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Use of branched-chain amino acids for reducing exercise-caused skeletal muscle damage

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Abstract
Introduction: Skeletal muscles damage (direct and vicarious) slows down the recovery processes in patients with injuries of the musculoskeletal system. It occurs in the early postoperative period as well. An increase in the rigidity of the skeletal muscle extracellular matrix can reduce pain, tissue swelling, and accelerate the recovery of contractility.
Objective: The analyses of the effect of branched-chain amino acids (BCAAs) intake on the expression of *IGF1* genes, type 1, 3 and 5 collagen, which are crucial in the composition of the skeletal muscle extracellular matrix, as well as on the muscle membrane damage against the background of chronic damage to skeletal muscles.
Material and methods: 12 young healthy male subjects, skiers aged 19 (18; 22) received a placebo treatment (maltodextrin, 100 mg/kg body weight/day; n = 6) or a mixture of amino acids (leucine, isoleucine, valine – 50:25:20 mg/kg body weight/day respectively; n = 6). The treatment was received daily against the background of a large amount of aerobic high-intensity training (up to 22 hours per week). Before and after the amino acids intake a biopsy of the *musculus vastus lateralis* was performed, and venous blood samples were taken during the experiment.
Results: The intake of leucine against the background of training led not only to a pronounced increase in the level of IGF1 protein in blood by 1.5 times (which corresponds to the literature data), but also to a trend towards an increase in the expression of *IGF1Ea* mRNA by 1.8 times in the skeletal muscle, and a decrease in the level of markers of muscle membranes damage – creatine phosphokinase (CPK) activity and myoglobin. In addition, changes in the IGF1-dependent collagen genes expression strongly correlated with changes in *IGF1Ea* expression, but not with IGF1 protein in blood (pooled group, n = 12).
Thus, the intake of leucine as a part of the essential amino acids can reduce damage to skeletal muscles caused by excessive physical activity, lack of physical activity, or direct trauma.
Conclusion: A 10-week BCAAs intake by individuals with documented chronic muscle membrane damage caused an increase of basal levels of IGF1 in blood and a trend towards increased *IGF1Ea* mRNA expression in skeletal muscle, and also caused a modest reduction in damage of skeletal muscle membrane.
Keywords: musculoskeletal system, skeletal muscle, damage, extracellular matrix, training, collagen
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Применение аминокислот с разветвленной боковой цепью для уменьшения повреждений скелетных мышц, вызванных физическими нагрузками

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Резюме
Введение: Повреждение скелетных мышц (прямое и викарное) замедляет процессы восстановления пациентов с травмами опорно-двигательного аппарата, в том числе в раннем послеоперационном периоде. Увеличение жесткости внеклеточного матрикса скелетной мышцы позволяет снизить болевой синдром, отек тканей, ускорить восстановление сократительной способности.

Цель исследования: Изучение влияния приема аминокислот с разветвленной боковой цепью на экспрессию генов *IGF1*, коллагенов 1-, 3- и 5-го типа, являющихся ключевыми в составе внеклеточного матрикса скелетной мышцы, и поврежденность мышечных мембран на фоне хронического повреждения скелетных мышц.
Материал и методы: 12 молодых здоровых мужчин 19 (18; 22) лет, спортсмены-лыжники в течение 10 недель принимали плацебо (мальтодекстрин, по 100 мг/кг массы тела/день; *n* = 6) или смесь аминокислот (лейцин, изолейцин, валин в количестве 50:25:25 мг/кг массы тела/день, соответственно; *n* = 6) ежедневно на фоне большого объема аэробных высокоинтенсивных тренировок (до 22 ч/нед.). До и после приема им выполнялась биопсия латеральной головки четырехглавой мышцы бедра, а также в ходе эксперимента осуществлялся забор венозной крови.
Результаты: Прием лейцина на фоне тренировок привел не только к выраженному росту уровня белка IGF1 в крови в 1,5 раза, что соответствует литературным данным, но и тенденции к приросту экспрессии мРНК *IGF1Ea* в 1,8 раза в скелетной мышце. Также снизился уровень маркеров поврежденности мышечных мембран – активности КФК и миоглобина. Кроме того, изменения экспрессии IGF1-зависимых генов коллагенов сильно коррелировали с изменением экспрессии *IGF1Ea,* но не IGF1 в крови (объединенная группа, *n* = 12).
Таким образом, прием лейцина в составе незаменимых аминокислот позволяет снизить повреждения скелетных мышц, вызванные избыточными физическими нагрузками, гиподинамией или прямыми травмами.
Заключение: Прием аминокислот с разветвленной боковой цепью в течение 10 недель лицами с подтвержденным хроническим повреждением мышечных мембран вызвал рост базального уровня IGF1 в крови и тенденцию к росту экспрессии мРНК *IGF1Ea* в скелетных мышцах, а также способствовал умеренному снижению поврежденности мышечных мембран скелетных мышц.
Ключевые слова: опорно-двигательный аппарат, скелетная мышца, повреждение, внеклеточный матрикс, тренировки, коллаген
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Introduction

