MPSresponse to the ingestion of WPH.The proposed lower muscle anabolic properties of SP as opposed to WP sources may be attributed to differences in amino acidcomposition,

especially leucine, and protein digestion and absorption kinetics. Given the small number of articles that compared WPH on MPS, additional research is needed to elucidate the physiological effects the WPH supplementation after endurance or resistance exercise.

3. Objetivo

Geral

Verificar o efeito da alimentação com proteína do soro do leite hidrolisado (WPH) e concentrado (WPC) em ratos Wistar exercitados até a exaustão.

Específicos

Após a aplicação da dieta por três semanas avaliar:

-Anabolismo muscular;

-Ativação da via 4EBP1;

-Concentração dos aminoácidos livre no músculo;

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5. APÊNDICES

5.1. Artigo 2: Whey protein hydrolyzed intake activates 4EBP1 signaling in rats after exercise- exhaustive.

O artigo científico deste item foi submetido à revista *INTERNATIONAL JOURNAL OF FOOD SCIENCES AND NUTRITION (*QualisB1 e FI: 2.31).
<https://www.tandfonline.com/action/authorSubmission?journalCode=iijf20&page=instructions >
Acesso em: 01 de março de 2019.

Abstract: Feeding whey protein (WP) combined with exercise enhances the magnitude of muscle protein synthesis (MPS) over that induced by exercise alone. We hypothesized that feeding with whey protein hydrolyzed (WPH) could increase the phosphorylation of p-4EBP1 in rats. This study has the objective investigate effect of supplementation with WPH, whey protein concentred (WPC) and casein (CAS) via 4E-BP1 phosphorylation, after exhaustive exercise in the treadmill in rats. Eighty four rats Wistar were distributed in twelve groups: WPH, WPC and CAS (sedentary, trained, sedentary-exhausted, trained-exhausted). Individual rats were euthanized at gastrocnemius muscle samples collected for measurement of the 4EBP1 phosphorylation and free amino acids in the muscle. WPH showed increased p-4EBP1 phosphorylation after exhaustive exercise compared with group who ingested WPC and CAS. The p-4EBP1 expression showed that animals fed WPC and WPH showing increase phosphorylation of 4EBP1 when compared to CAS in the sedentary condition. However, no significant difference was found in the trained group. We conclude that exhaustive exercise increases response in the stimulation of MPS, and enhance need of protein to stimulate the recovery of the musculature. And under these conditions, the ingestion of WPH caused significantly greater increases in MPS after exhaustive exercise compared with CAS and WPC.

Keywords: Exercise. 4EBP1 phosphorylation. Muscle protein synthesis. Whey protein.

INTRODUCTION

The physical exercise increases muscle protein synthesis (MPS) and muscle protein breakdown (MPB). When nutrient availability occurs the protein balance becomes positive and MPS occurs (GONZALEZ. et al., 2016). However, the necessity of nutrients after exercise results in the stimulation of the MPB and inhibits the MPS, causing the negative protein balance. The protein intake after exercise is important for stimulating the synthesis and muscle hypertrophy. However, the increase in muscular performance depends on adequate stimulation, with metabolic overload of intensity and volume, not only with the increase in protein consumption. Without appropriate muscle stimulation, the excess of ingested protein will be converted and stored in the form of body fat, going the opposite of the goal.The stimulation of the MPS after exercise depends on the quality of the protein (RASMUSSEN; PHILLIPS, 2003). Therefore, the MPS depends on the availability and absorption of the amino acids contained in this protein.

Several studies indicate that mammalian target of rapamycin (mTOR) is an important regulator of MPS. A variety of stimuli modulate the MPS via mTOR, such as hormones, growth factors, including mechanical and nutritional factors. Phosphorylation mTOR results in to enhanced mRNA translation, throughsignaling cascade ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor (eIF) 4E binding protein 1 (4E-BP1) (KAKIGI, et al.,2013). A 4E-BP1 regulates the activity of the complex eIF4E, preventing its connection in the complex eIF4F. Phosphorylated 4E-BP1 is a marker of activation mTOR signaling (QIN, JIANG, ZHANG, 2016).

