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Combined approach to counteract experimental cancer cachexia: eicosapentenoic acid and training exercise

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**TRAINING EXERCISE RATHER THAN EICOSAPENTAENOIC ACID ADMINISTRATION COUNTERACTS
CANCER CACHEXIA IN TUMOR-BEARING MICE**

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ABSTRACT

Cancer cachexia is a syndrome characterized by loss of skeletal muscle protein, depletion of lipid stores, anorexia, weakness, and perturbations of the hormonal homeostasis. Despite several therapeutic approaches were described in the past, effective interventions countering cancer cachexia are still lacking.

The aim of the present work was to verify the ability of eicosapentaenoic acid (EPA) to prevent the muscle depletion in Lewis lung carcinoma-bearing mice and to test the ability of progressive resistance training (PRT) to increase the EPA effect.

EPA alone did not prevent the muscle loss induced by tumor growth, while the combination with PRT induced a partial rescue of muscle strength and mass. The association of EPA and PRT reduced the dramatic PAX-7 accumulation and stimulated the increase of PCG-1 protein.

Overall, the present data suggest PRT as an effective tool that should be added on combined therapeutic approaches against cancer cachexia.

INTRODUCTION

Cancer patients frequently develop a condition of general wasting known as cachexia. This is a multifactorial syndrome that complicates patients' management, increases morbidity and mortality rates, reduces the tolerance to antineoplastic therapies, and results in poor quality of life [1]. Cachexia is characterized by muscle and fat wasting, anemia, anorexia and perturbations of the hormonal homeostasis. The pathogenetic mechanisms underlying cachexia are complex and only partially identified, thereby effective therapeutic strategies are lacking.

Several therapeutic approaches have been proposed to counteract cachexia. Among these are nutritional interventions, frequently including omega-3 polyunsaturated fatty acids (PUFA) [2], antioxidants [3], branched-chain amino acids [4], agonists of the melanocortin receptor [5], ghrelin [6] and anti-myostatin agents [7]. The omega-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to suppress the production of proinflammatory cytokines, of several molecules deriving from the arachidonic acid cascade, and of acute phase reactants such as C-reactive protein (reviewed in [8]; [9]). EPA administration to animals bearing the MAC16 tumor improves body weight loss and attenuates the catabolic effects of both LMF (lipid mobilizing factor) and PIF (proteolysis inducing factor) (see [10]). In particular, in the MAC16 model system EPA has been shown to inhibit the activity of the ubiquitin-proteasome-dependent proteolysis, thus reducing skeletal muscle wasting [11]. Finally, experimental studies suggest that omega-3 PUFAs may impair both tumor growth and metastatic spread, mainly by inducing apoptotic cell death, reducing pro-angiogenic factors and inhibiting oncogene expression (reviewed in [10]). By contrast, EPA proved ineffective in preventing cachexia in rats bearing the AH-130 hepatoma [12].

Clinical trials performed in malnourished cancer patients have shown that EPA administration improves body weight and lean body mass, likely in force of its anti-inflammatory properties [9],[13]. These observations, however, have not been completely confirmed by a large multicenter study performed on pancreatic cancer patients [14]. Finally, EPA administration *per se* has been demonstrated to exert no statistically significant benefit in the treatment of cancer cachexia [15].

In addition to nutritional interventions, other therapeutic strategies could effectively contribute to attenuate cancer-related muscle wasting. In particular, progressive resistance exercise training (PRT) has been proposed as a suitable tool, in view of recent observations suggesting that decreased physical activity plays a role in the onset of muscle atrophy in cancer patients [16]. Indeed, PRT stimulates the increase of muscle mass and strength, and might improve cancer-associated skeletal muscle wasting by

stimulating anabolic pathways as well as by down-regulating the activity of proinflammatory cytokines [17]. The molecular events that follow an acute bout of resistance exercise have been described by several studies (reviewed in [18]); by contrast, little is known about PRT.

