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An Enriched Collagen Peptide Formula but not Fish Collagen Improves Exercise Performance and Metabolic Status in Old Mice

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Abstract

Eighty twenty-four month old male mice, maintained under standardized conditions, were divided depending on dietary supply: A) standard age-balanced chow food and B) added with GPT-2218 (dosage to be worked out after preliminary data). Animals were weight-matched and each group randomly allocated to: s) a sedentary protocol or t) training protocol by applying endurance exercise. Namely, after adaptation in an ergometric treadmill for one week (5%, 6-8 m/min), the latter group was subjected to following schedule: 20 m/min, 8° slope, 50 min/day for the first week and 25 m/min, 8° slope, 50 min/day (corresponding to 75% of maximum VO_2) for the second week. A further group, either sedentary or trained, was supplemented with a generic fish collagen-peptide compound(C) claimed of stamina effect. So, altogether six groups were examined as follows: As (standard food, sedentary), At (standard food under training), Bs (supplemented-sedentary), Bt (supplemented under training), Cs and Ct. Parameters measured were VO_{2max}, liver and gastrocnemius tissue level of glycogen, gastrocnemius level of oxidative markers and inflammatory/anti-inflammatory balance. Results showed that as compared to At, where training showed only a trend improvement of VO_{2max}, Bt had a significant improvement ($p<0.05$ *vs* baseline and sedentary group). However, Bt showed significantly better performance than At ($p<0.05$ *vs* Bt and 0.01 vs baseline and sedentary group). Skeletal muscle concentration of TBARs significantly increased in both training group soon after exercise ($p<0.05$) but only Bt. At 2 h observation showed a significant, albeit partial, recovery ($p<0.05$ vs At). Concomitantly immediately after exercise, there was a drop of SOD (only in At reached a significance of $p<0.05$) and GSH-Px ($p<0.05$ *vs* baseline). Both these values in both groups recovered after 2 h ($p<0.05$). However, as for SOD only Bt recovery-values was significantly better ($p<0.05$ *vs* At). The forelimb strength was significantly improved by training ($p<0.05$) but at a higher rate in Bt group ($p<0.05$ vs At). Physical training brought about a significant increase/decrease of IL-1beta and IL-10, respectively in both groups ($p<0.05$). However, at 2 h post-exercise observation, these values recovered only in Bt group ($p<0.05$ vs At group). Hepatic glycogen stores were depleted only in At group ($p<0.05$), whereas both groups showed a significant decrease in skeletal concentration of glycogen ($p<0.05$). These values did not recover after 2 h in At group while a significant replenishment was recorded in Bt group ($p<0.05$). Overall, it appears that GPT-2218 enables a better training benefit on VO_2, together with a better hormetic effect of training on redox and inflammatory balance leading to a more efficient preservation of skeletal muscular glycogen and more consistent strength performance. Whatever the set of experiments and the parameters tested, the generic fish collagen-peptide did not yield any significant change, being comparable to the un supplemented group A. Further studies are awaited to scrutinize the impact of a GPT-2218 association with selective amino acids formulas.

Keywords: Collagen peptide; Celergen; Aging; Muscle

Introduction

Skeletal muscle mass in adults comes from a balance between protein synthesis and degradation ratio [1]. Our body triggers a complex set of biological responses to dynamically affecting such synthesis/degradation balance homeostasis. It has been shown that the adaptive response of muscle to disuse as well to ageing is represented by a reduction in glycogen content and protein synthesis. This is further detrimentally associated with an increase in protein degradation [2,3]. This invariably leads to a decline in muscle mass, efficiency and dysfunction in motor coordination [4]. This overall sarcopenic picture may heavily impair quality of life and increased risk of fractures due to falls [5]. Indeed, a change in body composition takes place from the third decade of life to different extent. Between the age of 30 and 60, it leads to an average gain of 0.45 kg of fat and loss of about 0.23 kg of muscle per year. Taken overall, the phenotypic aging feature in skeletal muscle mainly consists of accumulating oxidative byproducts of lipids, proteins and DNA, affecting the whole body and muscle in particular [6]. Proper nutrition and quality exercise is known to play an important role to partially counteract this phenomenon. However, it has been shown that under training the increase of muscle protein synthesis in older individuals, is about 30% less efficient, when compared with younger ones [7]. On top of this, it seems that older individuals may develop adaptations to resistance

