Eccentric resistance training and beta-hydroxy-beta-methylbutyrate free acid affects muscle PGC-1 alpha expression and serum irisin, nesfatin-1 and resistin in rats

SHIRVANI, Hossein, RAHMATI-AHMADABAD, Saleh, BROOM, David Robert <http://orcid.org/0000-0002-0305-937X> and MIRNEJAD, Reza

Published version

SHIRVANI, Hossein, RAHMATI-AHMADABAD, Saleh, BROOM, David Robert and MIRNEJAD, Reza (2019). Eccentric resistance training and beta-hydroxy-beta-methylbutyrate free acid affects muscle PGC-1 alpha expression and serum irisin, nesfatin-1 and resistin in rats. The Journal of Experimental Biology (JEB), 222 (10), jeb198424-jeb198424.

Eccentric resistance training and β-Hydroxy-β-methylbutyrate free acid affects muscle PGC-1α expression and serum irisin, nesfatin-1 and resistin

Hossein Shirvani[1], Saleh Rahmati-Ahmadabad[2*], David Robert Broom[3], Reza Mirnejad[4]

[1]Exercise Physiology Research Center, Life Style Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran.

[2]Department of Physical Education, Pardis branch, Islamic Azad University, Pardis, Iran

[3]Academy of Sport and Physical Activity, Sheffield Hallam University, Sheffield, United Kingdom

[4]Molecular Biology Research Center, Systems Biology and Poisoning Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran

Running head: Exercise and HMB-FA affects releasing peptides

Summary Statement: Eccentric resistance training and HMB-FA supplement may induce crosstalk between releasing peptides from other tissues and increases maximal strength. Their combination has greater effect compared to each intervention alone.

***Corresponding Author: Saleh Rahmati-Ahmadabad**
Department of Physical Education, Pardis Branch, Islamic Azad University, Tehran Province, Bumehen Highway, Pardis, Iran
Post Office Box: 1658174583
Tel: +98-21- 76281010-11, **Fax:** +98-21- 76281010
Email: salehrahmati@pardisiau.ac.ir
ORCID ID: 0000-0001-8751-1759

Abstract

The hypothalamus controls metabolism and feeding behavior via several signals with other tissues. Exercise and supplements can change hypothalamic signaling pathways, so the present study investigated the influence of eccentric resistance training and β-Hydroxy-β-methylbutyrate free acid supplement on PGC-1α expression, serum irisin, nesfatin-1 and resistin concentrations. Thirty-two male rats (8 weeks old, 200±17 g body mass) were randomized to control (CON), β-Hydroxy-β-methylbutyrate free acid (HMB) supplementation, eccentric resistance training (ERT), and β-Hydroxy-β-methylbutyrate free acid supplementation plus eccentric resistance training (HMB+ERT) groups. Training groups undertook eccentric resistance training (6 weeks, 3 times a week) and supplement groups consumed HMB-FA orally (76 mg/kg/day). Twenty-four hours after the last training session, rats were sacrificed after which serum and triceps brachii muscle were collected and sent to the laboratory for analyses. Two-way ANOVA and Pearson correlation were employed (significant level: $P< 0.05$). The results showed that eccentric resistance training increases skeletal muscle PGC-1α gene expression, as well as serum levels of irisin and nesfatin-1 ($P= 0.001$). Eccentric resistance training decreases serum concentration of resistin ($P= 0.001$). HMB-FA supplement increases skeletal muscle PGC-1α gene expression ($P= 0.002$), as well as serum concentartion of irisin and nesfatin-1 ($P= 0.001$). HMB-FA decreases the serum concentration of resistin ($P= 0.001$). Significant correlations were observed between PGC-1α gene expression and serum concentrations of irisin, nesfatin-1 and resistin. Generally, HMB-FA with eccentric resistance training may induce crosstalk between releasing peptides from other tissues and increases maximal strength. Their combination had a more substantial effect than each intervention in isolation.

Keywords:

Exercise; HMB supplement; Maximal strength; PGC-1α signaling pathway; Resistance training; Tissue crosstalk

Introduction

Energy homeostasis is an important aspect of bioenergetics which can be defined as an equilibrium of energy intake and energy expenditure (Lam and Ravussin 2016). The hypothalamus controls metabolism, feeding behavior (Timper and Bruning 2017) and body mass via several pathways that affect appetite including Peroxisome proliferator-activated receptor gamma coactivator (PGC-1α) (Hu et al. 2016, Park and Ahima 2015). PGC-1α is a key signaling pathway in the metabolism of carbohydrate, lipids and the regulation of cellular energy (Liang and Ward 2006). In addition, it stimulates mitochondrial biogenesis and promotes the remodeling of muscle tissue via changes to fiber-type composition (Zhang et al. 2017). It is plausible that PGC-1α affects irisin, nesfatin-1 and resistin which are peptides involved in energy homeostasis (Shirvani and Arabzadeh 2018).

The myokine Irisin is predominantly produced by skeletal muscle after physical exercise, and creates crosstalk between tissues. In particular, muscle-fat crosstalk changes the phenotype of white adipose tissue (converting white fat into brown fat) and induces body mass loss (Fukushima et al. 2016). Irisin has been reported to activate thermogenic programs in white adipose tissue and improve glycemia, which is dependent on PGC-1α (Bostrom et al. 2012). Thus, elevated irisin has been posited to be a possible anti-obesity agent (Spiegelman 2013). Nesfatin-1 is an anorexigenic protein likely to activate the melanocortin pathway and its involved in the regulation of blood glucose, improves insulin sensitivity, energy homeostasis, and metabolism (Dore et al. 2017, Myers 2006, Oh et al. 2006). Intracerebroventricular injection (ICV) of nesfatin-1 inhibited food intake in a dose-dependent manner results in a decrease in total body fat and body mass loss, while anti-nesfatin-1 has increased the intake of food in male rats (Oh et al. 2006). It was reported that nesfatin-1 promotes the differentiation of brown adipocytes through the PGC-1α (Wang et al. 2016). Hypothalamic resistin seems to be a key regulator of the brain-fat axis which regulates energy homeostasis (Rodriguez et al. 2018). ICV infusion of resistin reduced epididymal fats and increased peripheral insulin sensitivity (Park et al. 2008). Resistin modulates food intake, hypothalamic and peripheral lipid metabolism (Nogueiras et al. 2010). It was reported that resistin regulates fatty acid B oxidation by suppressing expression of PGC-1α (He et al. 2018).

