Full Length Research Paper

Prehydrolyzed dietary protein reduces gastrocnemial DNA without impairing physical capacity in the rat

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Previous studies have shown that hydrolyzed proteins exhibit antioxidant properties and may confer physical and physiological advantages when consumed by the exercising rat. The purpose of this study was to compare the effects of feeding either intact (I) and partly hydrolyzed (H) milk whey proteins on gastrocnemial DNA contents and protein metabolism in exercising Wistar rats. Protein synthesis and degradation, protein and DNA contents, and concentration of the serum insulin-like growth factor-1 (IGF1) were determined in six experimental groups according to the type of protein consumed [casein (C), whey protein isolate (I), hydrolyzed whey protein (H) and level of physical activity (sedentary (S) and trained (T)]. H produced significantly lower rates of protein synthesis and degradation and DNA contents in the gastrocnemius, while no differences were observed in the total muscle protein content and serum levels of IGF1. These results indicate that consumption of prehydrolyzed whey protein alters muscle metabolism resulting in less DNA, but maintains the muscle protein levels constant and sustain or improves physical performance, compared to the unhydrolyzed protein.

Key words: Dietary protein, hydrolyzed whey protein, peptides in muscle metabolism, phenylalanine, tyrosine, physical activity.

INTRODUCTION

Different food proteins may affect muscle metabolism in different fashions, as is known to occur when the milk whey proteins are compared with casein. One difference that has been reported between the whey protein concentrate compared with casein, is that the whey proteins stimulate the increase of fatty acid synthesis in the muscle, accompanied by a concomitant decrease in the liver, which was considered to be a positive effect on lipid metabolism (Morifuji et al., 2005). Hydrolyzing the protein alone could in itself alter the biological function of the protein, thus affecting metabolism (Meisel, 1998). It has been suggested, for instance, that the mere change of the physicochemical form in which the protein is presented to the animal suffices to influence the general metabolism, apparently as a result of the various peptides that are generated during partial enzymatic hydrolysis of the whey proteins (Morifuji et al., 2009; Nery-Diez et al., 2010; Faria et al., 2012). Alteration in glycogen metabolism has been perhaps the most often reported positive feature resulting from substituting a whey protein hydrolyzate for the native

ABBREVIATIONS: CS, Casein sedentary; **CT,** casein trained; **HS,** hydrolyzed whey protein sedentary; **HT,** hydrolyzed whey protein trained; **IS,** isolate whey protein sedentary; **IT,** isolate whey protein trained.
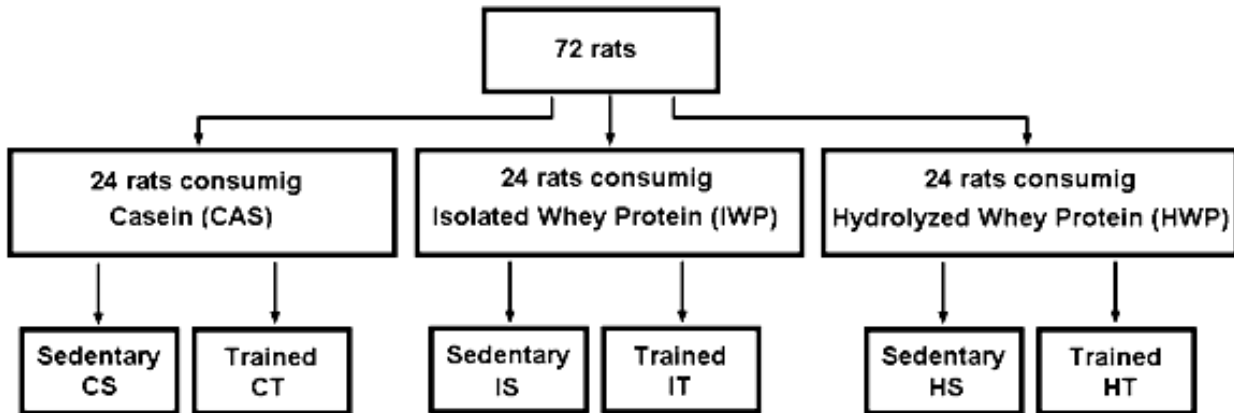
Figure 1. Grouping of animals by type of diet and physical activity. CS, casein diet and sedentary; CT, casein diet and trained; IS, whey protein-isolate diet and sedentary; IT, whey protein-isolate diet and trained; HS, whey protein-hydrolyzate diet and sedentary; HT, whey protein-hydrolyzate diet and trained.

