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ORIGINAL CONTRIBUTION

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Effect of soy- and whey protein-isolate supplemented diet on the redox parameters of trained mice

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Summary *Background* A number of clinical trials have successfully been performed using whey and/or soy proteins in the treatment of many diseases. They both have antioxidant properties, which appears to be a factor in aerobic physical performance as well. In addition, these are the most often used supplements that sportsmen take to increase their performance. *Aim of the study* To investigate the effect of whey and soy protein supplementation on redox parameters in the muscle, on body weight, and body composition in swimming-trained and non-trained animals. *Methods* The effect of whey and soy protein-isolate supplementation on muscle redox parameters, body weight, and body composition in trained and non-trained mice was investigated after a single exhaustive bout of exercise. Steady state free radical concentration measured using electron spin resonance (ESR) spectroscopy, reduced and oxidized glutathione ratio, thiobarbituric acid-reactive substances (TBARS), and protein carbonyl levels of the red leg muscle were measured. *Results* Free radical concentrations and glutathione composition of the tissue indicated that whey protein supplementation of the regular diet was able to prevent oxidative stress regardless of training. Soy protein supplementation decreased TBARS only in the muscle of untrained animals, while training *per se* lowered protein damage in all investigated groups. A mixture of soy and whey protein supplementation resulted in leaner animals after training, but had no synergistic effect on either of the measured redox parameters. *Conclusions* Athletes consuming these supplements could train with higher exercise intensity. The antioxidant effect of the two proteins is based on different mechanisms of action.

Key words soy protein – whey protein – oxidative stress – ESR – glutathione – exercise

Introduction

Whey and soy protein isolates are popular supplements in sport nutrition. Designed as high biological value protein supplements, they augment not only physical performance, but also the antioxidant defenses [1–3], and exhibit benefit in the field of exercise performance and enhancement [4–6]. In addition, a number of clinical trials have successfully been performed using whey and/or soy proteins in the treatment of cancer [7–9], HIV [10, 11], hepatitis [12, 13], cardiovascular diseases [1, 14], type II diabetes

with nephropathy [15, 16], osteoporosis [17, 18], hypercholesterolemia [19, 20], and stomach ulcerative lesions [21].

The biological components of whey [22, 23] or soy [24, 25] demonstrate a range of immunoenhancing and other beneficial properties. It is well known that physical exercise imposes oxidative stress on the body due to generation of reactive oxygen species (ROS), particularly the superoxide anion ($O_2^{\bullet -}$) [26–28]. Oxygen consumption may lead to an increase in ROS production, which may limit exercise performance. Therefore, the function of the antioxidant system appears to be a factor in aerobic physical performance. The glutathione (GSH) antioxidant machinery is one of the most important endogenous non-enzymatic protective systems. The thiol group of cysteine is responsible for the antioxidant effect of the GSH molecule [29, 30].

The primary mechanism by which whey is thought to exert its beneficial effects is by intracellular conversion of the amino acid cysteine to glutathione. A cysteine-rich whey protein isolate therefore represents an effective cysteine delivery system for GSH replenishment [31]. The mechanism by which soy protein is thought to exert its benefits appears to be linked to its content of isoflavones, an important class of bioactive phytoestrogens. Its effectiveness may be a function of the ability of subjects to biotransform soy isoflavones to the more potent estrogenic isoflavone, equol, which is superior to all other isoflavones in its antioxidant activity [32]. Moreover, diets containing soy protein prevent exercise-induced protein degradation of the skeletal muscle, possibly through inhibition of the calpain-mediated proteolysis [33].

A number of surveys showed that the use of nutritional supplements is widespread among athletes, and that a greater knowledge and awareness of their properties represents an important factor influencing their usage [34, 35]. The aim of our study was to investigate the effect of whey and soy protein supplementation on redox parameters in the muscle, on body weight and body composition in trained and non-trained animals. As far as we know, this is the first comparative study on the *in vivo* effect of whey *versus* soy protein on the investigated redox parameters.

Materials and methods

Animals and diets

The protocol and animal care was approved by the local Committee for the Care and Use of Laboratory Animals in accordance with the Hungarian Governmental Regulation No. 243/1998 (XII.31) based on the European Community guidelines (Helsinki Declaration and Directive 86/609/EEC).

