NUTRACEUTICAL PROPERTIES OF CHESTNUT FLOURS: BENEFICIAL EFFECTS ON SKELETAL MUSCLE ATROPHY.

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Atrophy protection by chestnut tocopherols

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Abstract

Plants contain a wide range of non-nutritive phytochemicals, many of which have protective or preventive properties for human diseases. The aim of the present work has been to investigate the nutraceutical properties of sweet chestnut flour extracts obtained from fruits collected from 7 geographic areas of Tuscany (Italy), and their ability in modulating skeletal muscle atrophy. We found that the cultivars from different geographic areas are characterized by composition and quantity of various nutrients and specific bioactive components, such as tocopherols, polyphenols and sphingolipids. The nutraceutical properties of chestnut sweet flours have been evaluated in C2C12 myotubes induced to atrophy by serum deprivation or dexamethasone. We found that the pretreatment with both total extract of tocopherols and sphingolipids is able to counterbalance cell atrophy, reducing the decrease in myotube size and myonuclei number, and attenuating protein degradation and the increase in expression of MAFbx/atrogin-1 (a muscle-specific atrophy marker). By contrast, polyphenol extracts were not able to prevent atrophy. Since we also found that γ-tocopherol is the major form of tocopherol in sweet flour and its content differs depending on the procedure of sweet flour preparation, the mechanisms by which γ-tocopherol as well as sphingolipids affect skeletal muscle cell atrophy have been also investigated. This is the first evidence that chestnut sweet flour is a natural source of specific bioactive components with a relevant role in the prevention of cell degeneration and maintaining of skeletal muscle mass, opening important implications in designing appropriate nutritional therapeutic approaches to skeletal muscle atrophy.

Introduction

Fruits and vegetables contain vitamins, minerals, fiber and potentially bioactive compounds known as phytochemicals. These compounds are not considered as nutrients but have protective or preventive properties for a variety of human diseases including cancer[1] cardiovascular diseases[2] and Alzheimer's disease[3] , mostly due to antioxidant and/or anti-inflammatory activities. Recent studies performed on Portuguese cultivars[4-6] and Croatian cultivars of *Castanea Sativa Mill* extracts[7] and on Mugello's marrons (Tuscany, Italy)[8,9] have evidenced chestnuts and marrons as an important natural source of antioxidants, particularly of polyphenols and vitamin E.

Polyphenols are the biggest group of phytochemicals that contribute to important characteristics of food such as taste, odor, colour and oxidative stability[10].

Many studies including epidemiological data suggest that long term consumption of food rich in plant polyphenols offers some protection against the principal chronic diseases, such as cancer, cardiovascular diseases, and neurodegenerative diseases[10], mainly related to oxidative stress response[11]. Vitamin E is a generic term for all tocopherol and tocotrienol derivatives. The most abundant form of vitamin E isoforms in mammals is α-tocopherol and it has long been recognized as a classic free radical scavenging antioxidant whose deficiency impairs mammalian fertility[12]. New biological activities including anti-inflammatory, antineoplastic, and natriuretic functions have been reported for other tocopherols, such as γ-tocopherol[12]. In particular, the tocotrienol subfamily of natural vitamin E possesses powerful neuroprotective, anticancer, and cholesterol-lowering properties; for example, nanomolar concentration of α-tocotrienol, but not of α-tocopherol, prevents neurodegeneration[13]. Epidemiological data suggest that γ-tocopherol is a better negative risk factor for certain types of cancer and myocardial infarction than is a α-tocopherol[1, 12, 14].

Oxidative stress induces unloading-mediated muscle atrophy that is prevented by polyphenols[15]. In addition, Vitamin E is a nutrient widely used to attenuate muscle injury associated with eccentric exercise[16].

Sphingolipids (SLs), components of the membranes of all eukariotic cells, participate in the control of a variety of important cell functions, such as cell growth, differentiation, inflammation, and apoptosis[17,18]. The first evidence of SL importance as functional nutrients was obtained by Merrill's group (reviewed in[17]) that reported that dietary sphingomyelin (SM) inhibits colon carcinogenesis in animal models. In addition, other studies have shown that consumption of SLs reduces serum low-density lipoprotein cholesterol and elevates high-density lipoproteins[17]. SLs are found in all foodstuff; however, only a few of the published studies reported the analysis of their content in food, found to be some hundreds of mg/kg, a relatively low concentration[17]. Degradation of the most abundant SLs, SM, by the action of several sphingomyelinases generates ceramide that

can also be formed from serine by *de novo* synthesis. Deacylation of ceramide leads to the formation of sphingosine that can be further phosphorylated by sphingosine kinases (SK) to yield sphingosine 1-phosphate (S1P)[18]. Among bioactive SLs, S1P acts as survival and trophic factor, increases cell proliferation and differentiation and inhibits apoptosis[18]. Recently, it has been demonstrated that SK1/S1P axis promotes myoblast differentiation[19], exerts a trophic action on denervated or damaged skeletal muscle fibres[19-20] and it is crucial for satellite cell activation and tissue regeneration[20-22].