protein (Morifuji et al., 2009; Faria et al., 2012). Morifuji et al. (2009) showed that the peptide Ile-Leu, from among seven possible dipeptides containing branched-chain amino acids from the milk-whey proteins, was capable of increasing the uptake of glucose by isolated rat muscle. The reports suggest that far from being unsafe, these alterations are consistent with the observed physiological advantages, such as greater endurance exercise performance, better post-exhaustion glycemic levels, and preserved blood total protein and albumin levels (Morifuji et al., 2009; Faria et al., 2012; Lollo et al., 2011).

The various advantages pointed out for the whey protein hydrolyzates are consistent also with a possible beneficial effect to the experimental animal, as has been the case of physical performance in rats undergoing endurance exercise (Morifuji et al., 2009; Pimenta et al., 2005) and promoting the synthesis of glycogen (Morifuji et al., 2009). Nery-Diez et al. (2010) additionally have suggested an anti-stress effect associated with the consumption of the whey protein hydrolyzate in rats undergoing endurance exercise.

Moreover, it has been observed that a whey protein hydrolyzate is capable of reducing the levels of creatine kinase in both exercising rats (Abecia-Soria, 2010) and soccer players (Lollo et al., 2011), which was originally understood to be the result of the antioxidant capacity of some bioactive peptides present in the hydrolyzate (Peng, Siong and Kong, 2009; Ren et al., 2008).

The present study was designed to investigate the possible effects produced by the consumption of a whey protein hydrolyzate, in comparison with those of a whey protein isolate. The comparisons included several parameters of muscle protein metabolism that included the rates of gastrocnemial protein synthesis and degradation, the concentration of serum insulin-like growth factor-1 (IGF1), and the gastrocnemial protein and DNA contents in Wistar rats. These parameters were assessed for the two types of protein under two states of physical activity,

sedentary and exercised, using four experimental and two casein control groups.

MATERIALS AND METHODS

Biological assay

Male Wistar (144 twenty-one-days old, specific-pathogen free) rats, bred at the Multidisciplinary Center for Biological Research, University of Campinas, SP, Brazil, were housed (~22°C, 55% RH, inverted 12-h light cycle) in individual growth cages, with free access to commercial chow (Labina, Purina, Brazil) and water at all times, until they became six weeks old. At 42 days and still consuming the commercial chow, the rats were tested for their running aptitude in order to exclude those that did not pass the initial stage of the protocol. The selected animals were then segregated into six groups according to diet and physical activity (Figure 1). The research methodology was approved by the Ethics Committee on Animal Experimentation (CEEA-UNICAMP, protocol 1340-1/2007).

Experimental diets

The diets were isonitrogenous (approximately 14% protein, dry basis), isolipidic and isocaloric (approximately 360 kcal/100 g) and formulated following the recommendations of the American Institute of Nutrition, AIN93-M diet (Reeves and Nielsen, 1993). The three diets differed among themselves with respect to the nature of the protein source, considering that in one case the whey protein was prehydrolyzed (Hilmar product 8360) and in the other, the protein was not (Hilmar product 9410). The prehydrolyzed whey protein exhibited a degree of hydrolysis of approximately 12.5%. Both the whey proteins were gifts of Hilmar Ingredients (Dalhart, TX, USA). The standard reference protein was casein. Food consumption was determined every other day and body mass evolution monitored once per week.

Training protocol

The rats were selected in the treadmill beforehand, considering as not fit those that stood for 30 s without departing from the baseline. The animals began training on the day they started to consume the experimental diets and lasted for nine weeks following the progres-

sive-resisted protocol of Hohl et al. (2009) Physical performance was determined by the last phase of this method, as the animals were brought to exhaustion. The sedentary rats were also subjected to a minimum of physical activity, running at the speed of 15 m/min for 10 min, three times a week.