Five-week-old pathogen-free NMRI (Crl:NMRI BR SPF) male mice weighing 18–20 g were obtained from ToxiCoop Ltd. (Budapest, Hungary). They were kept under standard conditions and fed a basal preformulated rat–mouse diet R/M-Z+H 15 mm (Ssniff Spezialdiaten GmbH., Germany) supplemented with different protein isolates. The preformulated diet containing 20% protein, was supplemented with either casein (control or C group), soy protein isolate (SP group), whey protein isolate (WP group), or a 1:1 mixture of soy and whey protein isolates (SWP group) to give a total protein content of 40% of the total weight of feed. Casein sodium salt was from Sigma (Budapest, Hungary), soy protein isolate Nutrisoy (82% of pure protein content), and whey protein isolate Diamond Whey (90% pure protein content) obtained by cross flow micro- and ultrafiltration were purchased from New Syform Ltd., Oderzo, Italy. The weight of animals and of the consumed feed was registered at the beginning and the end of each week.

Training protocol

Animals were divided randomly into nine groups, each containing eight mice. Four groups underwent endurance training in swimming. They trained thirty minutes on the first day, forty-five minutes on the second day, then one hour per day, six days per week. After the first three weeks, swimming time was increased to one and half hours for another three weeks. Four out of the remaining five groups did not undergo physical training, but animals were put to swim for 30–60 s, six days per week to make them emotionally accustomed to swimming. Animals were exercised in a plastic tank filled to a depth of at least 40 cm with tap water at a temperature of 34 ± 1 °C. On the final day of the experiment, all mice, but the untrained control group (control 0 group, C_0), performed a single acute bout of swimming exercise until exhaustion. Mice were put to swim with a piece of lead of 7.5% of their body weight tied to their tails. No mouse ceased the exercise because of any type of injury.

Tissue preparation

Immediately after the exhaustive exercise bout, mice were decapitated and exsanguination followed. The liver, and muscle from both hind legs were quickly removed and stored in liquid nitrogen. *Quadriceps femoris* red portion was used in our study, which is the main muscle group used during swimming. Tissue samples for antioxidant determination were minced thoroughly and homogenized with a Potter–Elvehjem

glass or a blade-homogenizer at 4 °C to a concentration of 100 g wet weight/ml of PBS. Body fat was also removed from inguinal and epididymial regions and weighed on an analytical balance (without further analysis).

Electron spin resonance (ESR) measurements of frozen tissue samples

Electron spin resonance spectroscopy is a direct method for detection of free radicals and was used to measure the *in vivo* generated free-radical concentration of tissue samples. Measurements were performed in a quartz finger-dewar filled up with liquid N_2 in a similar way to that described by us earlier [36].

Measurement of redox parameters

Thiobarbituric acid-reactive substance (TBARS) were determined based on the original description of Placer et al. [37] modified by Matkovics et al. [38]. The amount of protein carbonyls was measured by the original method of Levine et al. [39], which is based on the reaction of the carbonyl groups with 2,4-dinitrophenylhydrazine. Reduced (GSH) and oxidized (GSSG) glutathione was measured based on the original method of Sedlak and Lindsay [40] modified by Matkovics et al. [38]. To measure citrate synthase activity in the muscle, oxalacetate, and acetyl-coenzyme A were used as a substrate and a coenzyme, respectively. After the conversion, Ellman-reagent (10 mM) was used to measure the amount of sulfhydryl groups, and the absorbance of the complex was read at 412 nm [41]. Concentration of produced lactate was measured in blood plasma using a Lactate PAP kit (61192, bioMérieux® s.a., France), which is based on a lactate oxidase-peroxidase-chromogen reaction sequence of L-lactate giving a peak at 505 nm.

Statistical analysis

Distribution of data was determined using the Kolmogorov-Smirnov test and the results were statistically analyzed by unpaired Student's *t* or the non-parametric Mann–Whitney *U*-test using Statistica™ program package (Statsoft, Oklahoma, USA). The results are expressed as means ± SD, or medians and quartiles, respectively.

Results

Animals were fed with a preformulated regular diet supplemented with casein (C_0, C, and C_T, groups), soy protein isolate (SP and SP_T groups), whey protein isolate (WP and WP_T groups), or a 1:1 mixture of soy and whey protein isolates (SWP and SWP_T groups). Four out of nine groups underwent a six-week training in swimming (denominated with a T subscript: groups C_T, SP_T, WP_T and SWP_T), and all groups but one (C_0) were exposed to a final exhaustive exercise before termination of the experiment. Thus, C_0 can be regarded as an absolute control.