Skeletal muscle exhibits great plasticity in response to altered activity levels, ultimately resulting in substantial changes in tissue mass: an increase have been associated with resistance exercise or after anabolic agent administration, whereas a loss in skeletal muscle mass (atrophy) has been observed in several conditions, such as starvation, ageing and physical inactivity, as well as in disease states including cancer cachexia, diabetes and AIDS[23,24]. At present effective treatments that can prevent, attenuate or reverse muscle atrophy are not clearly defined. Emerging evidence implicates oxidative stress as a key regulator of cell signaling pathways, leading to increased proteolysis and muscle atrophy during periods of prolonged disuse [23]. The discovery of two muscle-specific E3 ubiquitin ligases, Muscle atrophy F-Box (MAFbx/ atrogin-1) and Muscle RING (Really Interesting New Gene) Finger-1 (MuRF1), prompted renewed expectation in identifying muscle-specific targets for therapeutic manipulation[24], as the two enzymes belong to the ubiquitin proteasome pathway, the predominant pathway involved in protein degradation in skeletal muscle.

Autophagy is also involved in skeletal muscle homeostasis: it can be detrimental and contribute to tissue degeneration, but can also be a compensatory mechanism for cell survival [25]. Autophagy is characterized by sequestration of cytoplasmic components in double-membraned autophagosomes and their fusion with lysosomes. Autophagosomal membrane nucleation is promoted by phosphatidylinositol-3-phosphate generated by the Beclin-1-interacting complex[25].

The aim of the present work has been to investigate the nutraceutical properties of sweet chestnut flour extracts obtained from fruits of seven different areas of Tuscany and to examine whether these extracts could modulate skeletal muscle cell atrophy in C2C12 myotubes, reported to be a good model for the study of skeletal muscle cell biology[19, 26-28].

Results

Composition of tuscan chestnut flour. Content of bioactive molecules

Since chestnut flour from Mugello area is obtained essentially from a single cultivar (Marrone Fiorentino) (Table 1), we used this sample for the chemical composition of this tuscan chestnut flour. Sweet chestnut flour was analysed and characterized for the content of proteins, fat, carbohydrates and ions. Data reported in Table 2 indicate that the four has an high content of carbohydrates, starch being the most representative and similar in content to Spanish chestnut cultivars[39]. Previous studies on Spanish and Portuguese chestnut cultivars showed a protein content of 4.9-7.4 g/100 g dry weight, a higher value respect to that reported in Table 2[40]. Moreover, in the same study the content of fat (1.7-3.1 g/100g dry weight) is lower of that found in Marrone Fiorentino. The high content of complex carbohydrates, associated with the low content of fat, makes the chestnut flour a healthy food representative of the Meditteranean diet. The chemical composition in metals was not significantly different from what previously reported[6]. From a nutritional point of view, chestnut flour can be an important source of minerals: potassium is present in a significant concentration as well as magnesium and calcium.

In order to investigate the nutraceutical properties of chestnut flour, the relative values of polyphenols, tocopherols and "functional" SLs were quantified (Table 3). Polyphenols vary from 1688 to 2799 mg/Kg as (gallic acid), whereas the content in SLs, similar in all the samples, ranges from 48 to 56 mg/Kg dry weight. Chromatographic analysis revealed the presence of several vitamin E isoforms. γ-Tocopherol was the major tocopherol; the profile was similar along all the assayed tuscan sweet chestnut flours (γ-tocopherol >>> δ-tocopherol > α-tocopherol). Tocopherol content, ranging from 18 to 114 mg/Kg, appears to be dependent from several parameters: the drying method (traditional vs other not traditional), cultivar tipology and geographic locations in the same area.

Effect of total polyphenol and tocopherol extracts on skeletal muscle cell growth

The effects of total polyphenol and tocopherol extracts on cell growth and viability have been quantified both by cell counting and MTS assay, a method that measures the ability of dehydrogenases in living cells to reduce MTS to formazan (Fig. 1). Both tocopherol and polyphenol extracts decrease cell growth in a time-dependent manner compared to control cells, but polyphenols have a more-pronounced effect than tocopherols. Of note, the cell number at day 4 is

not significantly different from day 1 in cells treated with tocopherol extract and only slightly with polyphenol extract (Fig.1A). The difference is more evident after four days of culture, when MTS reduction capability is reduced by polyphenol or tocopherol extract to 12% and 50% of the control cells, respectively (Fig.1B). Therefore, the reduction of formazan formation might be due either to a low number of cells metabolizing the MTS and to a decrease of mitochondrial activity in each cell.