Sample collection for analysis

In an attempt to minimize the consequences of both acute and chronic stress due to either training or diet, the animals were sacrificed by decapitation, following a 48-h recovery period. Immediately after sacrifice, the blood was collected in plain test tubes, centrifuged, and the serum stored at -20°C for the determination of serum IGF1. The gastrocnemius muscles, for the analysis of protein synthesis and degradation, were dissected, weighed and placed in a Krebs-Henseleit bicarbonate solution. The muscle tissues collected for protein determination were stored in liquid nitrogen until the time of analysis.

Determination of IGF1

The levels of serum IGF1 were determined by radioimmunoassay using the Lincoplex kit for rat/mouse IGF1 (cat # RMIGF1 87k), from Lincis ® Research Inc., according to the manufacturer's recommended procedure.

Muscle protein synthesis

The gastrocnemius muscles were placed in KHB solution (110 mM NaCl, 25 mM NaHCO$_3$, 3.4 mM KCl, 1 mM CaCl$_2$, 1 mM MgSO$_4$ and 1 mM KH$_2$PO$_4$, pH 7.4), supplemented with 5.5 mM glucose and 0.01% (w/v) of albumin. The muscles were pre-incubated for 30 min at 37°C with continuous oxygenation (95% O$_2$ and 5% CO$_2$), as described by Vary et al. (1998). Next, a new solution KHB supplemented with L-5µ Ci[^{3}H]-phenylalanine/mL was added for another 2 h. At the end of incubation, the tissues were homogenized in 10% of trichloroacetic acid (1:3 w/v), centrifuged at 10,000 x g for 15 min, at 4°C. The resulting granules were suspended in 1 M NaOH and incubated at 40°C for 30 min. The radioactivity from the β-emission was measured on a scintillation counter. The pattern of protein synthesis was calculated from the amount of radioactivity incorporated from phenylalanine during the 2 h of incubation, and was expressed in nanomoles of [^{3}H]-phenylalanine, per microgram of muscle protein/h.

Muscle protein degradation

Whole gastrocnemius muscles were placed in RPMI 1640 medium (Sigma-Aldrich, USA) and then pre-incubated for 30 min at 37°C, with continuous oxygenation (95% O$_2$; 5% CO$_2$), as described by Vary et al. (1998). This muscle was chosen rather than the soleus because of its significant involvement in the type of exercise imposed on the animals. The pattern of degradation was established by fluorimetric analysis, in terms of nanomoles of tyrosine released per microgram of muscle protein/h, as described by Waalkes and Udenfriend (1957).

Determining total muscle protein

Tissue samples weighing ~50 mg were submitted to protein extraction, in 250 µL of solution of 1M HClO$_4$ at 110°C for 30 min. Then the solution was centrifuged at 1006 x g for 30 min. The protein analysis was performed in accordance with the method of

Lowry et al., (1951). We used 10 µL of the supernatant from extraction, adding 1 mL of distilled water and 0.9 mL of solution A (0.4 g of Na, K tartrate, 20 g Na$_2$CO$_3$, 1 g NaOH and 200 mL of distilled water), followed by incubation in a water bath (50°C) for 10 min. After this, 0.1 mL of solution B (0.4 g of Na,K tartrate, 200 mL of distilled water, 0.2 g of CuSO$_4$.5H$_2$O, 0.08 g NaOH) was added to the system and allowed to stand at room temperature for 10 min. Next, 3 mL of solution C (10 mL of reactive Folin Ciocalteau and 140 mL of distilled water) were added and the mixture placed in a 50°C water bath for 10 min. Then, the solution was allowed to cool to room temperature and held for reading in a spectrophotometer, set at 650 nm.

Determination of DNA in muscle

Muscle samples (~50 mg) were submitted to protein extraction, in 250 µL of 1M HClO$_4$ solution at 110°C for 30 min. Then the solution was centrifuged at 1006 x g for 30 min. The analysis of the content of DNA was carried out in accordance with the method of Giles and Myers (1965). We used 0.5 mL of extract, adding 500 µL of 1M HClO$_4$ and 1.0 mL of solution of diphenylamine. The solution was vortex stirred, and transferred to a water bath (30°C) for 12 h. After cooling to room temperature the reaction mixtures were read in the spectrophotometer set at 595 nm.