Muscle damage, both direct and vicarious (due to training, immobilization, bed rest, excessive exercise) leads to edema, pain, and decreased muscle contractility [1]. This problem is relevant for people with impaired functionality (for example during rehabilitation after prolonged bed rest) performing ordinary physical activities. Damage of muscle membranes and extracellular matrix is one of the reasons behind delayed muscle soreness caused by physical activity [2, 3]. The increase of the extracellular matrix (ECM) stiffness, which pays a key role in transmitting force to tendons, may be a way of preventing damage and speeding up muscle recovery.

Insulin-like growth factor type 1 (IGF1) is one of the regulators of the expression of collagens and other ECM proteins [4]. Numerous studies involving patients with acromegaly and growth hormone (GH) deficiency as well as healthy volunteers, have shown that GH-induced increase/decrease in serum IGF1 leads to an increase/decrease in the expression of *IGF1, COL1A1, COL3A1, LOX* as well as to a change in the rate of collagen synthesis in *musculus vastus lateralis* and an increase/decrease in stiffness of the patellar ligament [5–8]. At the same time, work on fibroblasts demonstrated that it is IGF1, and not GH, that regulates collagen synthesis [9].

The source of IGF1 in blood is liver, which synthesizes and secretes it under the influence of GH [10]. In addition, the level of IGF1 in blood can be increased by the consumption of food rich in leucine [11, 12]. The increase in the amount of protein in food in combination with vitamin D3 after 6 months raises the level of IGF1 in the blood of elderly patients with hip fracture [13]. Moreover, an increase in *IGF1* gene expression in response to branched-chain amino acids (BCAAs) was found in primary porcine hepatocytes and HepG2 cells [14, 15]. At the same time, the effect of leucine on *IGF1* expression in skeletal muscle has practically not been studied. It is important to take into consideration that the effect of IGF1 at tissue level in the adult organism is primarily associated with locally secreted IGF1, since the bioavailability of systemic IGF1 decreases due to its binding to the IGFBP proteins [16].

In this study it is suggested that the intake of leucine as a part of amino acids against the background of chronic damage to skeletal muscles (a model of constant intensive aerobic training) will reduce the damage and activate ECM biogenesis, including through the IGF1-dependent signaling pathway. We have studied the effect of a 10-week BCAAs intake in volunteers who regularly perform intensive training (skiers who train 2 times a day, about 22 hours per week).

Objective

The analyses of the effect of branched-chain amino acids (BCAAs) intake on the expression of *IGF1* genes, type 1, 3 and 5 collagen, which are crucial in the composition of the skeletal muscle extracellular matrix, as well as on the muscle membrane damage against the background of chronic damage to skeletal muscles.

Material and methods

The research program was approved by the Biomedical Ethics Committee of the State Scientific Center of the Russian Federation – Institute for Biomedical Problems of the Russian Academy of Sciences (Protocol no. 411 dated December 5, 2015). All studies were carried out in accordance with the Declaration of Helsinki. All volunteers signed a written informed consent to participate in the study.