Since polyphenol and tocopherol extract interfered with cell growth, we evaluated the ability of both compounds to induce cell death by lactate dehydrogenase (LDH) assay. The slight effect of tocopherol and polyphenol extracts in releasing LDH activity into cell medium at 4 days of incubation (7±3% and 10±3%, respectively) compared with the effect of 0.1% Triton X100 (set to 100%) and the absence of apoptotic markers expression (cytosolic cytochrome C and mitochondria-associated Bax) at the concentration used (data not shown), indicate the lack of cytotoxicity, suggesting that C2C12 myoblasts likely undergo cell cycle arrest and, presumably, start the myogenic program.

Effect of total polyphenol and tocopherol extracts on starvation- and Dexa-induced muscle atrophy

In order to investigate the involvement of functional molecules identified in chestnut flour on skeletal muscle cell atrophy, we used C2C12 myoblasts, a well known cell model for studying myogenesis induction[19], the biology of terminal myogenic differentiated cells, and cell atrophy/hypertrophy. The contrast-phase images of C2C12 muscle myoblasts induced to differentiate as described in Methods is reported in Supplemental data (Fig 2). Myogenic differentiation is characterized by consistent and progressive modifications of cellular morphology such as tendency to single myoblast to elongate and fuse leading to the formation of syncytia with more nuclei (myotube) and modifications of the protein expression pattern with the appearance of typical protein markers of myogenic differentiation, such as sarcomeric actin, caveolin-3[19], phospho-p38 MAPK and the nuclear translocation of the transcription factor, myogenin[20] (Supplemental data). Following atrophy induction by serum starvation or treatment with Dexa, as reported in supplemental data, C2C12 myotubes show a clear modification of cell morphology with a significant decrease in myotube size. It is worthy to note a significant decrease to a third and to a half of the control value after treatment with 24 nM total polyphenol and tocopherol extracts, respectively. While 4 nM tocopherol does not have any significant effect, 4 nM polyphenol extract is still able to significantly reduce cell size.

Interestingly, the incubation with total polyphenol extract did not prevent the reduction of the cellular diameter elicited by Dexa or serum starvation (Table 4), whereas the pre-treatment with tocopherols is able to revert the atrophic phenotype measured as cellular diameter as well as myonuclei number. Table 4 also shows, as a positive control, the effect of protein synthesis inhibitor cycloheximide (3 μM), that promotes cell size and micronuclei number decrease, similarly to what observed with serum starvation and Dexa treatment.

In another set of experiments we have investigated the modifications on cell atrophy by measuring the expression of the protein marker MAFbx/atrogin-1 (Fig.2). When compared to control cells, the basal level of expression of MAFbx/atrogin-1 decreases after treatment with polyphenol and tocopherol extracts while, as expected, increases after Dexa addition (Fig.2A). In contrast, the treatment with polyphenol extract induces, whereas with tocopherol extracts reduces the MAFbx/atrogin-1 levels in atrophy-induced cells (Fig.2A). A significant reduction of ubiquitin ligase content is also found in serum-starved cells treated with total tocopherol (Fig.2B and polyphenol extract (Fig.2C). Successively, we have investigated the effect of pure γ-tocopherol, the main tocopherol found in chestnuts. Treatment with γ-tocopherol (24 nM) has a different effect compared to that promoted by total tocopherol extract on atrophic myotubes (Fig.2D). Indeed, the addition of the pure compound increases 3.1-fold the expression of MAFbx/atrogin-1 in control cells, whereas, added to Dexa-treated cells, it slightly reduces the expression of the atrophy marker (Fig.2D, see also 5A).

Effect of total polyphenol and tocopherol extracts on protein synthesis in C2C12 myotubes

In order to quantify the effect of polyphenol and tocopherol extracts on cell muscle atrophy we have also measured the incorporation of [^{3}H]-leucine into proteins (Table 5) in both starved- and Dexa -treated cells in the presence and absence of total extracts. The incubation with Dexa for 24 hours does not modify the incorporation of radioactivity into proteins in respect to control cells, whereas both polyphenol and tocopherol extracts increase it by approximately 1.7-fold and 2.0-fold, respectively, in agreement with their role in the promotion of myogenic program. The incubation with both Dexa and polyphenol extract does not significantly changes incorporation of [^{3}H]-leucine into proteins compared to polyphenol extract alone, but radioactivity content remains significantly different from Dexa-treated myotubes. In contrast, treatment with both Dexa and tocopherol extract changes protein radioactivity to 1.40-fold and 0.66-fold compared to Dexa and tocopherol-treated cells, respectively. Serum starvation induces a 48% reduction in the incorporation of radioactivity into proteins. In this experimental condition, the presence of tocopherol extract increases [^{3}H]-

leucine incorporation (1.37-fold), whereas polyphenol extracts do not significantly change protein synthesis rate compared to control cells. However, both extracts are able to prevent the effects of serum starvation.