Statistical analysis

The results were subjected to statistical analysis using the software Statistical Package for the Social Sciences (SPSS), version 16.0. The data were tested for normality (Kolmogorov-Smirnov test) and homogeneity available therein. For parametric data, two-way analysis of variance was used and means were compared (Tukey test), considering the value of $P \leq 0.05$ as a criterion for statistical significance. In the case of data that did not show normal distribution, the non-parametric Kruskal-Wallis test was used, with statistical significance set at $P \leq 0.05$.

RESULTS AND DISCUSSION

The present study was designed to investigate the possible effects produced by the consumption of a whey protein hydrolyzate, in comparison with those of a whey protein isolate. This is the first time that consumption of a diet with a hydrolyzed whey protein is reported to result in lower amounts of muscle DNA without a detriment to physical performance. At the end of the experiment, the rats were 113 days old and had completed nine weeks of training and consumption of the experimental diets. Age is an important consideration for metabolic studies and in particular for the metabolism of proteins. In the normal Wistar male rat, both DNA and protein increase in skeletal muscle, between 4 and 44 days after birth due to the intense protein synthesis necessary for cell growth (Winick et al., 1972). From there on, the contents of DNA may remain constant until about day 95, while protein continues to rise till day 140. Thus, treatment with different dietary proteins in combination with training is likely to affect muscle composition by changing the contents of DNA and protein.

The results show no observable differences in protein content among treatments CS, IS, HS, CT, IT and HT in the gastrocnemius (Table 1). In spite of the progressive

Table 1. Protein and DNA of gastrocnemius muscle, for the six groups of rats.

Gastrocnemius	Groups of rats, according to both diet and level of activity					
	CS	IS	HS	CT	IT	HT
Muscle protein (mg/100 mg)	12 ± 2	11 ± 3	11 ± 2	12 ± 2	11 ± 1.9	11 ± 1
Total protein content (mg)	230 ± 44	207 ± 58	207 ± 43	234 ± 39	219 ± 38	207 ± 27
*Total protein content (mg/g BW)	0.55 ± 0.1	0.53 ± 0.14	0.51 ± 0.08	0.63 ± 0.12	0.58 ± 0.08	0.55 ± 0.05
DNA in muscle (µg/100 mg)	23[a] ± 3	23[a] ± 5	17[b] ± 2.8	22[a] ± 1	21[ab] ± 3	17[b] ±3.2
Total content of DNA (µg)	444[a] ± 78	419[a] ± 67	326[b] ± 55	427[a] ± 25	406[ab] ± 67	311[b] ± 80
*Total DNA content (µg/g BW)	1.06[a] ± 0.19	1.07[a] ± 0.16	1.00[a] ± 0.16	1.15[a] ± 0.06	1.08[a] ± 0.16	0.83[b] ± 0.19
Mean weight of the animals ± SD (g)	455.42 ± 17.51	409.87 ± 8.63	441.03 ± 10.43	359.60 ± 5.02	384.73 ± 4.76	387.15 ± 8.63

*Total protein or DNA content normalized by body weight. The values represent means ± SD. CS, Control casein diet and sedentary; IS, whey protein isolate diet and sedentary; HS, hydrolyzed whey protein diet and sedentary; CT, control casein diet and trained; IT, protein isolate diet and trained; HT, hydrolyzed whey protein and trained. Means with different superscript letters along horizontal lines are different.

endurance training protocol to which the trained animals were subjected, the data suggest that neither protein concentration nor total muscle protein were affected by either physical activity or the state in which the dietary protein was administered to the animals. The weights of the gastrocnemius muscles also did not differ between groups. In the study of Pimenta et al. (2005), no significant changes in muscle protein content were also observed when comparing the whey protein isolate with the hydrolyzate (medium degree of hydrolysis ~8.5 - 9.0 %) in the diets of exercising young rats. Taken together, the results of this experiment suggest that the milk whey proteins, whether intact or prehydrolyzed, whether associated or not with physical endurance activity, did not result in an increase of the contents of muscle protein.