exercise by reducing muscle anabolism with 2.5 and 16% in muscle volume and muscle *vs* 6.2 and 27%, respectively as compared to younger control group [8,9]. Moreover, we have to consider that aging per se is associated to higher level of inflammatory parameters and these may be further elevated after moderate-intense training [10,11]. Selected nutrition supplementations when applied under specific condition have been shown to improve muscle build up. Leucine alone or in association with other branched-chain amino acids remarkably promotes protein synthesis via a mammalian target of rapamycin (mTOR)-mediated mechanism [12,13] and may also exert anti proteolytic properties. However, so far it is not fully resolved the issue whether these beneficial effects of leucine have to be entirely ascribed to its direct action or through intermediate metabolites [14]. Therefore, tailored addition of leucine metabolite to a well-balanced nutrition may prove to counter fight the tendency over the long term use of leucine supplementation to fully maintain its anabolic effect although this is still producing contradictory data [15]. Recently, it has been reported that collagen peptides may play a beneficial effect on the increase of muscle volume and strength in older individuals when associated with physical exercise. In a study enrolling elderly men (mean age 72) with sarcopenia joining a strength training program (3 days/week for 3 months), those supplemented with 15 g/day of collagen peptides had significantly larger growth in lean muscle mass and workload capacity than those given placebo [16]. The aim of the present study was to test a collagen-based multicomponent formula GPT-2218 (hydrolysed collagen, hydroximethyl-butirrate, creatine, L-leucine, vitamin C, E, B5, B6, B2, B1, A, D, B12, folic acid, zinc. Peptivis, Named Co., Italy) on functional and metabolic of old mice undergoing physical exercise.

Materials and Methods

Experimental animals, handling and supplementation

Eighty 24-month old male mice were housed in polypropylene cages (2-3 mice per cage) and maintained under standardized conditions: 12 h-light/dark circle, 22°C and 70% humidity. Standard laboratory chow diet and water were available ad libitum. Before the experiments, the mice were acclimatized for 1 week to the environment and diet. All animal procedures were performed to minimize animal discomfort following the international guidelines for animal research for experimental and other scientific purposes (National Research Council. Guide for the Care and Use of Laboratory Animals. Washington: National Academy Press; 1996). Food intake and water consumption were recorded daily, and all animals were weighed weekly. Blood were withdrawn at the start of the study to provide baselines. Each group was divided depending on dietary supply: A) standard age-balanced chow food and B) added with GPT-2218 (100 mg/kg). Each group was then assigned to one of the two treatments below indicated.

Exercise and monitoring (energy metabolism) method

At the end of this period, animals were matched for weight and randomly divided into two further groups: s) a sedentary control or t) a training group undergoing the endurance exercise training. In this latter subgroup, all animals were accustomised in an ergometric treadmill for one week (5%, 6-8 m/min, no incline, 10 min/day). After adaptation, B group animals were subjected to following schedule: 20 m/min, 8° slope, 50 min/day for the first week and 25 m/min, 8° slope, 50 min/day (averaging 75% of maximum VO_2) for the second week. So, altogether four groups were examined as follows: As (standard food, sedentary), At (standard food under training), Bs (supplemented)- sedentary and Bt (supplemented under training). Collaterally, we had a group of 10 mice supplemented with a French marine station-derived fish collagen and protein hydrolysate promoted as a stamina booster with also anti-inflammatory properties (Celergen, Swiss cap packaging, Switzerland).

Parameters tested

Body composition analysis: Body composition, i.e. lean mass and, fat mass were analysed by a dual-energy x-ray densitometer under general anaesthesia (isoflurane) using a PIXImus imager (GE Lunar, Madison, WI, USA). Data were analysed using the manufacturer-supplied software Lunar PIXImus, version 2.2 (Lunar PIXImus Corp., Madison, USA).

In-vivo muscle strength assessment: *In vivo* muscle strength was assessed as forelimb absolute grip strength with a low-force calibrated grip strength testing system (Panlab, Cornella, Spain) after mice were being familiarized for 2 days before the test by taking advantage of the animal's attitude to grasp a bar while suspended by its tail. This test consisted by prompting the mouse to grip a force transducer equipped with a metal bar (2 mm in diameter and 7.5 cm in length) with both forelimbs and pulling it by the tail (proximal to the body) parallel to the orientation of the strain gauge and the bar (Leiter JR.). In order to minimize human variability, the same operator measured all the mice in a group and in a blinded fashion, at the same time of day and in a protected environment. Force transducer meters were set to zero or reset before each measurement. The maximal force exerted by the mouse counter pull was calculated the degreeof tensile force. The absolute maximum grip strength was defined as the maximum tension recorded over five repetitions.