The aim of the present study has been to investigate if EPA effectiveness in the prevention of cancer-related cachexia might be enhanced by the association with PRT. The study has been performed on a well established model of cancer cachexia, namely mice bearing the Lewis lung carcinoma (LLC). The results obtained show that EPA administration to tumor hosts does not modify the wasting process, while the association with PRT results in partial restoration of both muscle mass and strength.

MATERIALS AND METHODS

All materials were supplied by Sigma (St. Louis, MO, USA), unless differently specified.

Animals and experimental design

C57BL/6 mice weighing about 20 g (Interfauna, Barcelona, Spain) and maintained on a regular dark-light cycle (light from 08:00 to 20:00), with free access to food and water during the whole experimental period. They were cared for in compliance with the *Policy on Humane Care and Use of Laboratory Animals* (NIH 1996). The experimental protocol has been approved by the Bioethical Committee of the University of Barcelona. All animal manipulations were made in accordance with the European Community guidelines for the use of laboratory animals.

Animals were randomized and divided into two groups, namely controls (C, n = 15) and tumor bearers (LLC, n = 24). Tumor-bearing mice were inoculated i.m. with 5×10^5 LLC cells. Both controls and LLC were divided into three sub-groups: untreated, treated with eicosapentaenoic acid (EPA), treated with EPA and submitted to PRT. EPA-treated groups received daily intragastric administration of 0.5 g/kg EPA in corn oil starting from the 4th day of tumor growth (the same dose used by [19]) while untreated groups received vehicle alone. As for PRT, mice were exercised on a Panlab/Harvard-Apparatus treadmill (Barcelona, Spain). Briefly, mice were got used to the treadmill for 5 days before tumor injection (starting from 6 m/min for 15min, increasing speed and time daily until reaching 60-70% of maximum oxygen consumption, corresponding to 14 m/min for 45 min) and exercised 5 days/week from the day of tumor implantation.

Animal weight and food intake were recorded daily. Tumor-bearing mice were sacrificed under anesthesia 14 days after tumor transplantation. Several muscles and tissues were rapidly excised, weighed, frozen in isopentane cooled with liquid nitrogen and stored at -80°C for further analysis.

Total physical activity

Total physical activity (IR ACTIMETER System and ACTITRAK software from PANLAB, Barcelona) was determined during the last 24 hours before the sacrifice of the animals in control and tumor-bearing animals using activity sensors that translate individual changes in the infrared pattern caused by movements of the animals into arbitrary activity counts [20]. For the measurements, animals remained in

their home cage. A frame containing an infrared beam system was placed on the outside of the cage; this minimised stress to the animals.

Grip force assessment

Skeletal muscular strength in rats was quantified by the grip-strength test [21,22]. The grip-strength device (Panlab-Harvard Apparatus, Spain) comprised a triangular pull bar connected to an isometric force transducer (dynamometer). Basically, the grip strength meter was positioned horizontally and the rats are held by the tail and lowered towards the device. The animals are allowed to grasp the triangular pull bar and were then pulled backwards in the horizontal plane. The force applied to the bar just before it lost grip was recorded as the peak tension. At least three measurements were taken per mouse on both baseline and test days, and the results were averaged for analysis.

ELISA

γ -interferon (IFN) serum levels were detected by a commercially available mouse ELISA kit, used according to the manufacturer instructions (Bender MedSystems, Vienna, Austria). Serum from each animal (50 μ l) was assayed in duplicate. Quantitative calibration was obtained performing a standard curve with recombinant mouse γ -IFN.

Western blotting

Samples from gastrocnemius muscle (about 50 mg) were homogenized in 10 mM HEPES, pH 7.5, containing 10 mM $MgCl_2$, 5 mM KCl, 0.1mM EDTA and 0.1% Triton X-100, with freshly added protease and phosphatase inhibitor cocktails, centrifuged at 3000 x g for 5 min at 4°C, and the supernatant collected as cytosolic extract. The pellet was resuspended in 20 mM HEPES, pH 7.9, containing 1.5 mM $MgCl_2$, 500 mM NaCl, 0.2mM EDTA and 25% glycerol, with freshly added protease and phosphatase inhibitor cocktails, incubated on ice for 30 min, centrifuged at 3000 x g for 5 min at 4°C, and the supernatant collected as nuclear extract. Protein concentration was assayed by the method of Lowry [23] using BSA as working standard. Equal amounts of protein (30 μ g) were heat-denatured in sample-loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), resolved by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The filters were blocked with Tris-buffered saline (TBS) containing 0.05% Tween and 5% non-fat dry milk and then incubated overnight with antibodies directed against: PGC-1 (Millipore, Billerica, MA, USA),