The DNA analyses, however, showed that by simply feeding the prehydrolyzed whey protein resulted in considerably lower concentrations and total contents of DNA in the gastrocnemius, as opposed to feeding the unhydrolyzed proteins, on a body weight basis (groups HS and HT were lowest; Table 1). Knowing that the levels of DNA resulting from consuming the hydrolyzate were significantly different from those of the isolate, the difference could then be due to the large number and nature of the peptides present in the prehydrolyzed protein, which were profusely released in the gut.

In previous experiments, we have shown that the performance of animals fed the hydrolyzate is equal or better than those fed either casein or the isolate protein (Nery-Diez et al., 2010; Pimenta et al., 2005). The fact that the content of DNA in the rats consuming the prehydrolyzed protein was consistently lower than in those consuming the whole proteins, did not mean that the hydrolyzate was disadvantageous to the muscle function because the muscle protein content did not differ between any two groups. In addition, a performance test done at the end of week eight gave the following exhaustion times (seconds): CT: 2,687 ± 397[b]; IT: 2,511 ± 70[b] and HT: 3,266 ± 779[a], thus attesting to the good performance capacity of group HT.

Other investigations involving physical exercise and the consumption of hydrolyzed whey proteins have shown that protein hydrolyzates effectively increase liver and muscle glycogen stores (Morifuji et al., 2005; Pimenta et al., 2005), in contrast to the unhydrolyzed protein. By minimizing the demand for endogenous protein for energy use, it is possible that the demand for newly biosynthesized proteins be accordingly lower, thereby resulting in an alleviation of the need to increase the myonuclear DNA.

From the results of protein synthesis measured by the incorporation of [^{3}H]-Phe in the gastrocnemius (Figure 2), it was observed that the trained group consuming the hydrolyzate (HT) incorporated significantly less phenylalanine than any of the sedentary groups, or even the CT group. It should be noted that although the rate of synthesis in group HT was much lower than that in group HS at 48 h (Figure 2), the rate of synthesis of the HT was still commensurate with its rate of degradation (Figure 3), while the rate of synthesis in the sedentary was about 25 times greater. It is also pertinent to note that this state of equilibrium refers to a time period in which there is an inertial positive balance of muscle mass due to the accelerated metabolism of exercise, followed by the cessation of a high demand for protein synthesis (Rennie and Tipton, 2000).