Protein-based supplements are used by physical activity practice, in search of rapid resources for muscle recovery and regeneration. WPs are widely used for high biological value, as well as amino acid composition and rapidly absorption kinetics.Studies have shown that exercise and ingestion of WPinduces activation MPS in humans, with stimulation phosphorylation of p70S6K and 4E-BP1 (MOORE, et al., 2011). Several studies showed that the consumption of WP is superior to that of soy protein (SP) and casein(CAS) with respect to MPS (VOLEK et al., 2013; ARISTIZABAl, et al., 2015).

Recent research studies the benefits of using WPH, since smaller peptides improve the time of digestion and absorption of these amino acids in the exercised muscles. In WPH the protein chains are broken into smaller particles (peptides) by hydrolysis, increasing the rate of absorption.Kakigi et al. (2013) verified that WPH stimulated a significant increase in mTOR, p70S6K1 and 4EBP1 expression after resistance exercise. However, Reitelseder et al. (2017) shown a similar for the induction of MPS via p70S6K phosphorylation, after supplementation with WPH and CAS.

The increased consumption WP supplementation by people practice regular exercise and the need additional research on the effects of protein hydrolyzed on muscle recovery and regeneration.Stimulated the development of this work, this study has the objective investigate effect of supplementation with WPH, WPC and CAS via 4EBP1 phosphorylation, after exhaustive exercise in the treadmill in rats.

MATERIALS AND METHODS

Experimental draw

A total of 84 male rats of Wistar specific pathogen free (SPF), recently weaned (21 days) from the Multidisciplinary Center for Biological Research (CEMIB / UNICAMP) were used. The research methodology was approved by the Ethics Committee on Animal

Experimentation (CEUA-UFGD, protocol 25/2017). The animals were kept at the Biological Testing Laboratory (LEB / DEPAN), with controlled temperature (22 ° C + 2), air humidity (50-60%) and inverted light / dark cycle (12 hours) with commercial diet until reaching body weight of 100g and water ad libitum. After this period, the animals consumed the experimental diet, control or standard.Amino acid profile of protein sources (Figure 1).

Amino Acid	CAS	WPC	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
Methionine	2.32	2.51	2.52
Cystine	0.16	1.48	1.6
Isoleucine	4.51	6.97	6.88
Leucine	7.62	10.15	10.14
Valine	5.36	5.81	5.68
Phenylalanine	3.89	2.86	2.78
Lysine	6.62	9.2	9.48
Total BCAA	17.49	22.93	22.7

Figure 1. Amino acid profile of protein. Data exhibited as a percentage.

The experimental diet followed AIN 93-G (American Institute of Nutrition; REEVES, et al., 1993) altered its protein source depending on the group, being whey protein hydrolyzed (WPH) or concentrated (WPC), and casein (CAS) for three weeks. The rats were distributed in 3 groups WPH, WPC and CAS and each group submitted in 4 different protocols: sedentary, trained, sedentary-exhausted and trained-exhausted (Figure 2).