Effect of polyphenols and tocopherols on skeletal muscle cell autophagy

Since autophagy is reported to be involved in protein breakdown in skeletal muscle atrophy, we have also analyzed the morphology of C2C12 myotubes following treatment with Dexa alone and in combination with total polyphenol or tocopherol extracts, and quantified the relative expression of Beclin-1, a known autophagic marker (Fig. 3). Notably, the treatment of atrophic myotubes with Dexa and polyphenol extract, but not tocopherol extract, leads to the appearance of autophagosomes, a hallmarker of autophagy and to an additive effect on Beclin-1 expression compared to Dexa alone. Total tocopherol extract does not prevent Dexa-induced autophagy.

Involvement of ERK1/2 on γ- tocopherol action

In order to have an insight into the mechanism of action of tocopherol, we have incubated C2C12 myotubes with γ-tocopherol and we have studied the activation of ERK1/2, recently reported to be an upstream regulator of Atrogin-1[25]. As reported in Fig. 4, in the presence of Dexa, γ-tocopherol is able to increase the level of phospho-ERK1/2, the active form of this MAPK, supporting a role of pure compound in the activation of ERK1/2 signaling.

Effect of sphingolipids on Dexa-induced skeletal muscle cell atrophy

Finally, we have investigated whether SL extract is able to modify the expression of the atrophy protein marker MAFbx/atrogin-1. As shown in Fig. 5A, although SL extract does not have any significant effect on the basal level of the atrophy protein, it is able to prevent the effect of Dexa, decreasing MAFbx/atrogin-1 levels of approximately 40% , similarly to what observed with total tocopherol extract (Fig. 3A), but not with γ-tocopherol (Fig. 3C and 5A).

Among the SLs, it is reported that the bioactive lipid S1P, derived from sphingosine kinase (SK) activation, plays a crucial role as trophic survival factor in skeletal muscle[19-20]. In order to investigate whether skeletal muscle cell atrophy is correlated to S1P content, we have treated C2C12 myotubes with the SK inhibitor compound II (iSK)[41] (Fig. 5B). We have observed that the inhibition of S1P generation promotes MAFbx/atrogin-1 expression, and slightly reduces Dexa

effect. In contrast, serum deprivation does not have any effect on the up-regulation of MAFbx/atrogin-1 expression elicited by iSK.

Experimental

Materials and chemicals

Chemicals, standards, cell culture reagents, Dulbecco's Modified Eagle's Medium (DMEM), fetal calf serum (FCS), protease inhibitor cocktail, bovine serum albumin, horse serum (HS) and dexamethasone (Dexa) were purchased from Sigma (St. Louis, MO, USA); C2C12 cells were obtained from American Type Culture Collection (ATCC CRL1772, Manassa, VA, USA); Coomassie Blue reagent was from Bio-Rad (Hercules, CA, USA); D-erythro-Sphingosine 1-phosphate (S1P) was from Calbiochem (San Diego, CA,USA); tocopherol set was obtained from Sigma (St. Louis, MO, USA). Sphingosine kinase inhibitor (SKI-II) was from Tocris (Bristol, UK); CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit was from Promega (Promega Madison, WI, USA); penicillin/streptomycin were from Invitrogen (Carlsbad, CA, USA); anti-MAFbx/atrogin-1 antibody, anti-beclin, anti-phospho-ERK1/2 antibodies from Cell Signalling (Danvers, MA, USA); (C20); anti-myogenin, anti-caveolin-3, anti-β-actin antibodies and secondary antibodies conjugated to horse radish peroxidase were from Santa Cruz (Santa Cruz Biotechnology, CA, USA); L-[3,4,5-^{3}H]-Leucine ([^{3}H]-leucine), (3.7-5.56 TBq/mmol) was from Perkin Elmer (Monza, Italy). Enhance chemiluminescence kit (Luminata Western HRP) were from Millipore (Merck Millipore Headquarters Billerica, MA) and high-performance chemiluminescence film was from GE Healthcare Life Sciences (Chalfont St. Giles, UK).

Sample preparation

Flours directly furnished by 12 producers from 7 different area of Tuscany (Supplemental data) immediately after milling have been kept at 16 ± 2°C till the time of analysis. Table 1 describes the areas of origin, the varieties of the fruits and the characteristics of the chain of production such as the mode of essication (wood essicator "*metato"* or air oven) the temperature and time of essication, and the type of mill (stone, or hammer, water mill or electrical mill).