Plasma lactate levels serve as an indicator of the exercise-induced physical stress. Figure 1 shows that lactate concentrations increased significantly in the plasma of all animals that underwent a final exhaustive exercise before death compared to basic values of the C_0 group. Steady state free radical concentrations were measured in the liver using ESR spectroscopy as an indicator of an overall oxidative stress of the organism. Data in Fig. 2 show that, similarly to the results of Fig. 1, all animals that performed a final

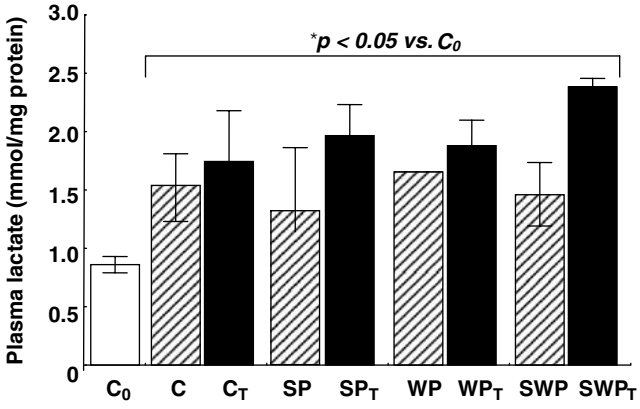

Fig. 1 Plasma lactate levels measured in different groups after an exhaustive bout of exercise. Results are expressed as means ± SD. *Significant differences compared to C_0 group, $n = 6–8$

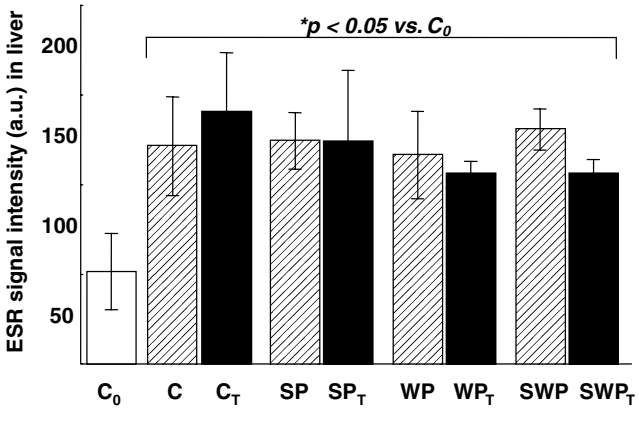

Fig. 2 Steady state free radical concentrations in the liver of mice fed with different diet, expressed as ESR signal intensities. Results are expressed as means ± SD. *Significant differences compared to C_0 group, $n = 6–8$

exhaustive exercise exhibited a significant oxidative stress compared to those in C_0 group.

Steady state free radical concentrations and the ratio between GSH and GSSG were used as markers of the redox state in the muscle. Figure 3 shows that exhaustive exercise caused a significant increase in free radical concentration of the muscle tissue in animals that consumed a diet supplemented with casein (groups *C* and C_T) or soy protein isolate (groups SP and SP_T) compared to basic values (C_0). Whey protein isolate-containing diet, however, prevented oxidative stress in the muscle induced by heavy exercise (groups WP, WP_T, SWP, and SWP_T). In none of these cases was soy protein supplementation or training alone able to lower free radical levels in the muscle tissue, while diet involving whey protein itself had a free-radical scavenging effect. Protein supplementation did not seem to have any effect on GSH/GSSG values of trained animals (Fig. 4). Only whey-containing supplementation caused a significant decrease in GSH/GSSG ratio in untrained animals after a final exhaustive exercise (WP and SWP groups) compared to control values (C_0 and *C*).

Protein carbonyl content and TBARS are additional markers of oxidative stress indicating the level of protein damage and lipid peroxidation, respectively. Soy and whey protein supplementation seemed to be effective in preventing lipid peroxidation after an exhaustive exercise without training (Fig. 5, *C vs.* SP, WP, or SWP groups). Training caused a surprising increase in the levels of TBARS in the soy and/or whey protein-fed groups compared to trained control (C_T) data, or to the respective untrained values. Interestingly, muscle tissue of animals eating casein appeared to be less susceptible to training-induced lipid peroxidation (*C vs.* C_T) than the other trained groups (C_T *vs.* SP_T, WP_T, or SWP_T). Protein carbonyl levels were lower in trained animals than in untrained ones: C_T, SP_T, WP_T, and SWP_T groups showed significantly lower protein carbonyl levels than their respective untrained controls (Fig. 6).