atrogin-1 (ECMbiosciences, Versailles, KY, USA) and Pax7 (developed by Atsushi Kawakami, obtained from the Developmental Studies Hybridoma Bank, University of Iowa). Peroxidase-conjugated IgG (Bio-Rad, Hercules, CA, USA) were used as secondary antibodies. Membrane-bound immune complexes were detected by an enhanced chemiluminescence system (Santa Cruz Biotechnology, USA) on a photon-sensitive film (Hyperfilm ECL, GE Healthcare, Milano, Italy). Protein loading was normalized according to tubulin (Sigma, St. Louis, MO, USA) expression. Quantification of the bands was performed by densitometric analysis using a specific software (TotalLab, NonLinear Dynamics, Newcastle upon Tyne, UK).

Data analysis and presentation

All results were expressed as mean \pm SD. Significance of the differences was evaluated by analysis of variance (ANOVA) followed by Tukey's test.

RESULTS

In comparison with control animals, mice bearing the LLC tumor markedly reduce their cumulative food intake; daily administration of EPA (0.5 g/kg; this dose effectively prevents cachexia in MAC16-bearing mice; see [19]) does not modify the decrease in food intake, that is partially corrected by treatment with EPA and exercise (C=64.8 g, LLC=50.0 g, LLC EPA EX=54.6 g; Fig. 1A).

In a voluntary grip strength test, LLC-bearing mice develop a force 30% lower than control animals (C=1.21±0.05 Newton, LLC=0.85±0.16 Newton; Fig. 1B). EPA administration does not prevent force reduction in LLC hosts, whereas its combination with exercise results in a limited but significant recovery (LLC EPA EX=0.98±0.21 Newton, $p=0.04$ vs LLC). Muscle strength in control groups is not affected by EPA or EPA+exercise (Fig 1B).

Analysis of the animal behaviour, evaluated for 24 hours in a continuous recording activity cage, shows a dramatic fall in the total activity of LLC-bearing mice (Fig. 2A); both fast and slow movements are reduced, the former more markedly than the latter (Fig. 2B). Despite the protection exerted against muscle weight loss, the combination of EPA and exercise reveals unable to prevent the decrease of both total and specific activity induced by LLC growth (Fig. 2A and 2B). Activity (total or specific) in control groups is qualitatively comparable to the corresponding LLC hosts (Fig. 2A and 2B).

Tumor growth in LLC-bearing mice is associated with loss of skeletal muscle mass (Fig. 3). Consistently with the loss of muscle strength, fourteen days after tumor transplantation the weight of both gastrocnemius and tibialis muscles is about 70% of controls (gastrocnemius: C=570±32, LLC=417±30; tibialis: C=163±13, LLC=118±14 mg/100g initial body weight). By contrast, the heart is only slightly affected and loses about 7% of control weight (C=466±25, LLC=434±31 mg/100g i.b.w.). EPA treatment does not counteract muscle atrophy in LLC-bearing mice (Fig. 3). When EPA treatment is associated with training exercise, LLC-induced gastrocnemius depletion is partially prevented (LLC EPA EX=455±24 mg/100g i.b.w., $p=0.02$ vs LLC; Fig. 3).

Among the other tissues analyzed, liver weight is unchanged in all experimental groups, while spleen mass increases about four fold in all LLC-bearing animals, independently from the treatment (Fig. 4). The systemic inflammation, suggested by the spleen hypertrophy, is confirmed by the increased circulating levels of γ -IFN (C=46±30, LLC=188±127 pg/ml). Similar to the spleen weight, EPA administration, alone or in combination with PRT, does not prevent the γ -IFN raise (LLC EPA=194±141,

LLC EPA EX=196±118 pg/ml). Finally, white perirenal adipose tissue virtually disappears in both untreated and treated LLC hosts (Fig. 4).