Traditional drying

The drying of chestnuts and/or marrons has represented for centuries the most common method of transformation of these fruits since it permitted to use the product all year round. The drying

process is still carried out in a traditional structure called "*metato*", located directly in the orchards or close to the producer's house (Supplemental Material).

The "*metato*" is a rectangular structure built in stone without using cement (the so called "a secco" technique: dry-stone technique). The only room is divided, at half height, by a layer of chestnut wooden boards. Through a window, located on the higher part of the structure, fruits are introduced and placed on the boards, ready for drying. Normally a 30 cm thick layer of chestnuts is left on the wooden board to be dried. In the lower part of the structure a fire exclusively made of chestnut wood and chaff is lighted. The fire must be rigorously kept without flame in order to maintain a constant heating of 35ºC-40ºC. The drying process lasts about 40 days. Considering that the loading into the "*metato*" is carried out progressively during the harvest, it is important to turn over the chestnuts each time (or on a weekly basis) a new load is added so that the fresh fruits are always placed as close as possible to the heat. After drying, the fruit is mechanically separated from the skin and the pellicle (threshing). The threshing is followed by a fruit selection for a further quality control.

Flour preparation

In some samples, grinding is carried out with traditional millstones and other samples have been obtained with modern techniques using heat pumps, thresher drums and hammer mills that improve the productivity. After the collection, the chestnut flour were analyzed as described in[29-30].

Determination of chestnut and chestnut flour chemical composition

The moisture content was determined by drying in an oven at 103°C for 6 h as described in Bellini et al.[29] The total nitrogen was quantified using the Kjeldahl method and the percentage of nitrogen was transformed into proteins by using the conversion factor of Mc Carthy (Nx5.3)[29].

For the fat determination, the sample was hydrolyzed by heating in the presence of 25% hydrochloric acid. The acidic solution, mixed with ethyl alcohol, was then extracted with ethyl ether and petroleum ether. The solvent was removed and the fat residue weighed[29]. For the evaluation of the percentage of saturated, monounsaturated and polyunsaturated fatty acids, fat extract was cooled and transesterified in the presence of potassium hydroxide methanolic solution. The methyl esters were separated by GC with FID detector and expressed as % m/m.

The determination of sucrose, fructose and glucose was carried out on the pulp of the fruit of flour by extraction in water, sonication of the solution (power 50-60%, room temperature) for 30

minutes and HPLC analysis (mobile phase acetonitrile: water 80:20, quaternary amine column Spheri Amino-5, 5µm 4.6 x 220 mm, refractive index detector)[29].

Determination of starch content was performed using the enzymatic kit from Boeringer Mannheim (Roche .Daemstadt, Germany). Ash analysis was conducted after incineration of samples at 525°C for approximately 8 h. For the determinations of metals, ash residues were dissolved in 1% nitric acid solution. The solutions were then analyzed by atomic absorption spectrophotometer Spectr AA220 (Varian, Polo Alto, USA).[29]

Preparation of phenolic extracts

A 20 g sample was weighed in a centrifugal tube and then added to 70 ml of 80 % methanol solution. The suspension was extracted in a ultrasonic bath for 15 min (90 % intensity, room temperature) in order to obtain better efficiency in the destruction of cell walls that contained phenolic compounds. The extract was separated by vacuum filtration and then transferred into a 100 ml flask, adding the necessary volume of deionized water to bring it to 100 ml. That solution was transferred into 250 ml round-bottom conical flask, added with 11 ml of hydrochloric acid at 37%, then hydrolyzed for 2 h at 90 °C to obtain aglycone form of the phenols. When the extract was cool, it was adjusted to pH 7.0 with sodium hydroxide 6 M and the volume was brought to 200 ml with 80% methanol[31].

Estimation of total phenolic content

The total phenolic content of the extracts was determined with Folin-Ciocalteu colorimetric reaction[32]. External calibration curve were performed using gallic acid as a standard. Five hundred µl of the phenolic extract and 2.5 ml of Folin-Ciocalteau reagent (diluted 1:10) were placed into a 5 ml flask and then, after 2 minutes, taken up to 5 ml with 75 g/l Na_2CO_3. The solution was mixed and incubated at 50 °C for 15 minutes. After cooling, absorbance was measured at 760 nm using a UV-VIS Cary 1-E spectrophotometer (Varian, Palo Alto, USA).