Studies have shown that after consumption of the whey proteins, the appearance of amino acids in plasma is fast, abundant and transient; a profile associated with an increase in protein synthesis (Boirie et al., 1997). Furthermore, leucine, which is an abundant amino acid in whey proteins, has an anabolic effect by working along the intracellular pathways involved in stimulating the synthesis of proteins (Blomstrand et al., 2006). Both leucine and whey protein hydrolyzates have been recognized as being insulinotropic (Power et al., 2009) and, because of this property, in addition to its high protein nutritional value, the physiological response to produce more muscle protein was instead permuted for a higher protein sparing effect, in turn resulting in a lower rate of protein
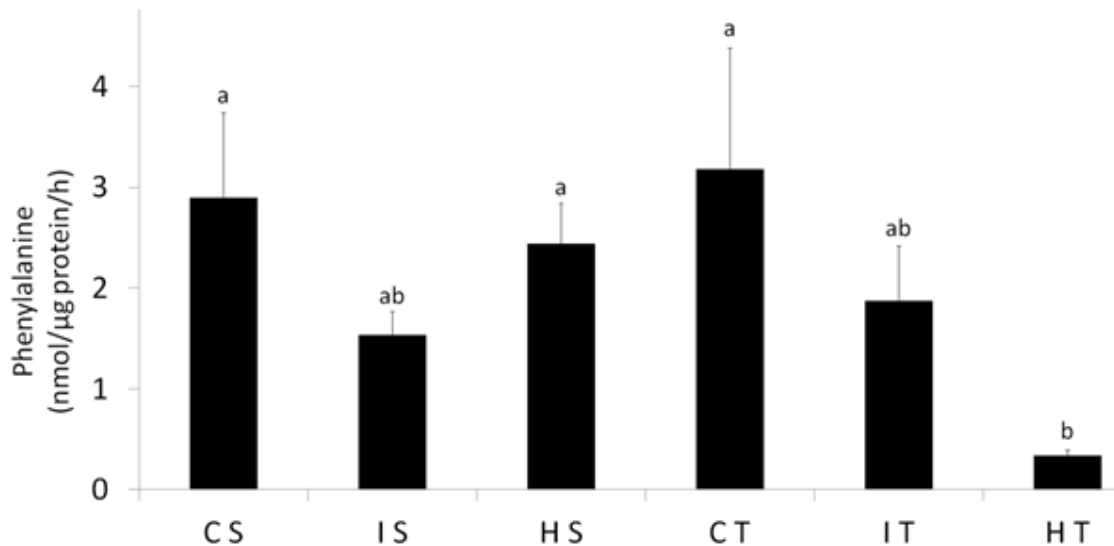
Figure 2. Phenylalanine incorporation in the gastrocnemius by groups. CS, Casein-Sedentary; IS, whey protein isolate-sedentary; HS, whey protein hydrolyzate-sedentary; CT, casein-trained; IT, whey protein isolate-trained; HT, whey protein hydrolyzate-trained). Values correspond to means ± SD. The non-parametric Kruskal-Wallis, with significance of $p < 0.05$ in A, and $p \leq 0.05$ in B was used.
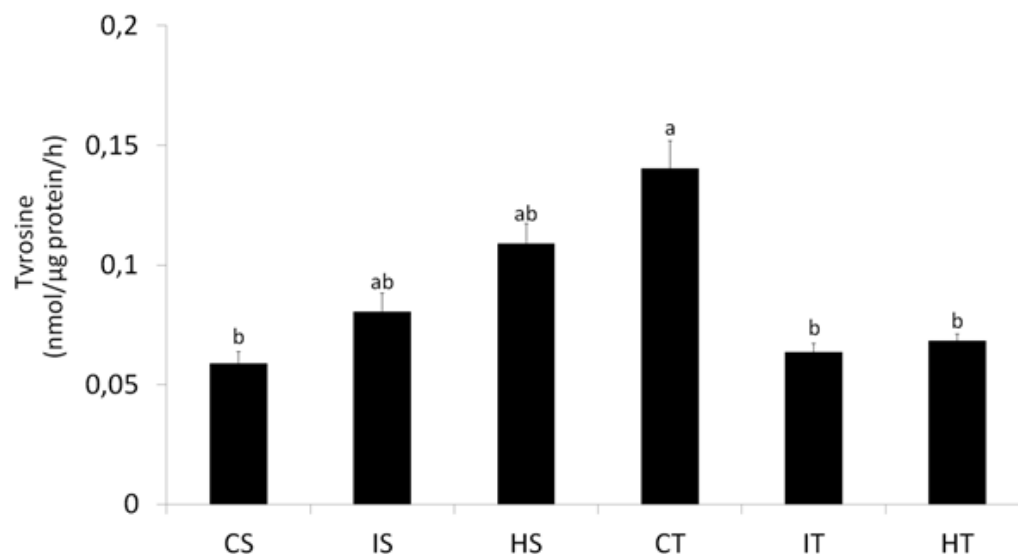

Figure 3. Tyrosine release from the gastrocnemius. (CS, Casein-Sedentary; IS, whey protein isolate-sedentary; HS, whey protein hydrolyzate-sedentary; CT, casein-trained; IT, whey protein isolate-trained; HT, whey protein hydrolyzate-trained). Values are means ± SD. The non-parametric Kruskal-Wallis analysis, at the 0.05 level of significance was used.