Tumor mass remains comparable between untreated and EPA-treated mice (Fig. 4). However, when EPA is coupled to exercise, a small but statistically significant tumor reduction in size can be observed (LLC=5.42±0.46, LLC EPA EX=4.42±0.92 g, p=0.013; Fig. 4).

Muscle wasting in cancer cachexia mainly relies on a hypercatabolic response in which the ubiquitin-proteasome system seems to play a major role [24]. Atrogin-1 is a muscle specific Ub-protein ligase (E3), critically involved in the enhancement of proteolysis [25], that is overexpressed in several conditions associated with muscle atrophy [26]. In apparent contrast with these reports, however, the present study shows that atrogin-1 protein expression in the gastrocnemius is comparable between untreated and treated LLC-bearing mice (Fig 5A). This observation suggests that atrogin-1 expression might not be the best marker of muscle proteolysis, as recently proposed by [27], and/or that other catabolic systems likely contribute to muscle wasting in LLC hosts.

Protein hypercatabolism apart, muscle wasting could also be associated with alterations in the myogenic process. Impaired myogenesis has been recently suggested to contribute to the onset of muscle wasting in experimental cancer cachexia [28]. Pax7 protein levels, measured in the nuclear extracts, markedly increase in LLC-bearing mice (Fig. 5B); such increase is prevented in part by the combination EPA+exercise.

Previous observations have shown that the levels of the peroxisome proliferator-activated receptor γ (PPAR γ) coactivator 1 (PGC-1), a factor that stimulates the switch from glycolytic to oxidative fibers, is reduced in different models of skeletal muscle atrophy, including cancer cachexia [29,30]. In the present experimental setting, however, PGC-1 protein levels do not decrease in tumor-bearing mice with respect to controls, while they are increased by EPA administration coupled to exercise (Fig. 5B).

DISCUSSION

In the present study, EPA, a widely studied PUFA, was combined with exercise training in order to enhance its hypothetical effectiveness in the prevention of experimental cancer cachexia.

Several studies proposed PUFA supplementation as an effective tool against different pathologies, including sepsis, coronary artery disease, asthma, inflammatory bowel disease and cancer (reviewed in [31]). The results obtained in the present study showed that EPA administration to LLC-bearing mice was completely ineffective in preventing tumor-induced muscle wasting. This is in contrast with a number of reports demonstrating a protective action of EPA in mice bearing the MAC16 tumor [19,32,33,34]. The discrepancy between the present results on LLC-bearing mice and those reported in the MAC16 hosts could reside in the intrinsic differences that characterize these experimental models. In this regard, studies performed on rats bearing the AH-130 hepatoma [12] and clinical trials involving cachectic patients affected by gastrointestinal or lung cancer [15], reported the ineffectiveness of EPA supplementation. Consistently, another fatty acid with antiinflammatory properties, the c9t11 conjugated linoleic acid, did not improve muscle depletion in Colon26-bearing mice [35].

In the clinical practice, exploitation of the potential benefits of PUFA administration is still largely limited by the poor knowledge of their mechanisms of action. In this regard, PUFA therapeutical effects are commonly accepted to rely on their antiinflammatory actions, exerted by reducing the production of inflammatory cytokines, such as tumor necrosis factor alpha, interleukin-1, and interleukin-6 [31]. In the present study, spleen weight, an indirect indicator of the systemic inflammatory state, was increased by four fold in LLC-bearing mice and not modified by EPA administration, suggesting that tumor-induced systemic inflammation was only marginally affected by the treatment. This hypothesis is further supported by the observation that circulating levels of γ -IFN, the mediator mainly involved in the pathogenesis of cachexia in LLC-bearing mice [36], increased in tumor hosts and were not modified by EPA.