Study organization

12 men (cross-country skiing, sports qualification from adult First-Class Sportsman to Candidate for Master of Sport, regular training experience of at least 3 years; average age 19 (18; 22) years, body weight 71.2 (68.6; 74.6) kg, height 1.78 (1.72; 1.80) m, body mass index 23.0 (22.4; 23.3) kg/m^2, (VO_{2max} (maximum rate of oxygen uptake) 4.69 (4.22; 4.97) l/min, total training 21–22 hours/week before and during the experiment)) were divided into experimental and control groups (6 people per group) in accordance with anthropometric and functional indicators.

The study was double-blind, placebo-controlled. Volunteers received a placebo treatment (maltodextrin 100 mg/kg body weight/day) or BCAAs (leucine:isoleucine:valine, in the amount of 50:25:25 mg/kg body weight/day respectively; Academy-T, Russia) daily in the morning after training. Prior to the experiment, the diet of volunteers in both groups did not differ and was sufficient. During the experiment, volunteers were advised to maintain a diet similar to that before the experiment. Additional intake of biologically active additives was not allowed. Volunteers did not take any kind of medication before and during the experiment. Before the beginning of the experiment and 3, 6 and 10 weeks after, venous blood samples were taken every morning on an empty stomach after a day of rest before the BCAAs intake. Before and 12 weeks after the beginning of treatment and training, a biopsy of the *musculus vastus lateralis* was performed by a fine-needle automatic microbiopsy using a Bard Magnum biopsy instrument (USA). Aerobic capacity was assessed 2–3 days after the biopsy during a treadmill test.

The concentration of IGF1 protein in blood serum was determined using an IMMULITE 2000 immunochemical automatic analyzer (Siemens, USA). Creatine phosphokinase (CPK) activity and myoglobin concentration in blood serum were measured using an AU680 biochemical automatic analyzer (Beckman Coulter, USA). Gene expression changes were assessed using a real-time polymerase chain reaction (PCR) technique. Muscle tissue samples were homogenized, then RNA was isolated from them using RNeasy Mini Kit (Qiagen, Germany) with subsequent assessment of the quality and concentration of RNA using a NanoDrop 2000 device (Thermo Fisher Scientific, USA). After the treatment with DNase I (Thermo Scientific Fermentas, Lithuania) and obtaining complementary DNA (cDNA) with the MMLV RT Kit (Evrogen, Russia) real-time PCR was performed (Rotor Gene Q (Qiagen)), HS-qPCR SYBR reagent kit (Evrogen, Russia). The mRNA expression of the target genes was assessed by the ΔCt method with the reference genes *RPLP0* and *GAPDH*. Primers are presented in Table 1.

The distribution of the studied variables was checked using the Shapiro–Wilk test and histograms; the variables distribution was abnormal, and therefore non-parametric

Table 1
Used primers
Таблица 1
Использованные праймеры

Transcript	Direction	Sequence, 5'–3'	Product size, bp
IGF1 signaling pathway			
IGF-1Ea	normal reverse	ATGCTCTTCAGTTCGTGTGTG GCACTCCCTCTACTTGCGTTC	285
IGF-1Ec (MGF)	normal reverse	ACCAACAAGAACACGAAGTC CAAGGTGCAAATCACTCCTA	281
IGF1R	normal reverse	GTGACGGGCTACGTGAAGAT CCCACAGTTGCTGCAAGTTC	148
INSR	normal reverse	CAGCGAGAAACTGCATGGTC GAAGACCCCATCCTTCAGGG	158
Intramuscular collagens			
COL1A1	normal reverse	CTCCAGGTGAAGCAGGCAAA AACCTCTCTCGCCTCTTGCT	90
COL3A1	normal reverse	TGTTCCACGGAAACACTGGT CGGCTGGAGAGAAGTCGAAG	162
COL5A1	normal reverse	ACAACAACCCCTACATCCGC TGACGCTTCACCGAAGTCAT	143
Reference genes			
RPLP0	normal reverse	CACTGAGATCAGGGACATGTTG CTTCACATGGGGCAATGG	77
GAPDH	normal reverse	CAAGGTCATCCATGACAACTTTG GTCCACCACCCTGTTGCTGTAG	496

statistical methods were used. Despite the small sample size, the chosen statistical methods (Mann–Whitney U test, Friedman's one-way analysis of variance with Dunn's post hoc test, Spearman's rank correlation coefficient) demonstrated its sufficiency and adequacy. All results are expressed as medians and interquartile ranges as "Me (Q1; Q2)". To assess the significance of differences between groups, the nonparametric Mann–Whitney U test was used. For all used methods, the threshold level of significance equaled 0.05.