Measurement of maximal oxygen consumption: After seven weeks of training, animals were transferred into a flow-through sealed treadmill chamber open-circuit system, VO_{2max} was calculated by letting a unidirectional flow of gas and the exercise test started on a 15% grade at the initial speed used for training. The velocity of treadmill was increased 3 m/min every 3 min till the mice were unable to catch up with the running speed. VO_{2max} was defined as the highest VO_2 reached during the exercise. Ambient air was pumped through the chamber at a flow rate of 5.5 l/min. Gas was sampled (500 ml/min) from a rear metabolic chamber and respiratory gas as measured by O_2 uptake and CO_2 production were determined through electronic gas analysers (Minato MG-360, Tokyo, Japan). The gas analyser was calibrated immediately before and after each test using standardized gases. The mice were measured in the same environment both before and after training.

Blood biochemistry and haematology

Tissue sampling and analysis (at baseline, end of exercise and 2 h later)

Markers of oxidative stress: Skeletal muscle and liver tissues were homogenized to examine redox parameters, such as thiobarbituric acid (TBARS), by spectrophotometry (532 nm) and expressed in nmol/mg/protein, as described by Draper et al. [17]. Superoxide Dismutase (SOD), glutathione peroxidase (GSH-Px), together with inflammation-related parameters, including IL-1βand IL-10. The levels of the above biochemical parameters in the skeletal muscle and liver were assessed

by ELISA (Jian-cheng Biotech. Sci. Co., Shanghai, China). Briefly, tissues were homogenized with the polytron homogenizer in 20 mM N-2 hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES buffer), 1 mM ethylene glycol tetra-acetic acid, 210 mM mannitol, 70 mM sucrose, pH 7.2 per g of tissue). After centrifugation at 12,000 g for 20 min at 4 ˚C the supernatant was retrieved and the reaction was started by adding diluted xanthine oxidase. After immediately incubation for 20 min, the absorbance was then read at 450 nm with the spectrophotometer. Glutathione peroxidase (GPx) was assayed in the liver sample homogenized in 8 volumes of cold buffer (50 mM Tris-HCl, pH 7.5, containing 5 mM EDTA and 1 mM 2-mercaptoethanol), next centrifuged 8500xg for 10 minutes at 40C. GPx activity was determined in supernatant using Bio-tech GPx-340TM Assay kit (O2XIS International, Inc., USA).

Glycogen analysis at muscular and liver tissue level

Liver and muscle samples (10 mg of snap frozen tissue) were incubated in 2 ml of KRBH-3% FA-free BSA with 0.2 µCi/ml of D-[U-14C] glucose. At the end of the incubation, the samples were digested in 1 M KOH and 100 µl of a solution by titrating the pH to 4.8 and 500 µl of acetate buffer (containing 0.5 mg/ml amyloglucosidase). After overnight hydrolyzation of the glycogen, the solution was neutralized and added 1 ml of TRA buffer (TRA 0.3 M, $MgSO_4$ 4.05 mM, KOH 1 N, pH 7.5, with hexokinase/glucose-6-phosphate dehydrogenase 250 U/ml, ATP 1 mM and NADP 0.9 mM) to convert glycogen to glycosylic units followed by enzymatic determination of glycosylic units and absorbance was read at 340 nM wavelength with the use of a glucose standard and normalized against both the free glucose sample controls and sample weight [18].

Statistical analysis

Data were expressed as mean and standard error of the mean and analysed statistically using one-way analysis of variance (ANOVA) followed by the Tukey test. The level of significance was set at 5% ($p<0.05$). The Statistical Package for the Social Sciences (SPSS®) 17.0 for Windows software was used for data analysis.

Results

Animals showed no side effects in eating up all the food, either supplemented or not, they were provided. Two mice supplemented with Celergen, developed a severe skin rash in one case and another some loose stools and fatigue.

Body weight, food intake, muscle weight and body composition

There was no significant difference in body weight between groups at baseline. Both aged groups showed no significant variation of their bodyweights over the course of the study (data not shown).