Estimation of total flavonoid content

Strata-X 6 ml/500 mg SPE cartridge (Phenomenex, Macclesfield, United Kingdom) was activated in order with 10 ml of ethyl acetate, 10 ml of methanol and 10 ml of 0.01 M hydrochloric acid. 10 ml of the phenolic extract were filtered through 0.45 µm RC syringe filter. 5 ml of the filtrate were

loaded into activated cartridge. 6 ml of 0.01 M hydrochloric acid were flushed through cartridge and then discarded. 40 ml of ethyl acetate were used to elute the flavonoid fraction. Flavonoid fraction obtained from SPE treatment was evaporated to dryness on a rotary evaporator, then dissolved in 10 ml of 80% methanol. Aluminum chloride colorimetric method was used for determination of flavonoids[33]. Seventy five μl of 50 g/lsodium nitrite were added to 2 ml of extract (appropriately diluted). Aluminum chloride (0.15 ml of 100 g/l) was added after 5 min; 0.5 ml of 1 M sodium hydroxide and 0.25 ml of water were added 6 min later. Absorbance was measured at 510 nm, using UV-VIS Cary 1-E spectrophotometer (Varian, Palo Alto, USA). External calibration curve was performed using quercetin as standard.

Preparation of tocopherol and sphingolipid extracts

The tocopherol extract was prepared performing the saponification procedure[34] that consists of mixing in a 250 ml round-bottom conical flask 20 g flour with a solution containing 30 ml absolute ethanol, 60 ml of 500 g/l potassium hydroxide, 15 ml of 100 g/l ascorbic acid and 30 ml sodium chloride saturated solution. The round flask were equipped with a reflux condenser and then heated at 70°C for 45 min. After cooling, the saponified mixture was extracted three times with 100 ml of 10% ethyl acetate in hexane. Extracts were collected and filtered through anhydrous Na_2SO_4. The extract was evaporated to dryness and reconstituted in 8 ml of heptane.

SL extracts were prepared from chestnut flours obtained from the specific areas. The sample was weighed in a screw tube and then added to 5 ml of $CHCl_3$: MeOH (1:2). The suspension was extracted in a ultrasonic bath for 10 min. The mixture was then incubated at 37.5 °C for 1 h in a closed screw tube, cooled down to room temperature and filtered. Saponification, used to remove phospholipids and neutral lipids from SLs, was performed using 1 M KOH for 6 h at 40°C with stirring, and successively the samples were neutralized and washed using the method of Folch et al. [35]. After solvent partition of SLs, HP-TLC analysis was performed accordingly to standardized procedures with some modifications[36]. Briefly, the plates (silica gel 60) were activated for 30 min at 120°C before use. Standards and reconstituted samples were applied onto the plate with a microdispenser and plate developed with mobile phase (chloroform:methanol:water, 65:25:4). To quantify each lipid species (SM, cerebrosides, galactosylceramide), calibration curves were obtained by running in parallel known amounts of purified lipid standards. The plates were visualized with sulfuric acid-$CuSO_4$-ammonium molybdate spray reagent followed by heating at 110°C for 15 min (SM) or with 3% cupric acetate in 8% phosphoric acid solution for 10 min at 180°C (ceramide) or with 0.5% α-naphthol in methanol-water, 1:1, dried at room temperature,

sprayed with sulfuric acid- water, 95:5, and heated at 100°C. Densitometry of standard and sample zones was performed and the SLs content determined by interpolation from the graph.

Estimation of total tocopherols content

Tocopherols were quantified according to the official NF ISO method 9936[37] by high-performance liquid chromatography (HPLC-1100 series, Hewlett-Packard) equipped with fluorescence detector (excitation λ 295 nm, emission λ 330 nm). Analysis of tocopherols was performed using a thermostated Phenomenex Luna silica column (25 cm × 4.6 mm i.d. × 5 μm), at 25 °C, with the corresponding analysis guard column. The HPLC mobile phase consisted of heptane/t-butyl methyl ether (94:6 v/v), eluted at a flow rate of 1.5 ml/min.

Cell culture and treatments

Murine C2C12 myoblasts were routinely grown in DMEM supplemented with 10% FCS. For myogenic differentiation experiments, cells were grown in until 95% of confluency and induced to differentiate by switching to differentiation medium (DM), DMEM containing 1-2% horse serum (HS) for the following 3-5 days. To obtain the phenotype of atrophic cells, C2C12 myotubes (MT) were maintained in a culture medium free of nutrients and growth factors (-FCS, starvation) for 24-48 hours, or treated with Dexa, a compound which enhances the degradation of proteins[25].

The analysis of the atrophic phenotype was performed a) by evaluating cell morphology under a phase contrast microscope and quantifying the number of myonuclei and the cell diameter (fiber size), b) by a molecular approach consisting in the evaluation by Western Blotting analysis of the expression of MAFbx/Atrogin-1, the ubiquitin ligase typically expressed in atrophic cells[24].

LDH activity determination

Cell toxicity was determined by lactic dehydrogenase (LDH) activity release in cell medium. Positive control of cell lysis was performed adding 0.1% Triton X 100 for 30 min to cell monolayer. Values were expressed as mean % ±SD of two independent experiments, each at least in duplicate.