Summarizing, EPA does not appear suitable to prevent cancer cachexia, despite being a good candidate for the treatment of other experimental wasting conditions such as diabetes [37] and arthritis [38]. The different effectiveness of EPA in counteracting the various types of muscle atrophies may depend on the underlying pathogenetic mechanisms. As an example, the redox imbalance differently contributes to muscle depletion in diabetes and in cancer cachexia, as shown by the observation that antioxidant treatment markedly prevented muscle atrophy in the former while only partially in the latter [39].

Several reports suggested training exercise as a strategy to mitigate muscle wasting in chronic pathological conditions (reviewed in [17]). The underlying mechanisms accounting for this protective effects are still not entirely clear. Exercise training was proposed to stimulate mitochondrial biogenesis, to increase muscle protein synthesis rate and to attenuate protein hypercatabolism [17]. Moreover, PRT proved effective in restoring lean body mass and function in patients with reumathoid arthritis-related cachexia, stimulating the enhancement of muscle IGF-1 levels [40].

Unfortunately, the usefulness of exercise in cancer patients received little attention in the past. The currently available data provide preliminary evidence that exercise training is a well-tolerated and safe complementary therapy that can mitigate several common treatment-related side-effects, including breath shortness and pain [41]. A common feature of cancer patient is fatigue. In this regard, a large body of evidence support the beneficial effect of exercise training in counteracting fatigue [42]. In the present work we provide the first evidence that PRT coupled to EPA administration partially prevented the loss of muscle mass and strength in tumor-bearing mice. The improvement of muscle depletion was associated with a small but significant decline in tumor burden in exercised, EPA-treated mice. This observation is consistent with previous data showing that PRT in Waker-256 bearing rats strongly reduced tumor growth [43], while swimming exercise attenuated colon carcinogenesis in the rat [44]. On the contrary, however, another study reported that tumorigenesis increased in p53-deficient mice exercised with a treadmill protocol similar to the one used in the present study [45]. PRT-induced suppression of tumor growth was hypothesized to depend on modulations of inflammation [46,47]. However, both spleen hypertrophy and γ -IFN circulating levels in LLC-bearing mice were not affected by the combination of exercise and EPA, suggesting that, at least in the present experimental conditions, the systemic inflammatory response is not involved in the inhibition of LLC growth.

The association of EPA with exercise in LLC-bearing mice resulted in attenuation of tumor-induced anorexia, associated with unchanged voluntary (extra-exercise) activity, that was close to untreated LLC bearers. In this regard, physical conditions in LLC hosts might be improved by exercise and EPA, leading tumor bearers to cope with the increased energy requirements by enhancing food intake rather than by further increasing the resting period,

already significantly higher than that observed in treated, exercised controls. On the other side, food intake remained comparable between control groups (EPA treated, exercised, and EPA untreated, sedentary). Such a discrepancy between trained, EPA treated controls and LLC hosts might reflect a different response to exercise. Indeed, voluntary activity was lower in trained than in sedentary controls, suggesting that healthy animals managed the enhanced energy requirements simply by recovering after exercise, without modifying food intake.

Apart from reduced food intake, that was shown to play a limited role in cancer-associated muscle depletion [26,35], other tumor-induced metabolic alterations could be targeted by PRT. Indeed, training exercise was shown to induce the restoration of lipid metabolism in tumor-bearing rats [48] and to modulate both protein synthesis and degradation rates in normal or wasting conditions (reviewed by [49]). Finally, PRT increased muscle mitochondrial biogenesis in patients affected by chronic kidney disease [50]. In the present work, PRT in association with EPA induced the increase of PGC-1 protein levels in LLC-bearing mice, possibly stimulating mitochondrial biogenesis and a shift from fast- to slow-twitch oxidative fibers, less susceptible to atrophy.

Recent data showed that muscle wasting in Colon26-bearing mice was associated with reduced myogenin levels and increased expression of Pax7, an inhibitor of the myogenic program [51], suggesting the occurrence of impaired myogenesis [28]. The results obtained in the present work confirmed the enhancement of Pax7 expression also in the skeletal muscle of LLC-bearing mice; this might suggest an increased satellite cell proliferation or their impaired differentiation. Loss of myonuclei, previously reported in Colon26-bearing mice [52], could lead to enhancement of satellite cell proliferation; such increase, however, could be vanished by the inability to complete the myogenic program, contributing to the atrophic phenotype. The present work provides the first evidence that PRT in association with EPA partially prevented muscle Pax7 increase in LLC hosts; this observation suggests that satellite cells might proceed into the myogenic program, thus partially explaining the protection exerted by such experimental protocol against LLC-induced muscle wasting.