Results

The effectiveness of 10 weeks of training for both groups of volunteers was expressed in the same increase in maximum running speed and VO_{2max}. However, the group taking BCAAs experienced a significant increase in running speed at the anaerobic threshold by 15.7% without an increase in oxygen consumption (figure 1A).

In the control group CPK activity was 197 IU/L, myoglobin content was 32 μg/L (figure 1B–1E). In the experimental group CPK activity was 237 IU/L, myoglobin content was 32 μg/l. It should be noted that at the beginning of the experiment, CPK activity in 4 out of 6 volunteers in the control group and in 5 volunteers in the experimental group was higher than the reference values of the corresponding set of reagents (10–171 IU/L, figure 1B). BCAAs intake led to a significant decrease in the CPK activity and to the difference in these values between the groups at the 6th week. It should be noted that in the experimental group the values were lower.

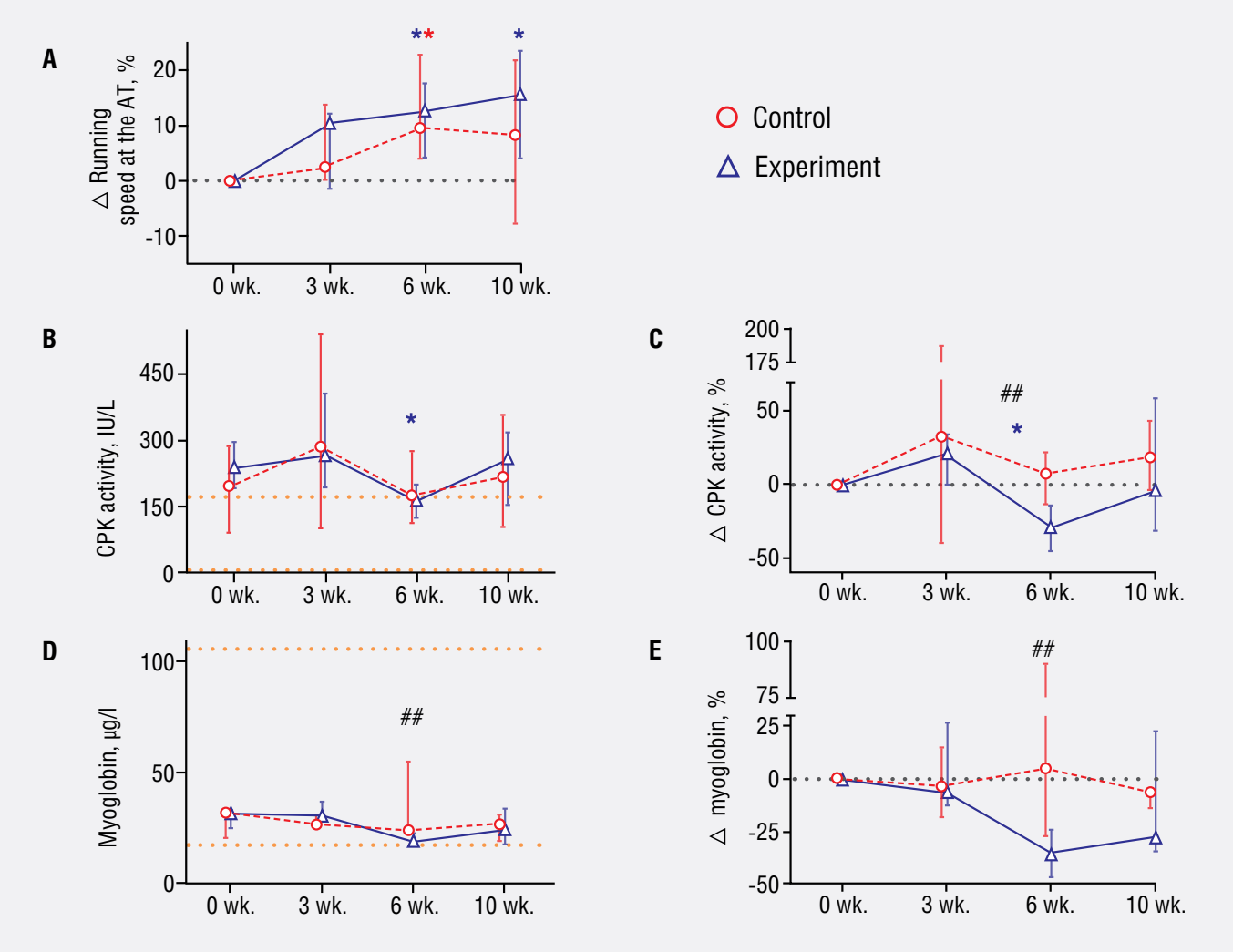

Figure 1. Changes in running speed at the anaerobic threshold (A), CPK activity levels (B – absolute values, C – differences from initial level) and myoglobin levels (D – absolute values, E – differences from initial level) during 10 weeks of training
Note: * corresponding color – difference from the initial level in the corresponding group, $p < 0.05$; ## – difference between groups, $p < 0.05$. The orange dotted line indicates the reference values for CPK activity and myoglobin levels