In the last decade, the use of supplements such as β-Hydroxy-β-methylbutyrate free acid (HMB) to promote fat loss and muscle growth has increased. HMB is an active metabolite of the nutritionally essential branched-chain amino acid (BCAA) leucine that has an anticatabolic role for muscle (reduces breakdown of muscle cell proteins) (He et al. 2016). There is evidence to support the inhibitory effects of HMB on dexamethasone-induced increase in protein

degradation and decrease in protein synthesis were regulated by p38/MAPK- and PI3K/Akt-dependent cell signaling, respectively (Aversa et al. 2012). It was demonstrated that leucine-polyphenol combinations stimulate irisin release and browning of adipose tissue (Brooke Baggett et al. 2013). To the authors knowledge, there has been no study investigating the effects of HMB on nesfatin-1 and resistin. Overall, HMB is effective in the regulation of many cellular processes such as protein synthesis and energy metabolism (Yin et al. 2010, Li et al. 2011, Duan et al. 2016, Wilson et al. 2013). HMB has numerous forms including HMB-FA and HMB-CA. HMB-FA is as dietary supplement in the free acid form and has more bioavailability compared to HMB-CA, which is a monohydrated calcium salt of the conjugate base (Wilson et al. 2013, Fuller et al. 2015). HMB supplementation has been shown to increase muscle size (Wilson et al. 2012), and enhances force production during recovery from an injury that is created by disuse-reloading (Alway et al. 2013).

Exercise has numerous influence on multiple gut peptides and consequently energy balance (Dorling et al. 2018). Studies have investigated different modes of exercise training on PGC-1α (P. C. Dinas et al. 2017, Jung and Kim 2014, Norheim et al. 2014), irisin (P. C. Dinas et al. 2017, Norheim et al. 2014, Samy et al. 2015), nesfatin-1 (Algul et al. 2017, Ghanbari Niaki et al. 2013, Ghanbari-Niaki et al. 2010, Mogharnasi et al. 2018) and resistin (Cobbold 2018, Shafiee and Sharifi 2017, Garcia-Hermoso et al. 2017). The effects of HMB on these factors has not been investigated widely. In addition, the combination of exercise and supplement may have different results then each intervation alone. The aim of the present study was to investigate the influence of eccentric resistance training and β-Hydroxy-β-methylbutyrate free acid supplement on PGC-1α expression, serum irisin, nesfatin-1 and resistin concentrations in rats.

Material and methods

Permissions

The present study was conducted with the written permission of the research deputy of Baqiyatallah University (ethical code: IR.BMSU.REC.1394.82) and was in accordance with National Institutes of Health (NIH) publication.

Animals and design

Thirty-two male rats (Sprague Dawley family, 8 weeks old, 200±17 g weight) were used in this cross-sectional study. Animals were kept in the Baqiyatallah University of Medical Science in the animal houses in special cages

where the floor was covered with clean wood chips. The temperature was 22 (± 2 ^{o}C), humidity between 45-50% with a lighting-dark cycle of 12 hours light followed by12 hours darkness. Special standard compressed food (Behparvar of Karaj) for laboratory rats (crude protein: 19.50-20.50%, fat: 3.5-4.5%, fibre: 4-4.5%, calcium 0.95-1%, phosphorus: 0.65-0.7%, salt: 0.5-0.55%, lysine 1.15%, methionine: 0.33%, threonine: 0.72, tryptophan: 0.25, energy: 16.16-17 mJ/kg) was provided at regaular times. The cages were fitted with urban filtered water in bottles of 500 ml. Rats were randomized into four groups (8 in each group) including control (CON), β-Hydroxy-β-methylbutyrate free acid supplementation (HMB), eccentric resistance training (ERT), and HMB supplementation plus eccentric resistance training (HMB+ERT). The training groups undertook eccentric resistance exercise training on a ladder while control groups activity was limited to light intensity activity (i.e. walking around the cage).

Thirty minutes prior to the exercise training the HMB groups orally consumed freely force fed the supplement (Beta-TOR, USA) at a dose of 76 mg/kg/day while non-supplement groups orally consumed a saline palcebo. The dosage equivalent in human studies is 3 to 6 g/day for an 80 kg person (Gallagher et al. 2000).

One-repetition maximum measurement

In the first session, one-repetition maximum (1RM) was considered as 50% of the rats body mass, as has been used previously (Gil and Kim 2015). On completion, the final load of the first session was recorded as the 1RM for the next session (Fig. 1).

Training protocol

Eccentric resistance exercise training was performed using a ladder (Manufactured by the Exercise Physiology Research Center, Life Style Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran). The ladder was made of wood with iron steps which had a height of 1.1 m, an inclination of 80 degrees and consisted of 26 steps in total. The ladder was designed to make the rats descend the ladder while imposing a constant load. This protocol has been used in previous research (Gil and Kim 2015). The rats performed 10 to 12 dynamic movements (repetitions) during each landing so the intensity is different. Rats exercised on the ladder with a free load for a week, standardized as a pre-training adaptation and to allow the rats to become accustomed to the exercise. After that, the rats performed the ladder descent exercise with a weighted backpack. The exercise was loaded as follows: one repetition of ladder exercise was conducted at 50%, 75%, 90%, 100% and 120% of 1RM, after which 30g was added

for each trial up to eight trials. Training ended before the 8th trial when rats showed signs of exhaustion, such as unable to descend, or were hanging from the ladder. Eccentric resistance exercise was performed three times a week for six weeks for a duration of 25 minutes per session.

Rat sacrifices, serum and triceps brachii muscle collection

Exactly twenty-four hours post the last training session, rats were anesthetized with intraperitoneal administration of a mixture of ketamine (supplied by Iranian company: Shiraz Iman Saba, Made in Holland, 30 – 50 mg/kg body mass) and xylazine (supplied by Iranian company: Shiraz Iman Saba, Made in Holland, 3 – 5 mg/kg body mass). Blood was collected into tubes and immediately processed for serum preparation during 10 min centrifugation at $1000 \times g$. Serum was then stored at -80 ^{o}C for future analysis. Triceps brachii muscle was excised, cleaned, divided into three pieces, washed in ice-cold saline, and immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Serum analysis

Serum concentrations of irisin and nesfatin-1 were analyzed using ELISA (BioVendor Laboratory Medicine, Brno, Czech Republic) standard operating procedures. The kit sensitivity for irisin and nesfatin-1 was 0.01 ng/ml and 14 ng/ml respectively. Irisin and nesfatin-1 kit inter and intra assay coefficients of variation were 10% and 8% respectively. Serum resistin concentation was analyzed by ELISA (Biovendor Research and Diagnostic Products, Czech Republic) standard operating procedures. The resistin kit sensitivity was 0.25 ng/ml. The inter and intra assay coefficients of variation were 7% and 5% respectively.