synthesis. One advantage of the milk whey proteins in physical activity is that their high leucine content usually produces a positive effect on muscle protein balance because, although endurance exercise may promote a negative balance (Johnson et al., 2004), leucine generally exerts the opposite effect, at both normal and supplemental levels (Garlick, 2005). Our results on the release of tyrosine from the gastrocnemius did not detect any considerable protein catabolism in the muscle (Figure 3), perhaps because leucine, besides stimulating protein synthesis, may also inhibit protein degradation (Garlick, 2005). Consistent with the above observations, the circulating serum IGF-1 levels showed no statistical difference between any two groups of the study (Figure 4), which means that levels were neither higher, nor lower than in the control. It has been suggested that
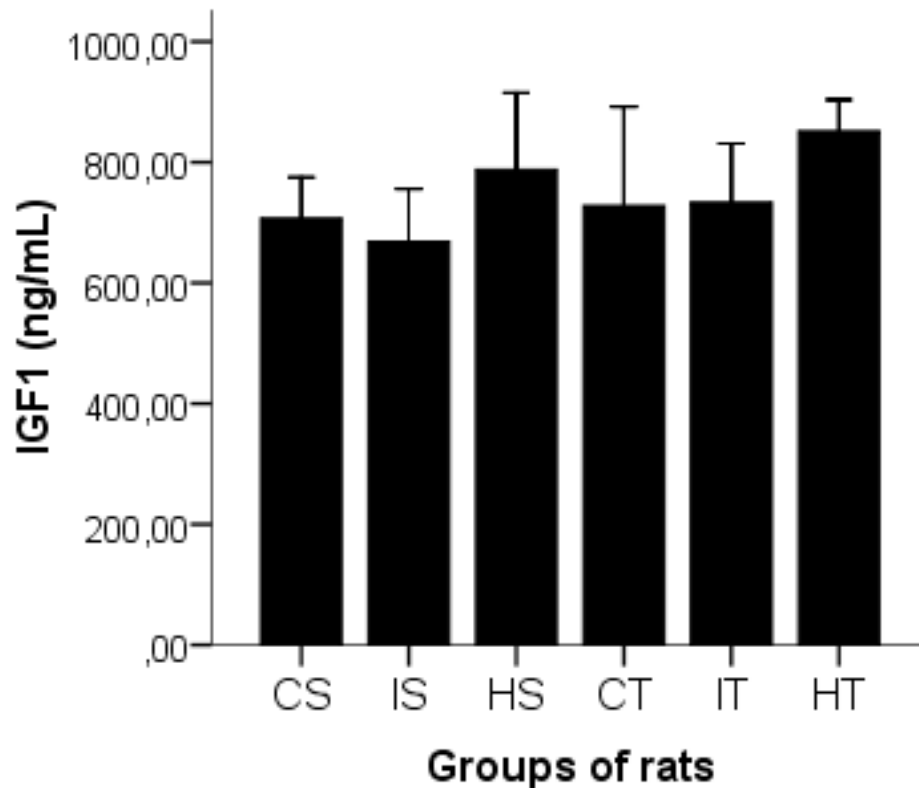
Figure 4. Free IGF-1 (insulin-like growth factor) determined in blood. CS, Casein-Sedentary; IS, whey protein isolate-sedentary; HS, whey protein hydrolyzate-sedentary; CT, casein-trained; IT, whey protein isolate-trained; HT, whey protein hydrolyzate-trained). The data for the six groups represent means ± SD. One-way ANOVA statistical analysis and Tukey, with significance <0.05.

reduction of IGF1 represents an adaptive response to DNA damage (Niedernhofer et al., 2006). On the other hand, heavy physical training is associated with elevated contents of DNA in the skeletal muscle, concomitant with elevated contents of IGF1 (Zentella and Massagué, 1992; Adams and Haddad, 1996). In this case, however, the good physical performance evidenced through the training protocol, in association with the unaltered levels of IGF1, may indicate that the animals were in normal health. Therefore, our results suggest that the observed low DNA levels and the invariant IGF1 levels did not reflect any pathological state or muscle damage, but rather an adaptive response to a muscle-protein sparing effect associated with the consumption of the hydrolyzed protein.

Conclusion

Data from this experiment suggest that consumption of a diet containing prehydrolyzed whey proteins as the sole source of protein can result in lower amounts of gastrocnemial DNA while maintaining normal levels of protein in the rat and the ability to perform physical work at above normal levels. Although this property appears to have a partial dependence on physical activity, the evidence presented here suggests that this is a distinctive characteristic of the hydrolyzate, not shared with the unhydrolyzed proteins, and that the health of the animal is not negatively affected.

Competing interests

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

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