Mice were weighed at the beginning and the end of every week during the six-week experiment. At the end, they were killed and inguinal and epididymial fat was excised and weighed. Citrate synthase enzyme activity was measured in excised muscle samples as an anabolic marker of tissue build-up.

Trained animals showed significantly lower body weights compared to their respective non-trained controls (Table 1). Total body weight did not change among non-trained groups, or among the trained ones. SWP_T group was the only exception, where the body weight gain was lower than in trained controls (C_T). Trained animals had lower fat body mass

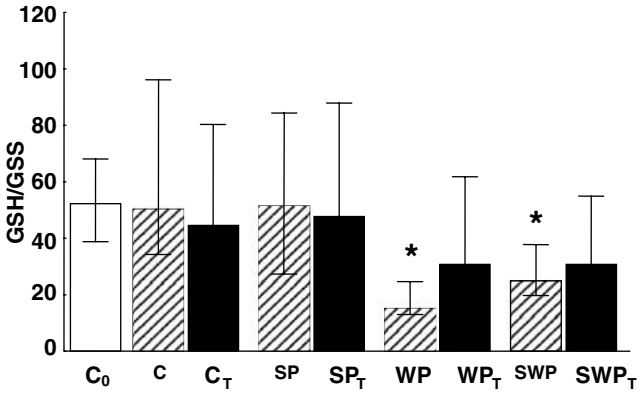

Fig. 4 Ratio between reduced and oxidized glutathione in the muscle of mice fed with different diets. Results are expressed as medians and quartiles. *Significant differences compared to *C* group, $n = 6–8$

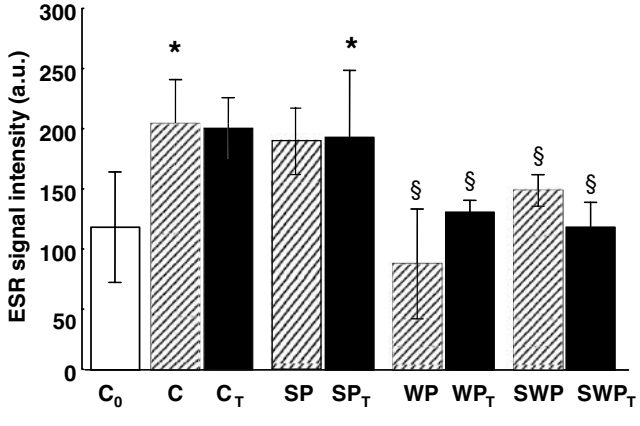

Fig. 3 Steady state free radical concentrations in the muscle of mice fed with different diet, expressed as ESR signal intensities. Results are expressed as means ± SD. *Significant differences compared to C_0 group; §significant differences compared to respective control groups (*C vs.* WP or SWP, and C_T *vs.* WP_T or SWP_T), $n = 6–8$

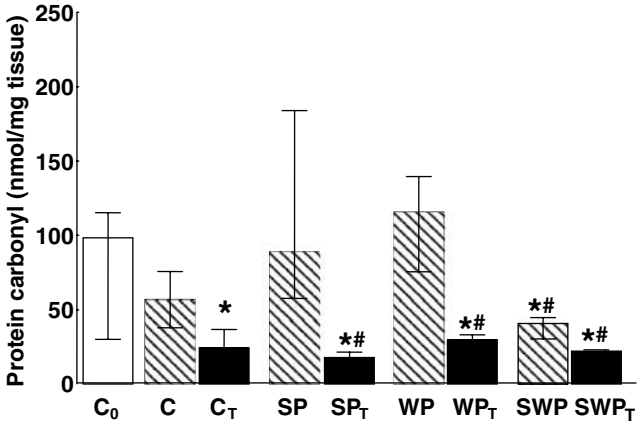

Fig. 5 Levels of TBARS in the muscle tissue of mice fed with different diets. Results are expressed as medians and quartiles. *Significant differences compared to *C* group, $n = 6–8$; #significant differences compared to C_T group; ^significant differences compared to respective untrained groups, $n = 6–8$