Redox measurement in gastrocnemius in sedentary and exercised animals without and with supplementation

Redox analysis of gastrocnemius showed that physical exercise in unsupplemented animals brought about a significant elevation of TBARs (Figure 1, $p<0.05$ *vs* baseline) which was paralleled by an inversely (r: -073, $p<0.05$) related decrease of SOD and GPx Figures 2 and 3 $p<0.05$ *vs* baseline). Same data were obtained in fish collagen-supplemented group, whereas the group supplemented with GPT-2218, showed no change in SOD and a lower increase of TBARs ($p<0.05$ *vs* fish collagen). While one week later, the whole redox panel ($p<0.05$ *vs* post-exercise) in GPT-2218 treated group, fish collagen supplementation did not provide any significant biochemical benefit (fish-collagen supplemented values: ns).

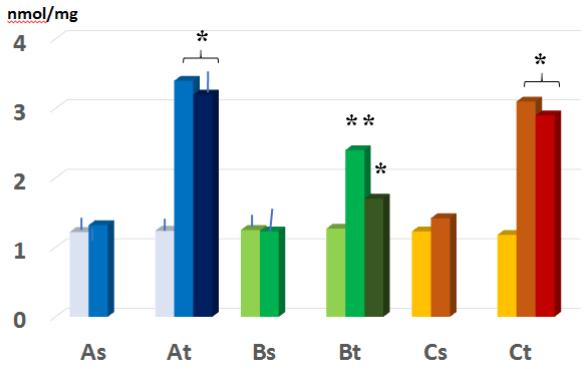

Figure 1: TBARs concentration in gastrocnemius: effect of GPT-2218
*$p<0.05$ *vs* baseline of all groups. **$p<0.05$ *vs* same timing testing in all other groups.

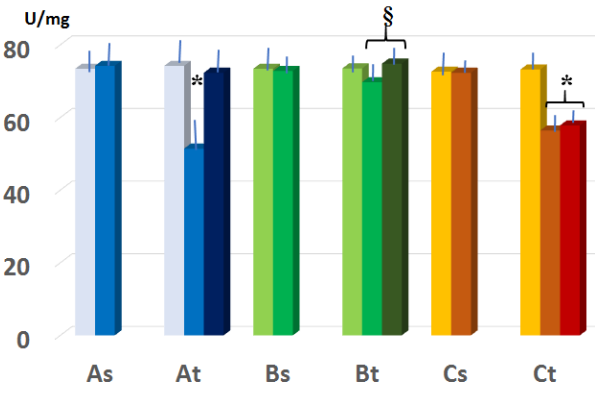

Figure 2: SOD concentration in gastrocnemius: Effect of GPT-2218
*$p<0.05$ *vs* baseline of all groups; § $p<0.05$ *vs* same timing in C group and *vs* values recorded immediately after exercise in A group.

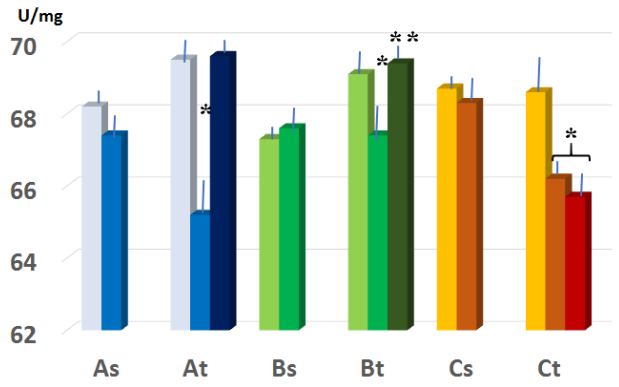

Figure 3: GPX concentration in gastrocnemius: effect of GPT-2218 *p<0.05 *vs* baseline of all groups; **p<0.05 *vs* same timing recorded in C group.

Modification of inflammatory/anti-inflammatory tissue level

As for the pro/anti-inflammatory panel, irrespective of the training and supplementation, the immediate post-exercise assessment showed a statistically significant decrease of IL-10 and increase of IL-beta (Figures 4 and 5, p<0.01 *vs* baseline). However, while what observed in the unsupplemented group and in the one fed fish collagen which after one week were still significantly abnormal (p<0.05 *vs* baseline), the group fed GPT-2218, showed a virtual recover to pre-exercise values (p<0.01 *vs* post-exercise values in unsupplemented and in fish collagen supplemented).