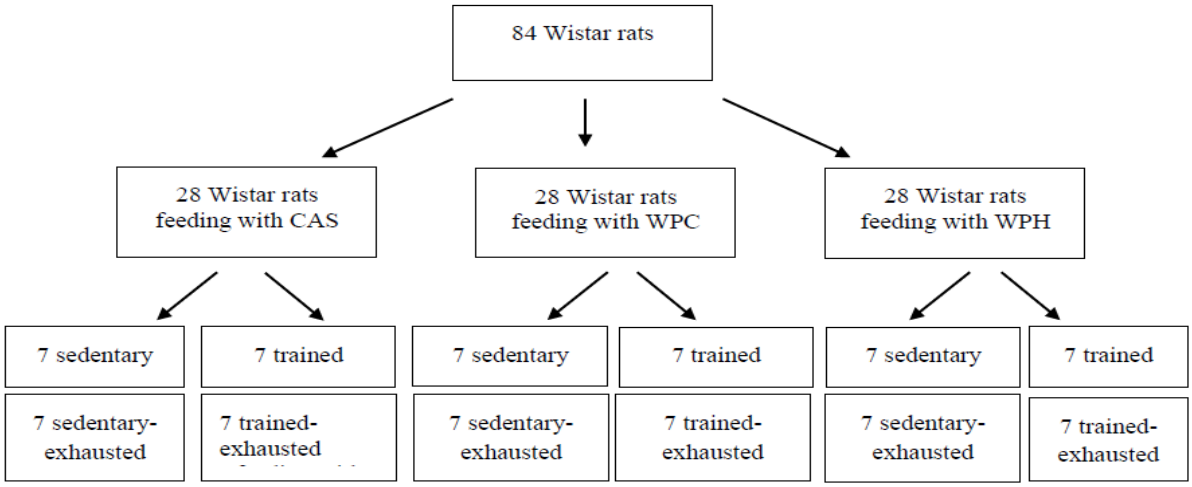

Figure 2. Distribution of groups.

After 3 weeks on experimental diet and training protocol the animals were sacrificed and blood and gastrocnemius muscle was collected 60 min after exhaustion.

Training protocol

The training protocol was suggested by Hohl et al. (2009). The groups of sedentary animals did not exercise, were only fed the experimental diets. The group of sedentary-exhausted animals were placed for 10 minutes of running at 12 m / min, three times a week, in the last week, for adaptation to the protocol. The group of trained animals were placed for exercise: 1st week) 10 minutes of running at 12 m / min; 2nd week) 30 minutes of running at 22 m / min; 3rd week) 45 minutes of running at 22.5 m / min. After, it followed the exhaustion criterion described by Nery-Diez et al. (2010). And then sacrificed.

Analysis

Food monitoring and weight evolution

Food intake was performed during the test, by monitoring consumption and food replenishment. The animals were weighed at the beginning of the experiment (4th and 7th day). After the beginning of the experimental diet, the weight evolution was followed, in which the animals were weighed every 2 days.

Gastrocnemius muscle analysis

Dosage of proteins

The 4EBP1 and p-4EBP1 protein was verified by Western Blot (WB) (KELLEY, et al., 1995). This method quantifies the proteins, separating them by weight (vertical elephoresis) and detection with specific antibodies. Samples were treated with Laemmli buffer containing 10 mM DTT. After electrophoresis, proteins were transferred to 0.45 nM nitrocellulose membrane (BioRad), incubated for 2 h in blocking solution to decrease nonspecific binding of the proteins. After 2h, the membranes were incubated with primary antibodies. After the incubation time, the primary antibodies were removed and the membranes were washed, after which time the membranes were incubated with HRP-conjugated secondary antibody (Invitrogen) for 2h. After washing, the membranes were again incubated in Super Signal developer solution (Pierce) and autoradiography was done by radiographic films (Kodak). The tonicity of the bands were verified by densitometry by the Scion Image program (Scion Corporation). The washes were performed in TBS plus 0.05% Tween, for approximately 5 minutes, three times.

Muscle Amino Acid Profile

For the determination of muscle amino acids, the groups were sampled, three animals from each group were randomly drawn, approximately 32 mg of sample was weighed and subjected to acid hydrolysis with 6N HCl solution in a special ampoule and oven-heated at 110 ° C for 22 hours. After hydrolysis, the material was collected, filtered and evaporated to dryness under reduced pressure (White, et al., 1986; Hagen, et al., 1989).

Statistical treatment

The SPSS 11.0 for Windows software was used for analysis of variance - multivariate ANOVA - using 5% for significance ($p < 0.05$) with Duncan's post hoc test.

RESULTADOS

Weight gain.Growth curves show that administration of WPC and WPH doesn't decrease the weight gain of animals during the experimental period when compared to animals fed standard diet AIN93, with CAS as a protein source. Figure 3 shows that during the experiment period, there was no significant difference in the weight gain of animals submitted to exercise training.