Рисунок 1. Изменения скорости бега на анаэробном пороге (А), уровней активности КФК (В – абсолютные значения, С – изменения относительно исходного уровня) и миоглобина (D – абсолютные значения, E – изменения относительно исходного уровня) в ходе 10 недель тренировок
Прим.: * соответствующего цвета – отличие от исходного уровня в соответствующей группе, $p < 0,05$; ## – различие между группами, $p < 0,05$. Оранжевым пунктиром указаны референсные значения для активности КФК и уровня миоглобина

There was an increase in the content of IGF1 protein in the blood serum against the background of taking leucine as a part of essential amino acids from the normal initial level by 42% at week 6 and by 47% at week 10, with a trend ($p < 0.1$) to a difference between groups at week 6 (figure 2A).

Expression of *IGF1Ea* mRNA isoform (figure 2B) and *COL5A1* collagen slightly increased ($p < 0.1$) in the experimental group, *COL3A1* also slightly increased expression ($p < 0.1$) in both groups (table 2). Changes in the *IGF1R* mRNA content were not found, *INSR* expression decreased in the control group ($p < 0.05$).

During the analysis of results of a combined group of volunteers ($n = 12$) a strong significant correlation was discovered between the expression of *IGF1Ea* mRNA and mRNA of *COL1A1, COL3A1* (together make up