Evaluation of gene expression

RNA extraction was performed by RNA purification kits (AccuZol, Bioneer, Cat. No: k3090, Korea) and 85 to 95 mg of triceps brachii muscle was used for each sample. Complementary DNA (cDNA) making was performed by cDNA synthesis kit (AccuPower RT PreMix) according to the manufacturer's instructions and oligo-$(dt)_{18}$ primers (0.25 μg per reaction). Real-time PCR was performed by light Cycler apparatus (Corbet Real time PCR machine, Australia). QuantiFast SYBR Green PCR Kit (Cat. No. 204052; Qiagen, GmbH, Germany) in using 15 μL reaction was used. The 15 μL reaction contained 0.5 μL single-strand cDNA, 7.5 μL Master Mix, 1 μL of the each forward

and reverse primers (5 pmol/µL), and 5 µL dH2O. PGC1α sense primer was 5′-GACCCTCCTCACACCAAAC-′3, and antisense primer was ′5- GCGACTGCGGTTGTGTATG -′3 (Shi et al. 2013). The β-actin sense and antisense primers were ′5-TATCGGCAATGAGCGGTTCC-′3 and ′5- CACTGTGTTGGCATAGAGG-3′, respectively (Rahmati-Ahmadabad et al. 2017), which were used as normalizer gene.

Statistical analysis

Real-time PCR cyclic threshold (CT) was analyzed by the Pfaffl method (Pfaffl 2001). All data was stored and analyzed using SPSS software, (IBM, version 24). The Kolmogorov–Smirnov test was used to assess data distribution and Levene's test was used to assess the equality of variances. Repeated measures ANOVA was used to identify any difference inrats' body mass for the duration of the study as well as changes in 1RM. In order to infer differences between groups, two way ANOVA and Tukey Post *hoc* test was used. Correlations were calculated using Pearson Product Moment correlation. Due to the low sample size non parametric tests inlcuding the Freidman test and spearman correlation were also conducted but this did not alter the interpretation of the findings so only the results of the parametric tests are presented. Effect size (ES) was reported to emphasize the size of the difference rather than confound the sample size. Significance was accepted if $P < 0.05$. Data are presented as mean ± standard deviation (SD) unless otherwise stated.

Results

There was no difference in body mass between groups (F (5, 140) = 0.40, P= 0.84; ES= 0.01) (Tab.1).

Table. 1: Rat body mass in control (CON), β-Hydroxy-β-methylbutyrate free acid supplementation (HMB), eccentric resistance training (ERT), and β-Hydroxy-β-methylbutyrate free acid supplementation plus eccentric resistance training (HMB+ERT) groups. N = 8 in each group.

Groups	Week 1 body mass (g)	Week 2 body mass (g)	Week 3 body mass (g)	Week 4 body mass (g)	Week 5 body mass (g)	Week 6 body mass (g)
CON	205.50±16.93	216.37±15.01	225.37±16.40	242.12±14.77	267.75±14.72	280.37±16.49
HMB	197.63±17.71	206.75±18.94	217.62±18.67	236.25±18.17	259.75±16.16	271.37±18.11
ERT	202.62±17.66	214.87±19.11	223.01±19.79	240.62±19.97	267.12±20.06	278.75±19.85
HMB+ERT	195.87±16.96	205.25±17.01	213.62±18.70	232.62±15.46	258.37±16.62	269.62±15.93

The mean weekly 1 RM of the exercise training groups initially (week 1, 2, 3) showed similar levels, as can be seen in Fig. 1. 1RM was significantly higher in HMB+ERT compared ERT group in week 4 (998.68± 97.98 Vs 1113.62± 81.30 g, F (1, 14) = 6.52, P= 0.02; ES= 0.31), 5 (179538± 180.56 Vs 2033.89± 183.61 g, F (1, 14) = 6.86, P= 0.02; ES= 0.32) and 6 (2150.56± 214.30 Vs 2433.63± 217.91g, F (1, 14) = 6.85, P= 0.02; ES= 0.33) (Fig.1).

Training groups had higher tissue PGC1α than non training groups (F (1, 28) = 93.74, P= 0.001; ES= 0.77) (Fig. 2A). PGC1α gene expression was significantly higher in HMB groups than non-supplement groups (F (1, 28) = 11.59, P= 0.002; ES= 0.29). Eccentric resistance training and HMB supplementation has the greatest PGC1α gene expression (F (1, 28) = 5.52, P= 0.02; ES= 0.16) (Fig. 2A).

For serum irisin, data analysis showed that there was a higher concentartion in training groups compared to non-training groups (F (1, 28) = 104.78, P= 0.001; ES= 0.78). (Fig. 2B). Results showed that serum irisin was significantly higher in HMB groups than control (F (1, 28) = 22.59, P= 0.001; ES= 0.44). The highest irisin was for HMB + ERT (F (1, 28) = 4.53, P= 0.04; ES= 0.13) (Fig. 2B).

For serum nesfatin-1, data analysis showed higher concentartion in training groups compared to non-training groups (F (1, 28) = 31.46, P= 0.001; ES= 0.52). (Fig. 2C). The results showed higher concentartions of serum nesfatin-1 in HMB groups than non-supplement groups (F (1, 28) = 34.76, P= 0.001; ES= 0.55). The highest serum nesfatin-1 concentration was in the HMB + ERT group (F (1, 28) = 18.87, P= 0.001; ES= 0.40) (Fig. 2C).

For serum resistin, data analysis showed that there was a lower concentrationin in training groups compared to non-training groups (F (1, 28) = 63.44, P= 0.001; ES= 0.69) (Fig. 2D). Results showed that serum resistin was significantly lower in HMB groups than non-supplement groups (F (1, 28) = 34.09, P= 0.001; ES= 0.54). The lowest serum resistin concentration was in HMB + ERT (F (1, 28) = 18.01, P= 0.001; ES= 0.39) (Fig. 2D).

Positive correlations between muscle PGC-1α gene expression and plasma irisin and nesfatin-1 were observed but there was a negative correlation with plasma resistin (Tab.2).

Table 2: Pearson's correlation coefficients of PGC-1α mRNA to other variables.