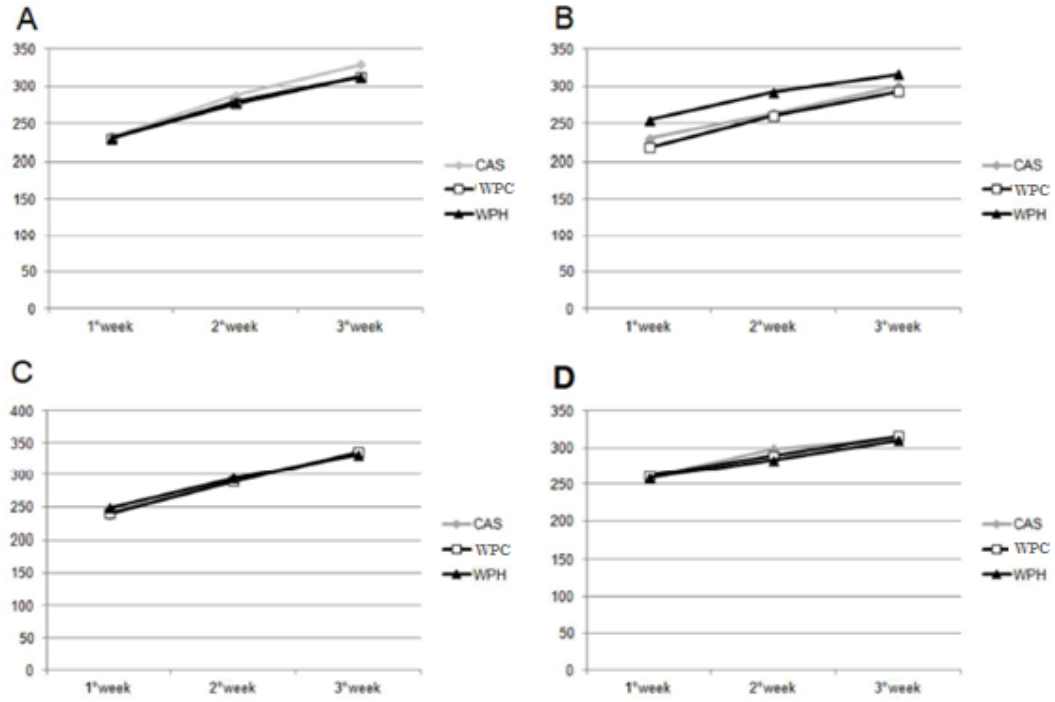

Figura 3. Mean weight of animals in grams over the course of the experiment lasting 3 weeks (n=8). CAS (caseína); WPC (Whey protein concentrate); WPH (Whey protein hydrolysate). A. Sedentário B. Exercised C. Sedentário Exausto D. Exercised Exausto.

Muscle protein synthesis. *4EBP1 and p-4EBP1 expression.*The 4EBP1 and p-4EBP1 activation weresignificantly increased by WPH after exhaustive exercise compared with CAS and WPC in both sedentary and trained conditions. p-4EBP1 phosphorylation was also higher in WPH and WPC than CAS in sedentary rats. There was no difference in p-EBP1 phosphorylation among all three proteins in trained rats (Figure 4 and 5).