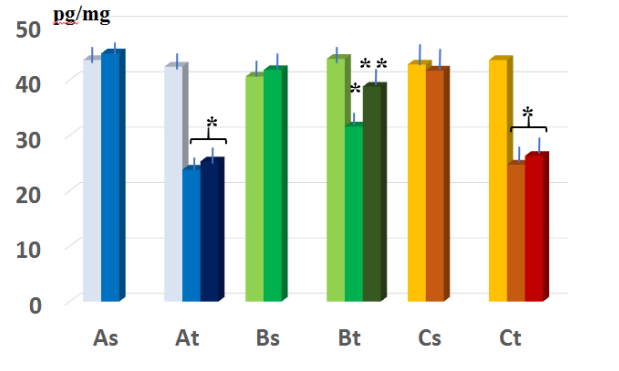

Figure 4: IL-10 concentration in skeletal muscle under training: effect of GPT-2218
*p<0.05 *vs* baseline of all groups; **p<0.05 *vs* same timing in all groups.

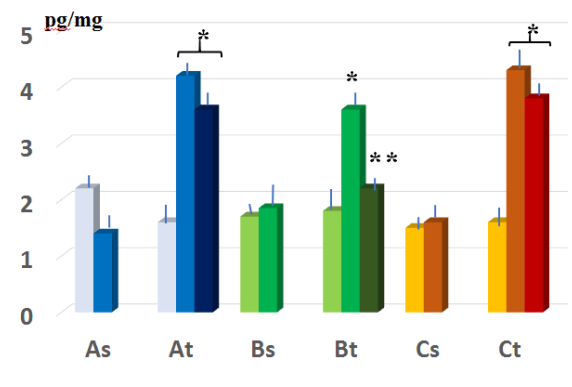

Figure 5: IL-1β concentration in skeletal muscle under training: effect of GPT-2218
*p<0.05 *vs* baseline of all groups; **p<0.05 *vs* same timing in all groups.

Muscle strength assessment

The analysis of forelimb strength variations during supplementation with or without physical exercise showed that training per se', within the short observation period significantly changed this parameter (Figure 6, p<0.05 *vs* baseline and *vs* sedentary group). The supplementeation with GPT-2218, but not the one with fish collagen, when associated to training significantly further enhanced the muscular performance (p<0.01 *vs* baseline and p<0.05 unsupplemented trained group and fish collagen-supplemented trained group).

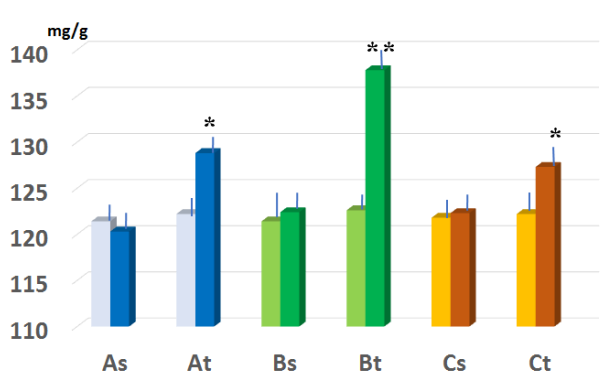

Figure 6: Effect of GPT-2218 on forelimb grip strength
*p<0.05 *vs* baseline of all groups; **p<0.05 *vs* same timing in all groups.

Effect of GPT-2218 on hepatic and muscular glycogen levels

As shown in Figures 7 and 8, hepatic and muscular glycogen levels was not affected in sedentary animals, irrespective of the supplementation (Figures 7 and 8). However, unsupplemented animals, soon after training had a significant decreased concentration (p<0.05 *vs* baseline) with an uncomplete but not significant recovery of

hepatic glycogen level but a significant drop in the muscular compartment ($p<0.05$ *vs* baseline) when tested 2 h afterwards. On the contrary, mice supplemented with GTP-2218, had a not significant decrease at the end of the exercise and 2 h later there was a significant recovery ($p<0.05$ *vs* immediate post-exercise value and *vs* sedentary value). When analysing the level of glycogen concentration in the gastrocnemius 2 h after the training session, GST-2218 supplemented mice showed a significant, albeit incomplete, recovery ($p<0.05$ *vs* sedentary and *vs* fish collagen-supplemented group) [19]. Overall, the fish collagen-peptide supplementation was ineffective to modify the above parameters, its data virtually mirroring the ones of unsupplemented group.