In conclusion, the results shown in the present study demonstrate that the association between EPA and PRT improved muscle wasting in LLC hosts, mainly by modulating some of the molecular

pathways involved in the pathogenesis of muscle depletion, encouraging to include PRT in the multi-disciplinary therapeutical approach that should be adopted to prevent cancer cachexia.

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FIGURE LEGENDS

Figure 1

Cumulative food intake (A) and voluntary grasping strength (B) in Control (C) and LLC bearing mice (LLC). Both groups were subdivided in untreated, EPA treated (EPA) and EPA treated plus exercised (EPA EX, see Materials and methods). Data (means \pm SD) expressed as percentages of controls. Significance of the differences: * $p < 0.05$ vs C; \$ $p < 0.05$ vs LLC.

Figure 2

(A) Spontaneous locomotor activity in Control (C) and LLC bearing mice (LLC) either untreated or EPA treated and exercised (EPA EX). Data (means \pm SD) expressed as percentages of controls. Significance of the differences: ** $p < 0.01$ vs C. (B) The total activity was subdivided in percentage of resting time, slow movements (between 2 and 5 cm/s) and fast movements (faster than 5 cm/s).

Figure 3

Gastrocnemius (GSN), tibialis and heart weight in Control (C) and LLC bearing mice (LLC). Both groups were subdivided in untreated, EPA treated (EPA) and EPA treated plus exercised (EPA EX, see Materials and methods). Data (means \pm SD) expressed as percentages of controls. Significance of the differences: *** $p < 0.001$ vs C; \$ $p < 0.05$ vs LLC.

Figure 4

Liver, spleen, perirenal white adipose tissue (WAT) and tumor weight in Control (C) and LLC bearing mice (LLC). Both groups were subdivided in untreated, EPA treated (EPA) and EPA treated plus exercised (EPA EX, see Materials and methods). Data (means \pm SD) expressed as percentages of controls. Significance of the differences: *** $p < 0.001$ vs C; \$ $p < 0.05$ vs LLC.

Figure 5

(A) atrogin-1 protein expression in the cytosolic fraction, (B) PAX-7 and PGC-1 protein expression in the nuclear fraction in the GSN of controls, LLC bearing mice (LLC) and LLC treated with EPA plus exercised (EPA EX, see Materials and methods). Densitometric quantifications were normalized according to tubulin levels. Data (means \pm SD) expressed as percentages of controls. Significance of the differences: * $p < 0.05$ vs C; ** $p < 0.01$ vs C; \$ $p < 0.05$ vs LLC.

Figure 1

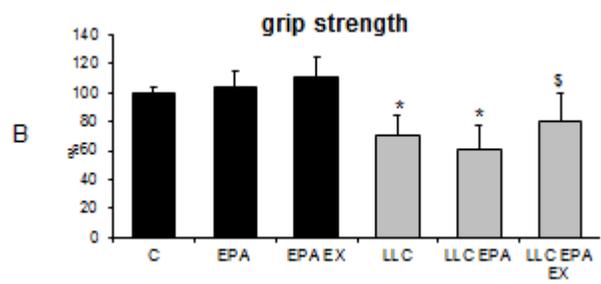
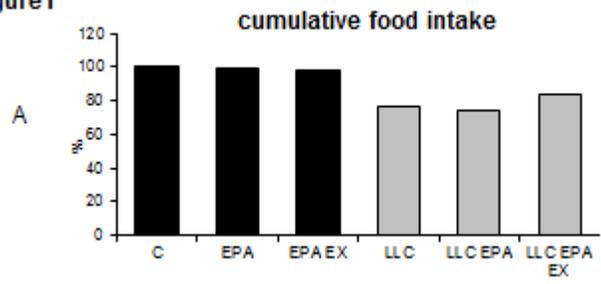