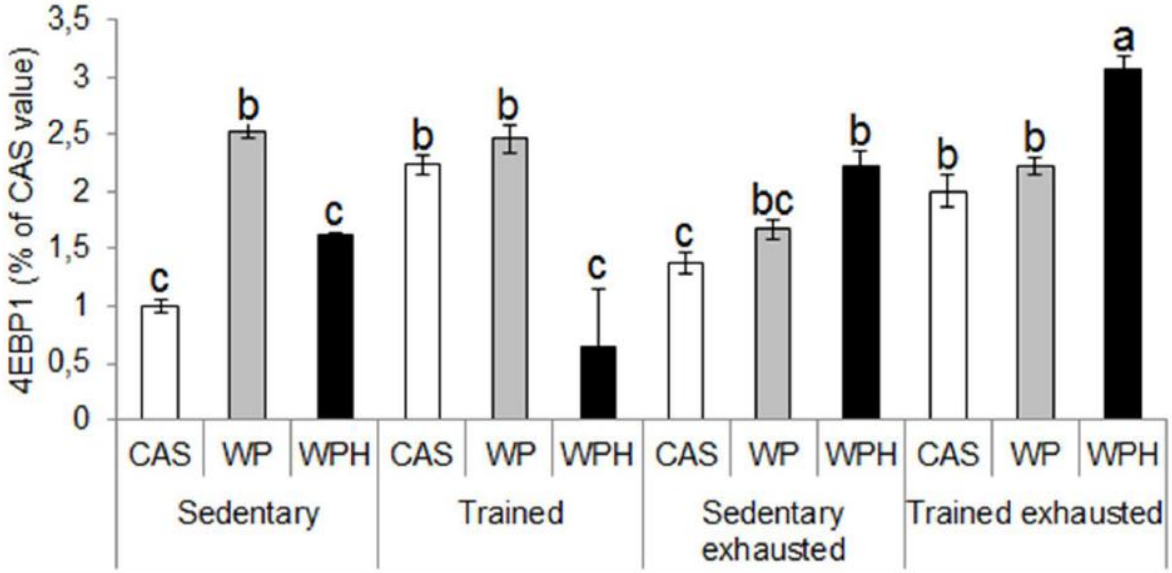

Figure 4. 4EBP1 total expression.Different letters indicate statistical difference ($p \leq 0,05$). CAS (caseína) WPC (Whey proteinconcentrate) WPH (Whey protein hydrolysate). A. Sedentário B. Exercised C. Sedentário Exausto D. Exercised Exausto.ANOVA multivariada de duas vias com *post hoc* Duncan.