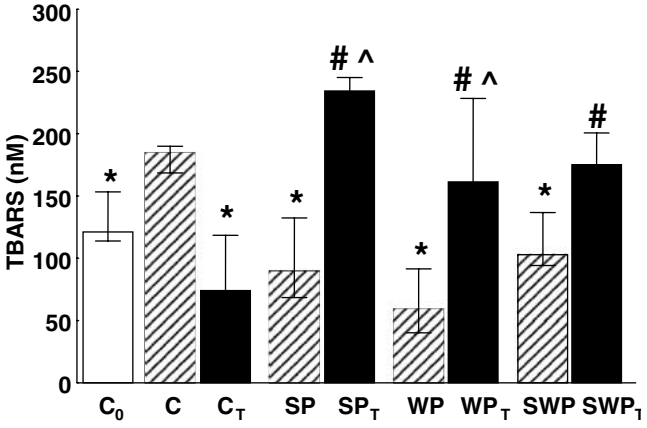

Fig. 6 Protein carbonyl levels in the muscle tissue of mice fed with different diets. Results are expressed as medians and quartiles. *Significant differences compared to C_0 group; #significant differences compared to C_T group; ^significant differences compared to respective untrained groups, $n = 6–8$

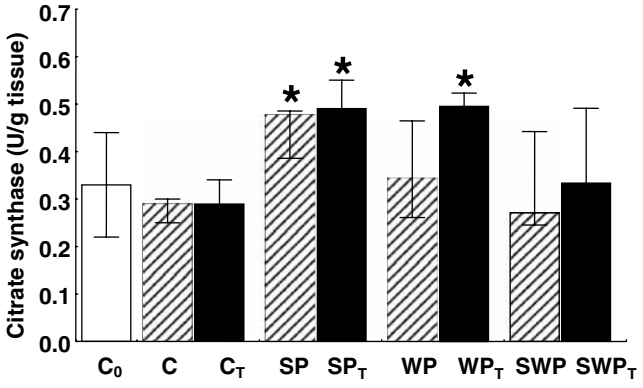

Fig. 7 Citrate synthase enzyme activity measured in the muscle tissue of mice fed with different diets. Results are expressed as medians and quartiles. *Significant differences compared to *C* group, $n = 6–8$

percentage *vs.* their respective untrained groups, i.e., body composition changed significantly by training. Body weights and the measured body fat of trained animals were significantly lower than those of untrained mice in spite of the *ad libitum* diet.

Citrate synthase levels (Fig. 7) showed that anabolic processes in muscle tissue of whey protein-fed animals were increased significantly by training. Soy protein supplementation, on the other hand, induced citrate synthase activity even without training. Lower body fat, more than muscle build-up, was the main reason for changes in body composition of SWP and SWP_T groups. Both soy (SP_T) and whey (WP_T) supplementation coupled with training produced an increase in muscle anabolic processes, probably through different mechanism because the 1:1 mixture of soy and whey proteins in the SWP_T group was unable to induce a similar increase in the activity of this anabolic marker enzyme. C_0 was the only group, which had no training, nor was exposed to the emotional stress of being submerged into water. Emotional stress due to swimming alone did not show significantly different effects on body weight and body composition as seen from data of C_0 *vs.* *C* groups in the Table 1.

Table 1 Body weight and amount of fat (% of body weight) in mice, with or without training and food supplements. Results are expressed as means ± SD

Group	Body weight (g)	Inguinal + epididymial fat (body weight %)
C_0	41.44 ± 2.73	4.98 ± 1.59
C	39.61 ± 2.02	3.82 ± 1.28
C_T	35.97 ± 1.01$^{\vee}$	2.27 ± 0.90$^{\vee}$
SP	39.85 ± 3.61	3.93 ± 1.52
SP_T	35.75 ± 1.83$^{\vee}$	2.42 ± 0.59$^{\vee}$
WP	39.09 ± 2.76	3.05 ± 0.82
WP_T	34.74 ± 1.68$^{\vee}$	2.39 ± 0.80
SWP	39.51 ± 1.05	3.41 ± 0.65
SWP_T	33.35 ± 2.09$^{\vee}$ $^{\#}$	2.12 ± 0.58$^{\vee}$

$^{\vee}$ $P < 0.05$ comparing trained to the respective untrained groups
$^{\#}$ $P < 0.05$ compared to C_T

Discussion

The focus of this study was to evaluate the effect of whey and soy protein isolate-supplemented diet on some redox and body composition parameters of mice exposed to an exhaustive swimming exercise after or without a six-week daily physical training. The organ of our interest was the skeletal muscle represented by red muscle tissue of the upper leg.