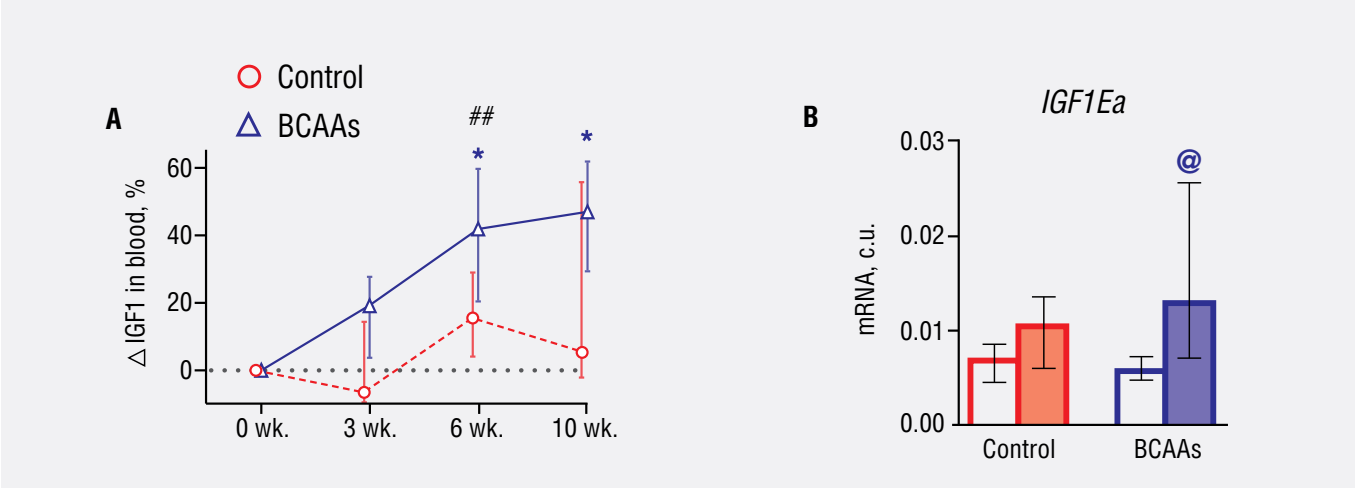

Figure 2. Changes in the content of the IGF1 protein in the blood (A) and the level of the IGF1Ea mRNA isoform in the skeletal muscle (B)
Note: * of the corresponding color – difference from the initial level in the corresponding group; ## – difference between groups, $p < 0.05$; @ – difference from initial level, $p < 0.1$
Рисунок 2. Изменения содержания белка IGF1 в крови (А) и уровня изоформы мРНК IGF1Ea в скелетной мышце (В)
Прим.: * соответствующего цвета – отличие от исходного уровня в соответствующей группе; ## – различие между группами, $p < 0,05$; @ – отличие от исходного уровня, $p < 0,1$

Table 2
Expression of *IGF1* mRNA isoforms of target genes in skeletal muscle in the control (C) and experimental (AA) groups. Data are presented as the ratio of target gene expression to reference gene expression (×1000)
Таблица 2
Экспрессия *IGF1* изоформ мРНК целевых генов в скелетной мышце в группе контроля (К) и эксперимента (АА). Данные представлены как отношение экспрессии целевых генов к экспрессии референсных (×1000)

Transcript	Control		Amino acids	
	before	after	before	after
IGF1Ea	6.9 (5.6; 7.5)	10.0 (7.2; 10.2)	5.8 (5.3; 6.3)	**10.3 * (8.8; 20.2)**
IGF1Ec (MGF)	0.05 (0.037; 0.067)	0.12 (0.062; 0.17)	0.049 (0.04; 0.055)	0.094 (0.058; 0.33)
IGF1R	0.24 (0.17; 0.32)	0.20 (0.085; 0.4)	0.34 (0.19; 0.58)	0.25 (0.13; 0.41)
INSR	6.1 (5.5; 8.4)	**2.2 ## (1.4; 7.2)**	3.4 (1.2; 5.4)	2.2 (6.9; 6.2)
COL1A1	0.46 (0.19; 1.0)	0.34 (0.22; 0.75)	1.6 (0.11; 3.4)	1.7 (0.83; 2.4)
COL3A1	150 (140; 190)	**730 * (160; 1300)**	190 (140; 270)	**430 * (250; 1800)**
COL5A1	2.8 (2.0; 3.1)	4.0 (3.1; 7.8)	2.5 (1.8; 2.8)	**5.1 * (3.3; 5.8)**

Note: ## difference between groups, $p < 0.05$; * tendency to the difference from initial level, $p < 0.1$
Прим.: ## различие между группами, $p < 0,05$; * тенденция к отличию от начального уровня, $p < 0,1$