Variable / Group	PGC-1α gene expression		
	Serum **Irisin**	Serum **Nesfatin-1**	Serum **Resistin**
CON	$r = 0.10$ $P = 0.42$	$r = 0.21$ $P = 0.32$	$r = 0.18$ $P = 0.32$
HMB	$r = 0.54$ $P = 0.12$	$r = 0.48$ $P = 0.12$	$r = -0.54$ $P = 0.14$
ERT	$r = 0.63$ $P = 0.09$	$r = 0.60$ $P = 0.10$	$r = -0.86$ $P = 0.05$
HMB+ERT	$r = 0.95$ $P = 0.01$ *	$r = 0.85$ $P = 0.01$ *	$r = -0.89$ $P = 0.01$ *

*$P<0.05$

Discussion

The findings of this study showed that eccentric resistance training resulted in greater skeletal muscle PGC-1α relative gene expression, increases serum concentrations of irisin and nesfatin-1 and decreases serum concentrations of resistin compared to control. In addition, HMB supplement resulted in increased skeletal muscle PGC-1α relative gene expression, increased serum concentrations of irisin and nesfatin-1, and decreased serum concentrations of resistin compared to control. The most important findings of the present study showed that a combination of eccentric resistance training and HMB supplement had a cumulative and greater effect on variables compared to exercise or HMB supplement alone.

There was a positive correlation between muscle PGC-1α gene expression with serum irisin and nesfatin-1 and a negative correlation with serum resistin. Resistance training and HMB supplementation increses 1 RM whilst no significant changes occurred in rat body mass. It appears that eccentric resistance training with and without HMB supplement can affect signalling pathways via crosstalk between tissues to increase strength.

Different modes of exercise training can affect PGC-1α gene expression, but resistance training has little effect on AMPK/PGC-1α pathway (Jacobs et al. 2014). Resistance training increases the phosphorylation of the anabolic Akt/mTOR signaling pathway, as well as the activation of the translation initiation regulators p70 S6k, 4E-BP1, and eIF2B (Atherton et al. 2005). In contrast, aerobic endurance exercise increased phosphorylation of AMPK and protein levels of PGC-1α (Atherton et al. 2005). However, in the present study, we observed enhanced PGC-1α

gene expression in response to eccentric resistance training due to the similarities with aerobic endurance training as both are able to act via the AMPK/PGC-1α pathway.

Results of previous studies indicates that physical training can increase irisin. Daskalopoulou et al. (Daskalopoulou et al. 2014) found plasma levels of irisin increased in response to increased exercise load by running on a treadmill in active, young people. Also, Boström et al. (Bostrom et al. 2012) highlighted that irisin increased after three weeks of aerobic training in rats and led to an increase in energy expenditure and improved glucose homeostasis. Huh et al. (Huh et al. 2012) demonstrated that after 30 minutes of speed activity, concentrations of irisin increased significantly. To the authors knowledge, this is the first study to report an increase in serum irisin concentation following chronic eccentric resistance exercise. in rats. The results of this study are consistent with the results of previous studies that investigate responses of other types of exercise training.

Plausible mechanisms for how exercise can increase irisin have been posited. Researchers have shown that exercise increases PGC-1α levels in skeletal muscle and increases the muscle-bearing FNDC5 membrane protein that results in the production of irisin (Schnyder and Handschin 2015). AMPKs activation during exercise is one of the factors for increasing PGC-1 α and irisin (Chavanelle et al. 2017). AMPKs activation leads to the phosphorylation of PGC-1α as FNDC5’s modifier and irisin secretion (Petros C. Dinas et al. 2017). Also, PGC-1α activates PPAR γ. PPAR γ is involved in energy metabolism and stimulates FNDC5 and irisin increase (Panati et al. 2016). It is highlighted that there is a relation between irisin amounts and precursor of FNDC5 and PGC-1α (Petros C. Dinas et al. 2017). The results of the present study showed a significant and positive correlation between PGC-1α gene expression and plasma concentrations of irisin. The eccentric resistance training is lilely to activate the PGC-1α activating signals, which may trigger a signal cascade to change the phenotype of the adipose tissue. Eccentric resistance training leads to energy consumption and heat production by increasing muscular tissue ratio to fat tissue and increasing UCP1 (Chavanelle et al. 2017) thus increasing PGC-1 α , FNDC5, and irisin (Petros C. Dinas et al. 2017).

Production and secretion of irisin from the muscle is also mediated by SMAD3 (mothers against decapentaplegic homolog 3). SMAD3 is a molecule that changes energy metabolism and regulates body mass. SMAD3 suppresses FNDC5 and PGC-1α in skeletal muscle and negatively regulate plasma irisin (Tiano et al. 2015). Exercise induces

phosphorylation of SMAD2 and Subsequently SMAD3 (Tiano et al. 2015). However, SMAD3 was not measured in the present study so future research should investigate this possible mechanism for increasing irisin in response to eccentric resistance training.

Ghanbari-Niaki et al. (2013) evaluated the effect of eight weeks of endurance training (five days a week for 60 minutes at a speed of 25 m/min with a zero gradient) on tissue nesfatin-1 gene expression and plasma levels of nesfatin-1 (Ghanbari-Niaki et al. 2013). Their results indicated that training increased the expression and plasma levels of nesfatin-1, which was related to plasma HDL concentration. Nesfatin is involved in the regulation of blood glucose, improves insulin sensitivity, energy homeostasis, and metabolism (Dore et al. 2017). The effect of exercise on nesfatin-1 has not been clearly recognized and not yet studied in response to eccentric resistance training. However, there are possible mechanisms available. Studies have shown that nesfatin-1 are affected by various factors (Li et al. 2014, Atici et al. 2017, Chaolu et al. 2011, Dore et al. 2017, J. F. Ge et al. 2015, Ayada et al. 2015). For example, it has been shown that starvation in rats decreases serum nesfatin-1 levels up to 18%. But conversely, it has been reported that nesfatin-1 concentrations returned to normal 1 to 12 hours after refeeding (Dore et al. 2017). In addition, some studies have shown that there is a direct relationship between nesfatin-1 and cortisol levels. Central injection of nesfatin-1 increased adrenocorticotropins (Jin-Fang Ge et al. 2015). According to previous studies, all of these factors are elevated as a result of eccentric resistance training protocols, which can be considered as a possible cause for increasing nesfatin-1 as a result of this method compared to studies that have not seen any changes. The adipose tissue also secretes various inflammatory cytokines that affect the expression and secretion of adipokines. For example TNF-α has different effects on adiponectin, leptin and nesfatin-1. Studies have shown that TNF-α, IL-6 and insulin increase the intracellular expression of nesfatin-1 in cultured fat cells (Ayada et al. 2015). These findings show that the expression and secretion of nesfatin-1 are regulated from different pathways.