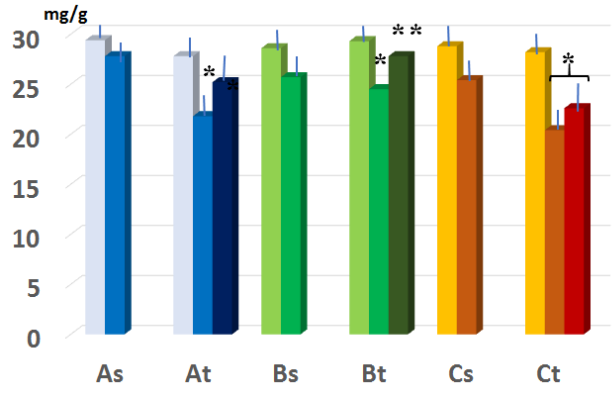

Figure 7: Hepatic glycogen concentration effect of GPT-2218
*$p<0.05$ *vs* baseline of all groups; **$p<0.05$ *vs* same timing in all groups.

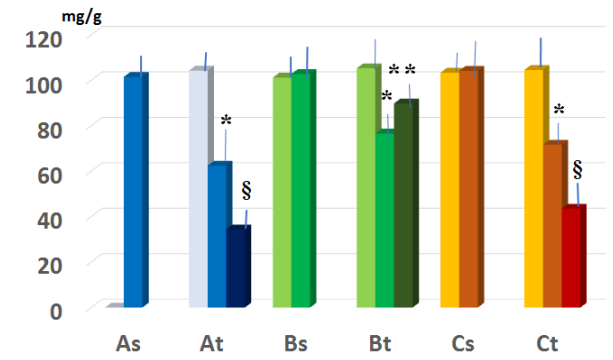

Figure 8: Glycogen concentration in gastrocnemius: effect of GPT-2218
*$p<0.05$ *vs* baseline of all groups; § $p<0.03$ *vs* values recorded immediately after exercise; *$p<0.05$ *vs* same timing in all groups.

Maximal oxygen uptake

Within the short training protocol, exercise per se did not bring any significant increase of this value in the unsupplemented groups (Figure 9). The supplementation of fish collagen-peptide was similarly ineffective, while GPT-2218 enabled a significant improvement of this value of about 10% ($p<0.01$ *vs* baseline, sedendary and fish collagen-supplemented group).

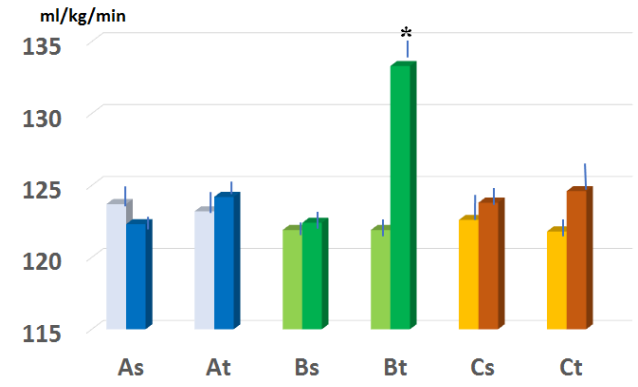

Figure 9: Effect of GPT-2218 on Maximal Oxygen Uptake
*$p<0.01$ *vs* baseline and other ecording time in all groups.