Plasma lactate levels and liver ESR measurements showed that all mice exposed to a final exhaustive swimming suffered a general oxidative stress (Figs. 1 and 2). Nevertheless, whey and/or soy protein supplementation of the average diet resulted in an antioxidant effect in the muscle. The effect on the measured redox parameters was different and depended on whether the animals were pre-trained or not. In untrained animals, whey protein isolate-supplemented diet could significantly prevent oxidative stress induced in the muscle by heavy exercise based on data of total steady state free radical concentration (Fig. 3) and of the lipid peroxidation marker, TBARS (Fig. 5). Soy protein isolate-supplementation on its own, on the other hand, had no significant effect on free radical concentrations of the muscle, but prevented an increase in lipid peroxidation (same figures), indicating that its antioxidant effect is based on a different mechanism than that of whey protein isolate.

Glutathione is a general indicator of the water-soluble antioxidant status of a tissue [42]. Based on the antioxidant properties of the used protein isolates,

we expected that their supplementation would prevent the decrease of GSH/GSSG in the muscle. In contrary to our expectations, GSH/GSSG ratio decreased in the muscle tissue of animals consuming whey protein isolate-supplemented diet and exposed to a final exhaustive exercise, but not in the other groups (Fig. 4). This coincided with lower free radical concentration values measured by ESR (Fig. 3). Thus, we speculate that oxidative damage induced by exhaustive exercise in the muscle tissue was prevented in whey protein-supplemented untrained animals at the expense of reduced glutathione.

Whey protein isolate used in this study consisted of 90% pure protein including β-lactoglobulin (56%), α-lactalbumin (15%), glycomacropeptide (20%), immunoglobulins (3%), bovine serum albumin (1.55%), and lactoferrin (0.125%). Many of these compounds are rich in cystine or glutamylcystine. The later one is easily transported into cells, representing a readily available substrate for GSH or other thiol-containing protein biosynthesis. Whey protein isolate contained 2.62% cystine compared to 0.97% of Supro® brand soy protein isolate and 0.3% of casein. Thus, it would have been logical to expect a higher GSH/GSSG ratio in animals fed by whey protein-isolate-rich diet. The fact that the opposite effect was registered suggests that the GSH was used up to fight the oxidative stress in the muscle or that its synthesis was decreased. Interestingly, the same phenomenon was observed in the muscle of mice at rest and after exercise after glutathione treatment [43]. But why would whey protein-fed animals use more intensively their GSH pool to fight oxidative stress than animals on other diets? One possible explanation is that they have been preconditioned by high dietary GSH levels to preferentially use this antioxidant source when necessary. But it cannot be excluded that whey cystein was used to synthesize, in addition to GSH, other thiol-containing antioxidants, like *N*-acetyl-L-cysteine, alpha-lipoic acid, or others [44, 45], decreasing the overall pool of GSH. We are currently investigating these possibilities.

Training for six weeks decreased protein damage regardless the diet (Fig. 6), preconditioning muscle tissue against the formation of the oxidative carbonyl product. Decreased fat ratio in the body weight presented in Table 1 and the anabolic marker citrate synthase [46, 47] showed that high amount of both soy- and whey-protein-containing supplementation can increase tissue build-up, especially under training (Fig. 7). This could mean that athletes consuming these supplements could train with higher exercise intensity. In fact, it has been shown that subjects on whey protein isolate-containing diet were able to generate greater power and increase the amount of work they could achieve during a fixed time [48].

To summarize our results, a soy and/or whey protein isolate-supplemented diet, containing 40% of the total weight of feed, resulted in a lower oxidative damage in the muscle induced by a single bout of exhaustive exercise in mice. The effect on oxidative stress markers was different depending on the protein isolate extract included into the diet, on the training status of the animals and on the nature of the marker. Whey protein isolate, a known cystein donor, had a preventive effect on oxidative markers, like steady state free radical concentration and TBARS, while the flavonoid-rich soy protein isolate prevented the formation of the protein damage marker in muscle of untrained animals. Training had a beneficial effect on the measured redox parameters only regarding protein carbonylation. This effect was independent from the diet indicating that training rather than the nature of supplementation was responsible for the observed protection. A mixture of soy and whey protein supplements had no synergistic effect on either of the measured parameters indicating that their mechanism of action must be different or that these proteins prevent each other's absorption or utilization by the organism, especially under training. The later could explain why body weights of trained animals fed with soy–whey protein supplements were lower than those in any other trained groups.

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