Some clinical studies have reported that there is a significant relationship between nesfatin-1 and insulin sensitivity (Khalili et al. 2017). Therefore, it is likely that exercise alters the concentration of insulin and cortisol, influencing blood glucose and nesfatin-1. These factors have not been examined in this study and warrant further investigation.

311 It has been shown that nesfatin-1 attenuated phosphorylation of S6K and S6 during brown adipocyte differentiation.
312 Nesfatin-1 via mTOR dependent mechanism promotes the differentiation of brown adipocytes. Activation of mTOR
313 induced by leucine or deletion of TSC1 decreased expression of brown adipocyte-related genes UCP1, UCP3,
314 PGC1α and PRDM16, as well as COX8B and ATP5B. Both leucine and TSC1 deletion blocked nesfatin-1-induced
315 up-regulation of UCP1, PGC1α, COX8B and ATP5B in differentiated brown adipocytes (Wang et al. 2016). Results
316 of the present study showed a significant and positive correlation between PGC-1 α gene expression and serum
317 level of nesfatin-1 which is likely because of mTOR activator elements that mentioned above.
318
319 Resistin, increases as a result of obesity due to a significant reduction in exercise and increase in energy intake
320 (Garcia-Hermoso et al. 2017). The present study also showed a significant and negative correlation between PGC-1α
321 gene expression and serum levels of resistin. It possible that regular moderate-intensity physical training suppresses
322 the expression of dual specificity protein phosphatase 1 (DUSP1), increases the expression of PGC-1α and reduces
323 the activities of JNK and ERK (Khadir et al. 2015). Khadir et al (2015) concluded that anti-inflammatory exercise
324 effects may be related to suppressing of NADPH oxidase, ERK1/2 and SAPK/JNK activities, and increases in SOD-
325 1 gene expression. In the presesnt study we observed a decrease in resistin after eccentric resistance training and
326 possible regulation by PGC-1α. Regarding the effects of HMB on PGC-1α, He et al. (2016) suggested that dietary
327 supplementation with HMB increases the gene expression of PGC-1α. They suggested that PGC-1α plays a key role
328 in the transformation of skeletal muscle fiber type. As a nitrogen-free metabolite, HMB improves skeletal muscle
329 function, as well as the health of the body in both animals and humans (He et al. 2016).
330
331 The present study showed that HMB enhances the positive effects of resistance training on strength (1RM). Lee et
332 al. (2012) showed that leucine (0.5 mM) increases stimulates expression PGC-1α by three- to fivefold in C2C12 cell
333 models (Li et al. 2012). Vaughan et al. (2013) reported that leucine (0.1–0.5 mM) dose-dependently enhanced PGC-
334 1α expression in skeletal muscle cells (Vaughan et al. 2013). A few studies demonstrated the effects of HMB on
335 irisin. Baggett et al. (2013) investigate the synergistic effects of leucine and its metabolites with polyphenols on
336 irisin in myotubes and diet-induced obese mice. They demonstrate that leucine-polyphenol combinations stimulate
337 irisin and PGC-1α (B. Baggett et al. 2013). To our knowledge, no research has examined the effects of HMB on
338 nesfatin-1 and resistin. The results of the present study showed that serum nesfatin-1 increases and serum resistin

decrease responses HMB supplement. The mechanism that HMB induced change in nesfatin-1 and resistin is not understood and requires further research.

Limitations

Blood collection were not performed each week because of the associated costs, making it impossible to identify how soon these changes may have occurred. The research was undertaken on a small sample of animals so effect sizes have been included as well as the significance of both parametric and non parametric tests. Caution should be exerted if generalizing the findingsto humans.

Conclusions

The most important findings of present study showed that a combination of eccentric resistance training and HMB-FA supplement has more effect on the primary outcomes measured compered to the exercise or supplement intervention alone. Exercise and HMB supplement could increases PGC-1α gene expression that may regulate the other releasing tissues and change serum concentrations of irisin, nesfatin-1, and resistin. In general, we found that eccentric resistance training with HMB supplementation could be affected by inter-tissue crosstalk that increases the strength. Further research is needed to determine the effects of other peptides that would have allowed the authors to make further inferences about cross talk.

Acknowledgment

The authors appreciate A. Ghanbari-Niaki PhD (Exercise Biochemistry Division, Faculty of Sport Sciences, University of Mazandaran, Babolsar, Mazandaran, Iran) for their helpful comments.

Author contributions

HSh designed this study. HSh and RM collected the materials and performed the experiments. SRA analyzed the data. SRA and DRB wrote the manuscript. All authors read and approved the final version of the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Funding

None.

Research involving human and animal participants

Thirty-two male rats (Sprague Dawley family, 8 weeks old, 200±17 g weight) were used in this study. The present study was conducted with the written permission of the research deputy of Baqiyatallah University (ethical code: IR.BMSU.REC.1394.82) and was in accordance with National Institutes of Health (NIH) publication.

Figure legends

Figure 1: The 1 RM of eccentric resistance training (ERT), and β-Hydroxy-β-methylbutyrate free acid supplementation plus eccentric resistance training (HMB+ERT) groups. N = 8 in each group.

Figure 2: The Real-time PCR of skeletal muscle tissue PGC-1α relative mRNA expression (A), serum Irisin (ng/ml) (B), nesfatin-1 (ng/l) and resistin (ng/ml) (D) in control (CON), β-Hydroxy-β-methylbutyrate free acid supplementation (HMB), eccentric resistance training (ERT), and β-Hydroxy-β-methylbutyrate free acid supplementation plus eccentric resistance training (HMB+ERT) groups. N = 8 in each group

References

Algul, S., Ozdenk, C. and Ozcelik, O. (2017) 'Variations in leptin, nesfatin-1 and irisin levels induced by aerobic exercise in young trained and untrained male subjects', *Biol Sport,* 34(4), 339-344.

Alway, S. E., Pereira, S. L., Edens, N. K., Hao, Y. and Bennett, B. T. (2013) 'beta-Hydroxy-beta-methylbutyrate (HMB) enhances the proliferation of satellite cells in fast muscles of aged rats during recovery from disuse atrophy', *Exp Gerontol,* 48(9), 973-84.