Conclusion

The ability to perform muscular work is critical for maintaining health and performing activities of daily living and the reduction of muscle mass and its functional ability to exert workload during ageing is one of the main factors causing frailty in the elderly [20]. It is reported that, from the age of 60, there is an acceleration of muscle mass loss averaging 2% annually [19]. Moreover this phenomenon is paralleled by a concomitant muscle strength decay estimated at 3% yearly [21]. Such progression of sarcopenia is affected by changes such as modified lifestyle habits, reduction in anabolism, increased low-grade inflammation and altered redox balance, all conjuring up for a significant muscle catabolism [22,23]. From an anatomic viewpoint, this is mirrored by a reduction in total muscle cross-sectional area of nearly 40% between 20 and 60 years of age [24]. Being the main source of adenosine triphosphate, glycogen is the major secondary form of long-term energy storage and its tissue levels are reliable markers of workload-related expenditure [25]. Indeed, it can be quickly mobilized to meet a sudden need for blood glucose during exercise [26] and its depletion when performing long-lasting intensive exercises, may lead to severe exhaustion. If such phenomena progresses to a constant further consumption of blood glucose, the liver glycogen, is debulked by glucagon to glucose and lactate builds up causing considerable fatigue [27]. Collagen peptides are gaining a growing interest in the medical field. As a matter of fact, collagen is the most common protein of the extracellular matrix and it beneficially affects skeletal muscle, by supporting tensile strength and elasticity [28]. Unlike what shown for muscle mass, the synthesis rate of collagen in skeletal muscle is known to decay about tenfold already from 1 to 24 months of age [29]. Moreover, its apparent age-related increase is indeed due to a defective resorption rather than a true neo-collagen synthesis, thus invariably bringing to a detrimental effect on the overall muscle physiology [30]. Only very recently has been convincingly reported by Zdzieblik et al. [16] that a specific quantitative collagen peptide supplementation could exert favourable body composition changes in elderly subjects and, namely, on muscle mass when undergoing a rationale and physically affordable exercise training. This prompted us to further deepen the our study by using a complex formula either using this collagen peptide and further endowed with a mixture of selected

amino acids, vitamins, minerals and trace elements (GPT-2218). Our results showed that this intervention exerted significant benefits on all parameters tested unlike and well beyond any generic fish collagen peptide-antioxidant formula. As for antioxidant protection exerted by GPT-2218, one can refer to the radical scavenging property of its peptide moieties potentiated by the presence of specific amino acids within its sequence. Indeed, they may hand over protons to electron-deficient radicals, such as it has been shown primarily for Tyr, Trp and Met, and, to a lesser extent, also by Phe, Cys and His [31-33]. The failure of the antioxidants component (lutein, resveratrol, coenzyme Q10 and selenium) of the generic, albeit much stamina-endowing claimed, fish collagen-peptide to bring about any traceable redox effect can be ascribed to its likely poor bioavailability or defective inner titre). The same doubts can be casted as for the uneffective anti-inflammatory action of this fish collagen mixture whereas, on the contrary, GPT-2218 showed a significant beneficial modulation of pro/anti-inflammatory cytokines. It is likely that, an efficient antioxidant activity of GPT-2218, enabled a reduced catabolic cytokine release and a faster anti-inflammatory cytokine counterbalancing recovery. Furthermore, it is known that peripherally generated ROS can move across the cell membrane of myocyte bringing about a dysregulaion of systemic redox balance as a whole. In this respect, as main organ in metabolic homeostasis, the liver is significantly affected by ROS during and after physical exercise [34,35]. Although we do not have data taken during the endurance exercise training period, one can envisage that. Given the moderate magnitude and duration causing a likely limited exercise-associated ROS generation, this may have triggered redox-sensitive signalling [36] with beneficial preconditioning phenotype effect of GPT-2218 [37,38]. If so, this can help explaining also the observed trend higher immediate post-exercise values of hepatic and muscular glycogen stores when compared to their sedentary counterpart. Again, this trend did not appear when the generic fish collagen peptide complex was used.

All the above biochemical modulations can be advocated to be among the factors explaining the significant physical performance improvement in GPT-2218-supplemented mice either as pure strength output and better aerobic capacity developed while tested by intensive exercise. Besides the already mentioned importance to buffer the exercise-induced increase of ATP demands burdening mitochondrial electron transport chains, other beneficial factors especially in aging [39] are the robust anti-atrophic effect of leucine, independent of its conversion to HMB, as shown in the immobilization model of sarcopenia [40] and suppress protein breakdown in the body [41,42]. A further beneficial component of GPT-2218 is HMB which has been reported to stimulate protein synthesis via activation of mammalian target of rapamycin pathway [43]. Moreover HMB is capable to mitigate protein degradation via, firstly, inhibition of ubiquitin proteasome pathway as well as inhibiting proapoptotic mechanisms in catabolic states [44]. Although we did not analyse the detailed enzymatic machinery of collagen synthesis, our data hold some interesting when considering that a recent study with green tea extract failed to exert a significant glycogen replenishment effect [45]. Overall, although these data come from an experimental setting, are worth of interest in clinics especially when considering the limiting age-factor in maintaining a proper muscle structure and lower responsiveness to training [46-49].

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