Figure2

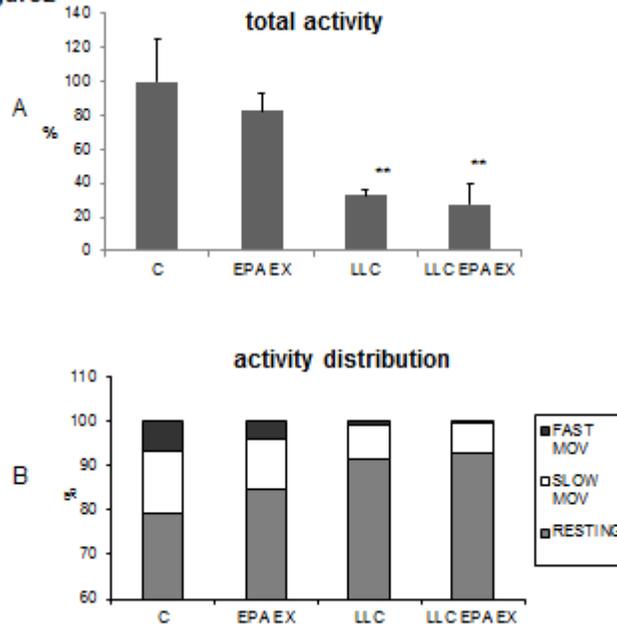


Figure3

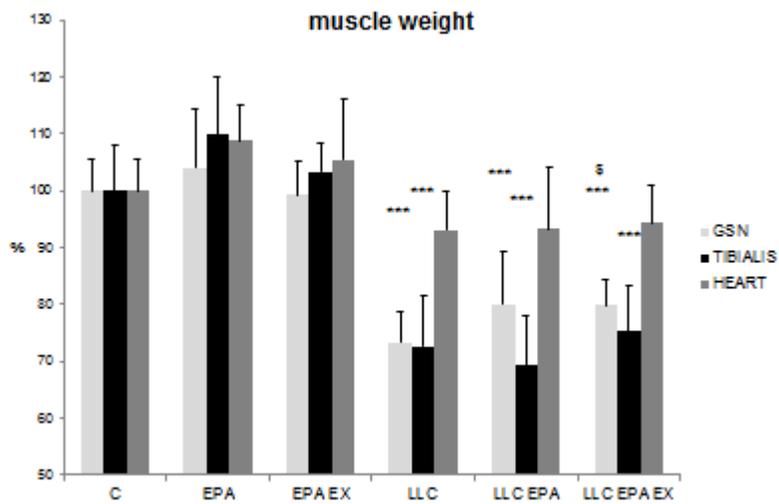


Figure4

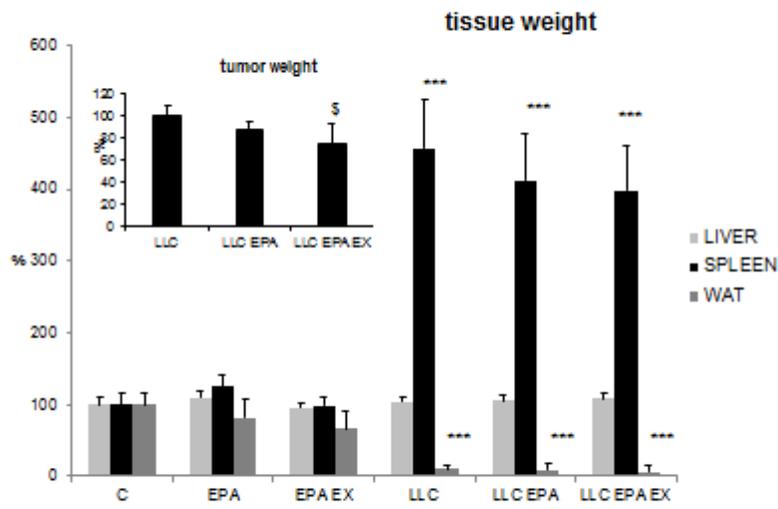


Figure5