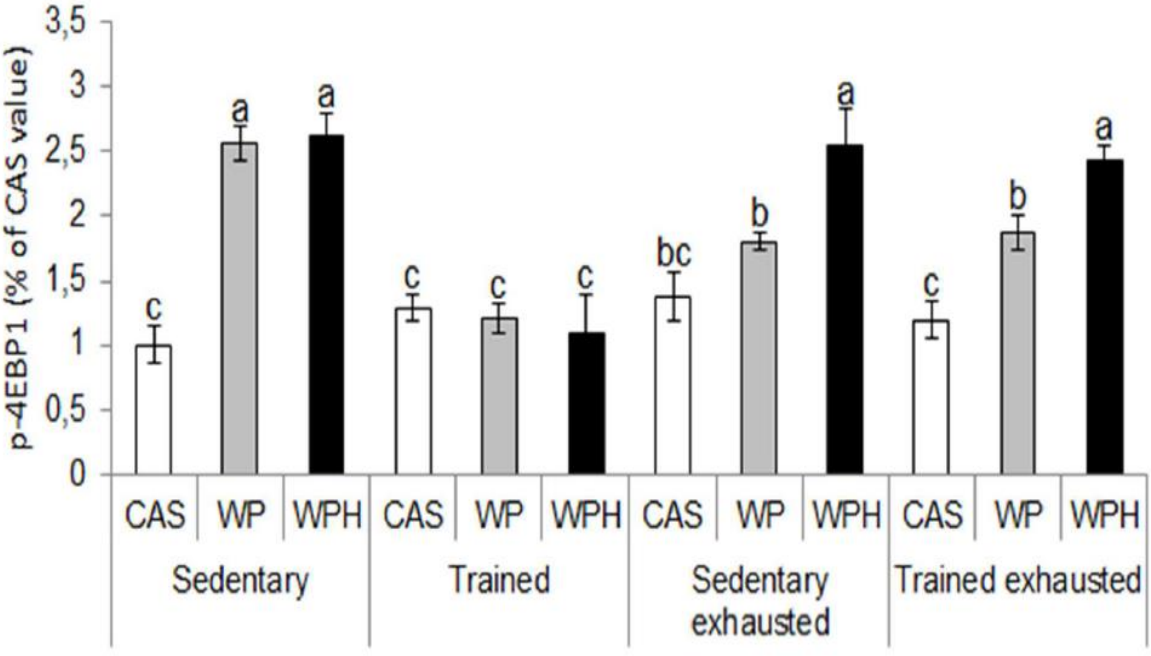

Figure 5. p-4EBP1 expression. Different letters indicate statistical difference ($p \leq 0,05$).CAS (caseína); WPC (Whey proteinconcentrate); WPH (Whey protein hydrolysate). A. Sedentário B. Exercised C. Sedentário Exausto D. Exercised Exausto.ANOVA multivariada de duas vias com *post hoc* Duncan.

MuscleFree Amino Acids.Free BCAA concentrations in muscle were increased in the groups fed with WPH than WPC and CAS in sedentary, exercise and exhaustive conditions. Leucine concentrations in the muscle increased after WPH ingestion compared com WPH and CAS in sedentary, exercise and exhaustive conditions (Figure XX). AAs concentrations in the muscle increased after WPH ingestion compared com CAS in sedentary, exercise and exhaustive conditions (Figure 9).

WPH>WPC>CAS	WPH>WPC≈CAS	WPH≈WPC>CAS	WPH>CAS*
Met	Leu	Trp	Cys
Val	Ile	Phe	Ala
Tyr	Pro	Ser	Tau
Asp	His		
	Gly		
	Asn		

Figure 6. Muscle Free Amino Acids.

DISCUSSION

The main finding this study was that ingesting Whey proteins (WP) activated p-4EBP1 phosphorylation in rats trained or sedentary after exhaustive exercise. The p-4EBP1 expression showed that animals fed WPC and WPH showed increased phosphorylation of 4EBP1 when compared to CAS in the sedentary condition. However, no significant difference was found in the trained group. Specifically, WPH showed increased p-4EBP1 phosphorylation after exhaustive exercise compared with group who ingested WPC and CAS.

The increase of training intensity led to an increase of p- 4EBP1 signaling activation.The exercise-exhaustive group was more effective in increasing this intracellular signaling, resulting in increased MPS than trained group.The exhaustive exercise triggered a greater response in the stimulation of MPS, through the p- 4EBP1 and consequently a greater need of protein to stimulate the recovery of the musculature. So, adaptation to training tends to decrease muscle damage. As shown by Higino et al. (2016), repeating a training protocol up to six weeks after the first initial session, generates less damage. The data together show that the induction of MPS depends on the manipulation of training variables, such as volume and intensity, since trained rats show little change in MPS,

independent of the protein source. Suggesting that, the additional consumption of protein would not result in improved muscle recovery.

Several studies suggested that mTOR activated an important regulatory in MPS. The evidence suggests mTOR phosphorylation regulates translation initiation via 4EBP1 and p70S6K1 activation (FINGAR, et al., 2004). The via 4EBP1 disconnect this protein to the initiating factor eIF4B, initiating the translation (DRUMMOND, et al., 2010). Some studies have verified that after exercise and/or protein ingestion increases the phosphorylation of the 4EBP1 and was correlated with the increase in MPS (DRUMMOND, et al., 2010; HAMARSLAND, et al., 2019). Therefore, 4EBP1 phosphorylation after protein intake and muscle contraction induced by physical exercise increases MPS.

This study, the animals fed with WP increased MPS in the sedentary condition than animals fed with CAS. CAS is the protein used as a protein source in this type of animal (AIN93-G), although it is a slow digestion protein, it contains all the amino acids necessary for the growth and evolution of these animals, so it was used as a control protein. These findings have been observed by other researchers (TANG, et al., 2009).The main explanation for this finding is that WP has more biological requirements than CAS, such as greater digestibility and utilization of its amino acids, facilitating the use of this protein for growth (HARAGUCHI et al. 2014).