Atherton, P. J., Babraj, J., Smith, K., Singh, J., Rennie, M. J. and Wackerhage, H. (2005) 'Selective activation of AMPK-PGC-1alpha or PKB-TSC2-mTOR signaling can explain specific adaptive responses to endurance or resistance training-like electrical muscle stimulation', *FASEB J,* 19(7), 786-8.

Atici, E., Mogulkoc, R., Baltaci, A. K. and Menevse, E. (2017) 'The effect of thyroid dysfunction on nesfatin-1 and adiponectin levels in rats', *Horm Mol Biol Clin Investig,* 32(3).

Aversa, Z., Alamdari, N., Castillero, E., Muscaritoli, M., Rossi Fanelli, F. and Hasselgren, P. O. (2012) 'beta-Hydroxy-beta-methylbutyrate (HMB) prevents dexamethasone-induced myotube atrophy', *Biochem Biophys Res Commun,* 423(4), 739-43.

Ayada, C., Toru, U. and Korkut, Y. (2015) 'Nesfatin-1 and its effects on different systems', *Hippokratia,* 19(1), 4-10.

Baggett, B., Bruckbauer, A. and Zemel, M. (2013) 'Synergistic Effects of Leucine and its Metabolites with Polyphenols on Irisin in Myotubes and Diet-induced Obese Mice', *The FASEB Journal,* 27(1_supplement), 637.11-637.11.

Baggett, B., Bruckbauer, A. and Zemel, M. (2013) 'Synergistic Effects of Leucine and its Metabolites with Polyphenols on Irisin in Myotubes and Diet-induced Obese Mice', *The FASEB Journal,* 27(1).

Bostrom, P., Wu, J., Jedrychowski, M. P., Korde, A., Ye, L., Lo, J. C., Rasbach, K. A., Bostrom, E. A., Choi, J. H., Long, J. Z., Kajimura, S., Zingaretti, M. C., Vind, B. F., Tu, H., Cinti, S., Hojlund, K., Gygi, S. P. and Spiegelman, B. M. (2012) 'A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis', *Nature,* 481(7382), 463-8.

Chaolu, H., Asakawa, A., Ushikai, M., Li, Y. X., Cheng, K. C., Li, J. B., Zoshiki, T., Terashi, M., Tanaka, C., Atsuchi, K., Sakoguchi, T., Tsai, M., Amitani, H., Horiuchi, M., Takeuchi, T. and Inui, A. (2011) 'Effect of exercise and high-fat diet on plasma adiponectin and nesfatin levels in mice', *Exp Ther Med,* 2(2), 369-373.

Chavanelle, V., Boisseau, N., Otero, Y. F., Combaret, L., Dardevet, D., Montaurier, C., Delcros, G., Peltier, S. L. and Sirvent, P. (2017) 'Effects of high-intensity interval training and moderate-intensity continuous training on glycaemic control and skeletal muscle mitochondrial function in db/db mice', *Sci Rep,* 7(1), 204.

Cobbold, C. (2018) 'Type 2 diabetes mellitus risk and exercise: is resistin involved?', *J Sports Med Phys Fitness*.

Daskalopoulou, S. S., Cooke, A. B., Gomez, Y. H., Mutter, A. F., Filippaios, A., Mesfum, E. T. and Mantzoros, C. S. (2014) 'Plasma irisin levels progressively increase in response to increasing exercise workloads in young, healthy, active subjects', *Eur J Endocrinol,* 171(3), 343-52.

Dinas, P. C., Lahart, I. M., Timmons, J. A., Svensson, P.-A., Koutedakis, Y., Flouris, A. D. and Metsios, G. S. (2017) 'Effects of physical activity on the link between PGC-1a and FNDC5 in muscle, circulating Irisin and UCP1 of white adipocytes in humans: A systematic review', *F1000Research,* 6, 286.

Dinas, P. C., Lahart, I. M., Timmons, J. A., Svensson, P. A., Koutedakis, Y., Flouris, A. D. and Metsios, G. S. (2017) 'Effects of physical activity on the link between PGC-1a and FNDC5 in muscle, circulating Iotarisin and UCP1 of white adipocytes in humans: A systematic review', *F1000Res,* 6, 286.

Dore, R., Levata, L., Lehnert, H. and Schulz, C. (2017) 'Nesfatin-1: functions and physiology of a novel regulatory peptide', *J Endocrinol,* 232(1), R45-R65.

Dorling, J., Broom, D. R., Burns, S. F., Clayton, D. J., Deighton, K., James, L. J., King, J. A., Miyashita, M., Thackray, A. E., Batterham, R. L. and Stensel, D. J. (2018) 'Acute and Chronic Effects of Exercise on Appetite, Energy Intake, and Appetite-Related Hormones: The Modulating Effect of Adiposity, Sex, and Habitual Physical Activity', *Nutrients,* 10(9).

Duan, Y., Li, F., Li, Y., Tang, Y., Kong, X., Feng, Z., Anthony, T. G., Watford, M., Hou, Y., Wu, G. and Yin, Y. (2016) 'The role of leucine and its metabolites in protein and energy metabolism', *Amino Acids,* 48(1), 41-51.

Fukushima, Y., Kurose, S., Shinno, H., Thi Thu, H. C., Takao, N., Tsutsumi, H., Hasegawa, T., Nakajima, T. and Kimura, Y. (2016) 'Effects of Body Weight Reduction on Serum Irisin and Metabolic Parameters in Obese Subjects', *Diabetes Metab J,* 40(5), 386-395.

Fuller, J. C., Sharp, R. L., Angus, H. F., Khoo, P. Y. and Rathmacher, J. A. (2015) 'Comparison of availability and plasma clearance rates of beta-hydroxy-beta-methylbutyrate delivery in the free acid and calcium salt forms', *Br J Nutr,* 114(9), 1403-9.

Gallagher, P. M., Carrithers, J. A., Godard, M. P., Schulze, K. E. and Trappe, S. W. (2000) 'Beta-hydroxy-beta-methylbutyrate ingestion, Part I: effects on strength and fat free mass', *Med Sci Sports Exerc,* 32(12), 2109-15.

Garcia-Hermoso, A., Ceballos-Ceballos, R. J., Poblete-Aro, C. E., Hackney, A. C., Mota, J. and Ramirez-Velez, R. (2017) 'Exercise, adipokines and pediatric obesity: a meta-analysis of randomized controlled trials', *Int J Obes (Lond),* 41(4), 475-482.