Viability MTS assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-sulfophenyl)-2H-tetrazolium (MTS) assay[20]. Briefly, C2C12 cells in 96-well plates were plated at

50% confluence and synchronized in serum-free medium for 24 h. Then, cells were incubated in medium containing 10% FCS, and treated with bioactive molecules or specific inhibitors for the indicated times and incubated for further 24 h. MTS assay kit was utilized to measure production of formazan, which is directly proportional to the cell viability. Optical density at 490 nm wavelenght was determined using an ELISA microplate reader (Bio-Rad, Hercules, CA, USA).

Protein synthesis measurement by [^{3}H]-leucine incorporation

To measure the rate of protein synthesis, C1C12 differentiated cells (24-well plates), treated or not with Dexa or starved as indicated for each specific experimental condition, were incubated in Leu-labeling medium RPMI (without Leu), and labeled with 0.037 MBq /ml of [^{3}H]-leucine for 2 hours. At the indicated time points, the monolayer was washed with phosphate buffered saline and incubated in ice cold 10% trichloroacetic acid (TCA) for 30 min at 4°C. The TCA-precipitable material was washed with cold 5% TCA, and then solubilized in 0.1 M NaOH. [^{3}H]-leucine incorporation was measured by liquid scintigraphy in a beta counter (Beckman). Decays per minute (DPM) were normalized by total protein for each well.

Western blotting analysis

Immunoblotting was performed using enhanced chemiluminescence as previously reported[27, 38]. Cells were lysed for 30 min at 4 °C in a buffer containing 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1 mM EDTA, 6 mM EGTA, 15 mM $Na_4P_2O_7$, 20 mM NaF, 1% Nonidet and protease inhibitor cocktail (1.04 mM AEBSF, 0.08 μM aprotinin, 0.02 mM leupeptin, 0.04 mM bestatin, 15 μM pepstatin A, 14 μM E-64) essentially as described in[38]. Cell extracts were centrifuged for 15 min at 10,000x*g* at 4 °C. Proteins (10-25 μg) from lysates were resuspended in Laemmli's sodium dodecylsulfate (SDS) sample buffer. Samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting analysis as previously described[38]. The blots were first blocked in 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBST) at room temperature for 1 h, and then incubated in the same blocking solution containing primary antibodies overnight at 4°C. After sufficient washes with TBST, blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h. Blots were again washed in TBST and, thereafter, signals were detected by using Luminata Western HRP reagent. Finally, blots were exposed to high-performance chemiluminescence film. Densitometric analysis of the bands was performed using NIH IMAGE (ImageJ software, Bethesda, MD, USA) and Quantity-One (Imaging

and Analysis Software by Bio-Rad Laboratories, Hercules, CA) and band intensity was reported as relative percentage (means ± SEM), obtained by calculating the ratio of specific protein on β-actin or β-tubulin intensity and normalizing to control, set as 100.

Statistical analysis

For all the experiments at least three samples were analysed and all the assays were carried out in triplicates. The results are expressed as range of mean values and standard error (S.E.M.) or standard deviation (S.D.). The differences between the chestnut extracts were analysed using one-way analysis of variance (ANOVA), considering statistical significant $P < 0.05$.

Discussion

The present study points out that tuscan chestnut sweet flour is a natural source of specific bioactive components (tocopherols and SLs) able to counteract the degenerative processes (atrophy and autophagy) responsible for the loss of skeletal muscle mass. The basic composition and nutraceutical properties of sweet chestnut flours obtained from fruits collected from 7 different areas of Tuscany (Italy) were found to be very similar; the differences seemed to be dependent on several parameters from the methodologies used to obtain the flours to the tipology of cultivar or regions of origin. The chemical composition was also similar to that published for fruits of other territorial origins reviewed in[6]. In the present manuscript, we also show the vitamin E profile that appears to be similar along all the assayed tuscan sweet chestnut flours (γ-tocopherol > δ-tocopherol > α-tocopherol > β-tocopherol), and to what has been reported from other chestnut fruits[4].

It is worth noting that the content in tocopherols measured in sweet chestnut flours (18-114 mg/kg) is higher than that found in fruits by Barreira et al.,[4] and de Vasconcelos et al.[6], that have reported a value of 4 and 4-27 mg/kg for Portuguese chestnuts, respectively, likely due to the loss of water during flour preparation, or other factors such as harvesting year, sampling or methodological analytical procedures.

Although almonds, hazelnuts, walnuts, pistachios and pecans are not generally used to produce flour, the comparison between the composition of chestnut flours and these fruits indicates a quite dissimilar qualitative vitamin E profile from almond or hazelnut, but very similar to that obtained from walnut, pistachios or pecans[42].