In the current study, the ingestion the WP presented no significant difference in the phosphorylation of p-4EBP1 in trained animals. The animals are used in these experiments because of their genetic similarity to humans, facilitation of diet control and profundity analysis of synthesis parameters, which facilitates the understanding of metabolic alterations(Leon, 2005). Contrary to this finding other authors verified that the consumption of WP has a significantly greater effect on MPS in animals or individuals trained (TANG, et al., 2009; CANDOW, et al., 2006; PENNINGS, et al., 2011). The group of trained rats practiced constant exercise, so exercise was not effective in stimulating MPS.

The consumption of WP results in a significant increase in the phosphorylation of p-4EBP1 in the groups after exhaustive exercise. This result has been described previously by other researchers (MOORE, et al., 2011) but not in the studied situation (post-exhaustion). We observed that ingestion of WPH in a significant increase in the phosphorylation of p-4EBP1 after exhaustive exercise than WPC and CAS. However, Reitelseder et al. (2017) found a similar effect of MPS induction after consumption of WPH and CAS in elderly

individuals who participated in a single resistance exercise session, but elderly people are less sensitive to the anabolic effects of diet and exercise (KUMAR, et al., 2012).

The animals fed with WPH increased p-4EBP1 phosphorylation after exhaustive exercise than animals fed with WPC. Since WPH is produced from WPC containing the same concentrations of amino acids, however, after hydrolysis these peptides would be broken down into several sizes, which would facilitate digestion by the gastro intestinal tract and, consequently, a greater availability of these amino acids to the MPS. However, no studies were found comparing the effect of both proteins on MPS in the studied condition.

The ingestion of an ‘‘unbalanced’’ AAs profile results in less free AA concentrations in the systemic circulation to support the postprandial increase in MPS (VAN VLIET,et al., 2015). Protein supplementation with WP increases the circulating amino acids, making the protein balance positive. Some articles found a significantly higher increase in the concentration of AAs after the consumption of WP than after the consumption of SP and CAS (TANG, et al., 2009; KANDA, et al., 2016; ANTHONY, et al., 2007). WP contains abundant EAA, SP and CAS contains fewer EAAs than WP. Thus the WP increased the concentration of EAAs (especially LEU) in the blood, facilitating absorption by the muscle, because it activates mTOR phosphorylation via protein transporters enhancing MPS. In the current study, ingestions of WPH increase the concentration of free muscle EAAs (especially LEU) than CAS and WPC.

We concluded, the consumption of WPH results in a significant accelerates MPS in the groups of rats that practiced exercise until exhaustion compared with CAS and WPC. We found that the use of protein supplementation is necessary only when the metabolic stimuli are superior to adapted, and under these conditions, the use of WP, a protein of rapid digestion and absorption, cause a superior result in repair and reconstructionmuscle.This increase in MPS could have been caused by a rise in concentration of AAs, especially leucine, facilitating absorption by the muscle. The combined data indicates that WPH may be more anabolic than WPC or CAS.

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6 ANEXOS

6.1PARECER DE APROVAÇÃO DO COMITÊ DE ÉTICA

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
PRÓ-REITORIA DE ENSINO DE PÓS-GRADUAÇÃO E PESQUISA

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Dourados-MS, 28 de novembro de 2017.

CERTIFICADO

Certificamos que a proposta intitulada "***Efeitos do consumo de proteína de soja e probiótico nas lesões musculares"***, registrada sob o protocolo de nº 25/2017, sob a responsabilidade de *Pablo Christiano Barboza Lollo e Suelen Maiara Medeiros da Silva* – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo *Chordata, subfilo Vertebrata* (exceto o homem), para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovada pela Comissão de Ética no Uso de Animais (CEUA/UFGD) da Universidade Federal da Grande Dourados, em reunião de 18/08/2017.

Finalidade	*( ) Ensino (X) Pesquisa Científica*
Vigência da autorização	*15/01/2018 a 01/05/2019*
Espécie/linhagem/raça	*Rattus norvegicus - Wistar*
Nº de animais	56
Peso/idade	21 dias
Sexo	machos
Origem	Universidade Federal da Grande Dourados-UFGD

Melissa Negrão Sepulvida

Melissa Negrão Sepulvida
Coordenadora CEUA