Ge, J.-F., Xu, Y.-Y., Qin, G., Peng, Y.-N., Zhang, C.-F., Liu, X.-R., Liang, L.-C., Wang, Z.-Z. and Chen, F.-H. (2015) 'Depression-like Behavior Induced by Nesfatin-1 in Rats: Involvement of Increased Immune Activation and Imbalance of Synaptic Vesicle Proteins', *Frontiers in Neuroscience,* 9, 429.

Ge, J. F., Xu, Y. Y., Qin, G., Peng, Y. N., Zhang, C. F., Liu, X. R., Liang, L. C., Wang, Z. Z. and Chen, F. H. (2015) 'Depression-like Behavior Induced by Nesfatin-1 in Rats: Involvement of Increased Immune Activation and Imbalance of Synaptic Vesicle Proteins', *Front Neurosci,* 9, 429.

Ghanbari-Niaki, A., Kraemer, R. R. and Soltani, R. (2010) 'Plasma nesfatin-1 and glucoregulatory hormone responses to two different anaerobic exercise sessions', *Eur J Appl Physiol,* 110(4), 863-8.

Ghanbari-Niaki, A., Rahmati-Ahmadabad, S. and Ansari-Pirsaraei, Z. (2013) 'Effects of aerobic training on tissue nesfatin-1/nucleobindin-2 mrna, plasma nesfatin-1 and high-density lipoprotein concentration in female rats', *IJHPA,* 4(2), 1-7.

Ghanbari Niaki, A., Mohammadi Joojadeh, F., Zare Kookandeh, N., Najafi, S., Chaichi, M. J., Rodbari, F. and Bayat, H. (2013) 'Liver and plasma nesfatin-1 responses to 6 weeks of treadmill running with or without zizyphus jujuba liquid extract in female rat', *Int J Endocrinol Metab,* 11(2), 95-101.

Gil, J. H. and Kim, C. K. (2015) 'Effects of different doses of leucine ingestion following eight weeks of resistance exercise on protein synthesis and hypertrophy of skeletal muscle in rats', *J Exerc Nutrition Biochem,* 19(1), 31-8.

He, F., Jin, J. Q., Qin, Q. Q., Zheng, Y. Q., Li, T. T., Zhang, Y. and He, J. D. (2018) 'Resistin Regulates Fatty Acid Beta Oxidation by Suppressing Expression of Peroxisome Proliferator Activator Receptor Gamma-Coactivator 1alpha (PGC-1alpha)', *Cell Physiol Biochem,* 46(5), 2165-2172.

He, X., Duan, Y., Yao, K., Li, F., Hou, Y., Wu, G. and Yin, Y. (2016) 'beta-Hydroxy-beta-methylbutyrate, mitochondrial biogenesis, and skeletal muscle health', *Amino Acids,* 48(3), 653-64.

Hu, F., Xu, Y. and Liu, F. (2016) 'Hypothalamic roles of mTOR complex I: integration of nutrient and hormone signals to regulate energy homeostasis', *Am J Physiol Endocrinol Metab,* 310(11), E994-E1002.

Huh, J. Y., Panagiotou, G., Mougios, V., Brinkoetter, M., Vamvini, M. T., Schneider, B. E. and Mantzoros, C. S. (2012) 'FNDC5 and irisin in humans: I. Predictors of circulating concentrations in serum and plasma and II. mRNA expression and circulating concentrations in response to weight loss and exercise', *Metabolism,* 61(12), 1725-38.

Jacobs, B. L., Goodman, C. A. and Hornberger, T. A. (2014) 'The mechanical activation of mTOR signaling: an emerging role for late endosome/lysosomal targeting', *J Muscle Res Cell Motil,* 35(1), 11-21.

Jung, S. and Kim, K. (2014) 'Exercise-induced PGC-1alpha transcriptional factors in skeletal muscle', *Integr Med Res,* 3(4), 155-160.

Khadir, A., Tiss, A., Abubaker, J., Abu-Farha, M., Al-Khairi, I., Cherian, P., John, J., Kavalakatt, S., Warsame, S., Al-Madhoun, A., Al-Ghimlas, F., Elkum, N., Behbehani, K., Dermime, S. and Dehbi, M. (2015) 'MAP kinase phosphatase DUSP1 is overexpressed in obese humans and modulated by physical exercise', *Am J Physiol Endocrinol Metab,* 308(1), E71-83.

Khalili, S., Shekari Khaniani, M., Afkhami, F. and Mansoori Derakhshan, S. (2017) 'NUCB2/Nesfatin-1: A Potent Meal Regulatory Hormone and its Role in Diabetes', *Egyptian Journal of Medical Human Genetics,* 18(2), 105-109.

Lam, Y. Y. and Ravussin, E. (2016) 'Analysis of energy metabolism in humans: A review of methodologies', *Mol Metab,* 5(11), 1057-1071.

Li, F., Yin, Y., Tan, B., Kong, X. and Wu, G. (2011) 'Leucine nutrition in animals and humans: mTOR signaling and beyond', *Amino Acids,* 41(5), 1185-93.

Li, H., Xu, M., Lee, J., He, C. and Xie, Z. (2012) 'Leucine supplementation increases SIRT1 expression and prevents mitochondrial dysfunction and metabolic disorders in high-fat diet-induced obese mice', *Am J Physiol Endocrinol Metab,* 303(10), E1234-44.

Li, J. B., Nishida, M., Kaimoto, K., Asakawa, A., Chaolu, H., Cheng, K. C., Li, Y. X., Terashi, M., Koyama, K. I., Amitani, H., Sakoguchi, T., Ushikai, M., Ikeda, S., Aoyama, K., Horiuchi, M., Li, J. Z. and Inui, A. (2014) 'Effects of aging on the plasma levels of nesfatin-1 and adiponectin', *Biomed Rep,* 2(1), 152-156.

Liang, H. and Ward, W. F. (2006) 'PGC-1alpha: a key regulator of energy metabolism', *Adv Physiol Educ,* 30(4), 145-51.

Mogharnasi, M., TaheriChadorneshin, H., Papoli-Baravati, S. A. and Teymuri, A. (2018) 'Effects of upper-body resistance exercise training on serum nesfatin-1 level, insulin resistance, and body composition in obese paraplegic men', *Disabil Health J*.

Myers, M. G., Jr. (2006) 'Keeping the fat off with nesfatin', *Nat Med,* 12(11), 1248-9.

Nogueiras, R., Novelle, M. G., Vazquez, M. J., Lopez, M. and Dieguez, C. (2010) 'Resistin: regulation of food intake, glucose homeostasis and lipid metabolism', *Endocr Dev,* 17, 175-84.