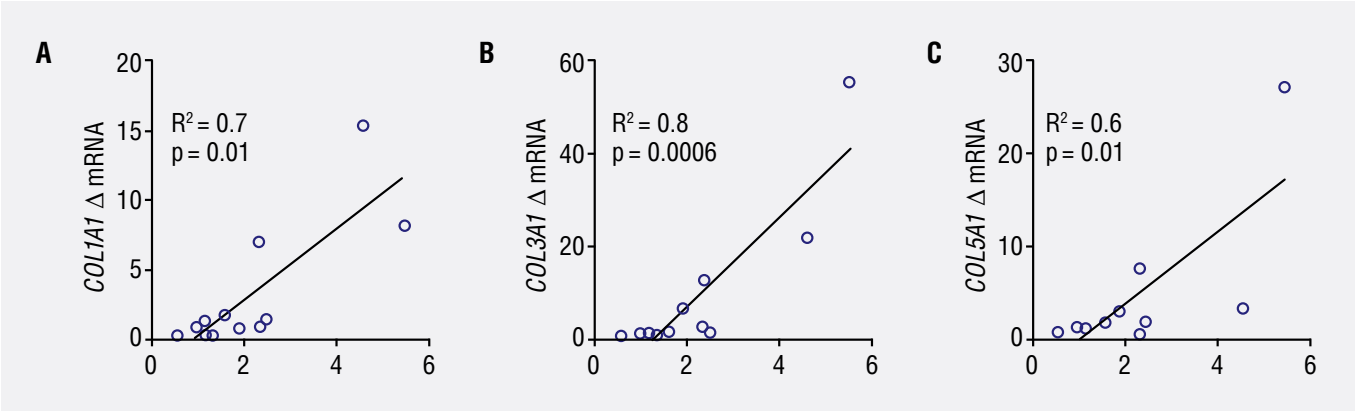

Figure 3. Correlation of COL1A1 (A), COL3A1 (B), and COL5A1 (C) mRNA expression with IGF1Ea mRNA expression in skeletal muscle
Рисунок 3. Корреляция экспрессии мРНК COL1A1 (A), COL3A1 (B) и COL5A1 (C) с экспрессией мРНК IGF1Ea в скелетной мышце

more than 50% of the mass of the skeletal muscle ECM) [17], *COL5A1* in skeletal muscle (figure 3) which was not observed during the comparison of correlation of these genes with the level of *IGF1* in blood.

Discussion

The leucine intake as a part BCAAs by the volunteers against the background of intense physical activity led to an increase in the concentration of IGF1 protein in blood by 1.5 times. This result is similar to those obtained in other studies on animals and volunteers [11–13]. However, the trend towards an increase in the content of *IGF1Ea* mRNA in the experimental group in human skeletal muscle has not been previously presented (Table 2). The obtained strong correlation coefficients of collagen and *IGF1Ea* emphasize the importance of IGF1 in the autocrine/paracrine regulation of skeletal muscle ECM stiffness and its resistance to damage.

Increased basal levels of CPK and myoglobin activity in all subjects indicate chronic muscle fiber damage in volunteers due to continuous intensive aerobic training. The fact that the activity level of CPK and, to a lesser extent, myoglobin after 6 weeks decreased in the experimental group in comparison to the control group indicates an increase in resistance of muscle fiber membranes to damage. It correlates with the results of the studies examining the effect of a 5-week protein intake (33.5 g/day) on long-distance runners. A lower level of CPK basal activity and a significantly lower increase in this indicator after a 42 km marathon were observed against the background of BCAAs intake, as well as a higher performance in a 12-minute test (distance covered) [18]. The discovered increase in the speed at the anaerobic threshold without an increase in the oxygen consumption intake can be associated with the ECM stiffness increase (epimysium, perimysium and endomysium) [19–21]. Our results on the running efficiency increase in the experimental group are consistent with data that indicate a decrease muscle membranes training damage.

Conclusion

In our study of the effects of the essential amino acids on skeletal muscle damage, it has been noticed that 10 weeks of leucine, isoleucine, and valine intake in young athletes with documented chronic muscle membrane damage caused an increase in basal blood levels of IGF1 and a trend towards an increase in *IGF1Ea* mRNA expression in skeletal muscles. The results of our study show that long-term use of these amino acids is associated with reduced muscle damage and increased running speed in athletes performing intensive aerobic training. The close correlation found between *IGF1* gene expression and expression of genes encoding extracellular matrix proteins in skeletal muscle suggests that the reduction in muscle damage against the background of amino acids intake is mediated by IGF1-dependent regulation.

We believe that in order to obtain more pronounced protective properties of amino acids in cases of muscle damage, the dose increase and/or prolongation of amino acids intake duration may be necessary. Further research is needed to test this hypothesis. In addition, it seems promising to study the effect of amino acids intake on muscle damage in individuals with impaired functionality during normal physical activity.

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