Norheim, F., Langleite, T. M., Hjorth, M., Holen, T., Kielland, A., Stadheim, H. K., Gulseth, H. L., Birkeland, K. I., Jensen, J. and Drevon, C. A. (2014) 'The effects of acute and chronic exercise on PGC-1alpha, irisin and browning of subcutaneous adipose tissue in humans', *FEBS J,* 281(3), 739-49.

Oh, I. S., Shimizu, H., Satoh, T., Okada, S., Adachi, S., Inoue, K., Eguchi, H., Yamamoto, M., Imaki, T., Hashimoto, K., Tsuchiya, T., Monden, T., Horiguchi, K., Yamada, M. and Mori, M. (2006) 'Identification of nesfatin-1 as a satiety molecule in the hypothalamus', *Nature,* 443(7112), 709-12.

Panati, K., Suneetha, Y. and Narala, V. R. (2016) 'Irisin/FNDC5--An updated review', *Eur Rev Med Pharmacol Sci,* 20(4), 689-97.

Park, H. K. and Ahima, R. S. (2015) 'Physiology of leptin: energy homeostasis, neuroendocrine function and metabolism', *Metabolism,* 64(1), 24-34.

Park, S., Hong, S. M., Sung, S. R. and Jung, H. K. (2008) 'Long-term effects of central leptin and resistin on body weight, insulin resistance, and beta-cell function and mass by the modulation of hypothalamic leptin and insulin signaling', *Endocrinology,* 149(2), 445-54.

Pfaffl, M. W. (2001) 'A new mathematical model for relative quantification in real-time RT-PCR', *Nucleic Acids Res,* 29(9), e45.

Rahmati-Ahmadabad, S., Azarbayjani, M. and Nasehi, M. (2017) 'The Effects of High-Intensity Interval Training with Supplementation of Flaxseed Oil on BDNF mRNA Expression and Pain Feeling in Male Rats', *Annals of Applied Sport Science,* 5(4), 1-12.

Rodriguez, M., Pintado, C., Molto, E., Gallardo, N., Fernandez-Martos, C. M., Lopez, V., Andres, A. and Arribas, C. (2018) 'Central s-resistin deficiency ameliorates hypothalamic inflammation and increases whole body insulin sensitivity', *Sci Rep,* 8(1), 3921.

Samy, D. M., Ismail, C. A. and Nassra, R. A. (2015) 'Circulating irisin concentrations in rat models of thyroid dysfunction -- effect of exercise', *Metabolism,* 64(7), 804-13.

Schnyder, S. and Handschin, C. (2015) 'Skeletal muscle as an endocrine organ: PGC-1α, myokines and exercise', *Bone,* 80, 115-125.

Shafiee, Z. and Sharifi, G. (2017) 'Comparing the effect of resistance, aerobic, and concurrent exercise program on the level of resistin and high reactive protein C of overweight and obese women', *International Archives of Health Sciences,* 4(1), 1-6.

Shi, J., Jiang, B., Qiu, Y., Guan, J., Jain, M., Cao, X., Bauer, M., Su, L., Burkly, L. C., Leone, T. C., Kelly, D. P. and Liao, R. (2013) 'PGC1alpha plays a critical role in TWEAK-induced cardiac dysfunction', *PLoS One,* 8(1), e54054.

Shirvani, H. and Arabzadeh, E. (2018) 'Metabolic cross-talk between skeletal muscle and adipose tissue in high-intensity interval training vs. moderate-intensity continuous training by regulation of PGC-1alpha', *Eat Weight Disord*.

Spiegelman, B. M. (2013) 'Banting Lecture 2012: Regulation of adipogenesis: toward new therapeutics for metabolic disease', *Diabetes,* 62(6), 1774-82.

Tiano, J. P., Springer, D. A. and Rane, S. G. (2015) 'SMAD3 negatively regulates serum irisin and skeletal muscle FNDC5 and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1alpha) during exercise', *J Biol Chem,* 290(12), 7671-84.

Timper, K. and Bruning, J. C. (2017) 'Hypothalamic circuits regulating appetite and energy homeostasis: pathways to obesity', *Dis Model Mech,* 10(6), 679-689.

Vaughan, R. A., Garcia-Smith, R., Gannon, N. P., Bisoffi, M., Trujillo, K. A. and Conn, C. A. (2013) 'Leucine treatment enhances oxidative capacity through complete carbohydrate oxidation and increased mitochondrial density in skeletal muscle cells', *Amino Acids,* 45(4), 901-11.

Wang, Y., Li, Z., Zhang, X., Xiang, X., Li, Y., Mulholland, M. W. and Zhang, W. (2016) 'Nesfatin-1 promotes brown adipocyte phenotype', *Sci Rep,* 6, 34747.

Wilson, J. M., Fitschen, P. J., Campbell, B., Wilson, G. J., Zanchi, N., Taylor, L., Wilborn, C., Kalman, D. S., Stout, J. R., Hoffman, J. R., Ziegenfuss, T. N., Lopez, H. L., Kreider, R. B., Smith-Ryan, A. E. and Antonio, J. (2013) 'International Society of Sports Nutrition Position Stand: beta-hydroxy-beta-methylbutyrate (HMB)', *J Int Soc Sports Nutr,* 10(1), 6.

Wilson, J. M., Grant, S. C., Lee, S. R., Masad, I. S., Park, Y. M., Henning, P. C., Stout, J. R., Loenneke, J. P., Arjmandi, B. H., Panton, L. B. and Kim, J. S. (2012) 'Beta-hydroxy-beta-methyl-butyrate blunts negative age-related changes in body composition, functionality and myofiber dimensions in rats', *J Int Soc Sports Nutr,* 9(1), 18.

Yin, Y., Yao, K., Liu, Z., Gong, M., Ruan, Z., Deng, D., Tan, B., Liu, Z. and Wu, G. (2010) 'Supplementing L-leucine to a low-protein diet increases tissue protein synthesis in weanling pigs', *Amino Acids,* 39(5), 1477-86.

Zhang, L., Zhou, Y., Wu, W., Hou, L., Chen, H., Zuo, B., Xiong, Y. and Yang, J. (2017) 'Skeletal Muscle-Specific Overexpression of PGC-1alpha Induces Fiber-Type Conversion through Enhanced Mitochondrial Respiration and Fatty Acid Oxidation in Mice and Pigs', *Int J Biol Sci,* 13(9), 1152-1162.