The high γ-tocopherol dominance in chestnut flour may represent an important feature for the nutraceutical properties of this product, since γ-tocopherol is known to be the most effective isoform in scavenging free radicals and nitrogen oxygen species and to have anticancer and anti-inflammatory action[1, 43].

Table 3 indicates that γ-tocopherol content was in the same range for all geographic areas considered (89-96%). We have found some statistical differences in γ-tocopherol content among the analysed samples, also of the same area, suggesting an influence of the drying parameters in preserving the content of bioactive compounds.

Numerous studies performed using animal models have confirmed that prolonged skeletal muscle inactivity/or atrophic conditions promote oxidative stress, leading to an increase of reactive oxygen species (ROS) produced in both inactive and contracting skeletal muscles. When ROS production in cells exceeds the antioxidant capacity to maintain the normal redox balance, a pro-oxidant state occurs altering redox signaling.

The here reported findings demonstrate that long-term incubation with chestnut flour tocopherol and polyphenol extracts decreases myoblast proliferation. Since no apoptotic hallmarks are found, the decrease in cell growth appears due to cell cycle arrest and, likely, in the induction of myogeneic differentiation (E.M., personal communication).

A growing number of *in vitro* studies reveal that selective antioxidants can delay disuse muscle atrophy. Notably, it is worth noting that total tocopherol extracts from tuscan chestnut sweet flour protect from skeletal muscle cell atrophy and this protection appears not to be due to a general antioxidant action, since the total polyphenol extract, that are also considered antioxidants, fails to protect C2C12 cells either from changes in morphology than from reduction in protein synthesis. In addition, chestnut flour tocopherol extract does not affect Dexa-induced autophagy, whereas, polyphenol extract potentiates the effect of Dexa on Beclin-1 expression and on the acquisition of an autophagic phenotype, indicating a more severe cytotoxic effect after treatment with this latter bioactive molecule. Notably, although both total tocopherol extract and γ-tocopherol can prevent Dexa-induced atrophy, each of the compounds differently affects Atrogin-1 expression. In particular, pure γ-tocopherol alone increases expression of the atrophy marker MAFbx/atrogin-1 and alters cell morphology in accordance with recent studies that have cast doubt on the benign effects of long-term antioxidant supplementation[44]. In contrast, although γ-tocopherol accounts for more than 90% of the tocopherol extracts, the latter has a positive effect both in control and in atrophic myotubes. On the other hand, the total extract may contain other marginal compound(s) responsible for the beneficial effects.

Therefore, tocopherol mixtures, obtained from natural products, might offer beneficial advantages in some patients and in aging populations because they possess enhanced anti-inflammatory activity compared to pure compounds, as also reported in various studies1,43. Moreover, the properties of tocopherol chestnut extracts likely able to counteract ROS-mediated processes and/or directly exert antioxidant effects, might be useful for ameliorating skeletal muscle performance. This in vitro study needs to be confirmed in animal models since the bioavailability of the active compounds could preclude the putative beneficial effects. Moreover, the translation from animal models to humans, already occurred for other molecules such as curcumin, will allow to address the pharmacokinetics, safety, and efficacy of this nutraceutical in human health. Since chestnust flour is a gluten-free flour, the nutraceutical properties reported in this study and others[5-9] allows to consider this functional food as a part of a diet for people affected by coeliac disease.

A novel and relevant finding of the present study is the presence of SLs in chestnut flour and the protective role played by SLs extracts on skeletal muscle cell atrophy. Although, SLs represent an important source of bioactive molecules, only a few of the published studies reported the

analysis of their content in food, found to be some hundreds of mg/kg[17]. The content of SLs approximately 50 mg/kg dry weight is a relatively low concentration, but appears to be sufficient to prevent MAFbx/atrogin-1 expression.

SLs are first and/or second messengers involved in the regulation of cell fate, inflammation and migration[18] and are known to play an important role in maintaining cellular redox homeostasis through regulation of NADPH oxidase, mitochondrial integrity, and antioxidant enzymes[45]. It is worth noting that diseases which result in muscle weakness, such as heart failure, are characterized by alteration in SLs metabolism[46]: elevated sphingomyelinase (SMase) activity that, in intact muscle, increases mitochondrial ROS that contribute to diminished muscle force[47].

Bioactive SLs (ceramide, S1P and ceramide-1-phosphate) play a crucial role in skeletal muscle cell biology, and, since they are interconvertible molecules[18,46], they often exhibit opposite actions also in skeletal muscle cells[26,36]: S1P is a prosurvival and promyogenic factor[19-21] whereas ceramide inhibits myogenesis[48].

The characterization of the specific bioactive lipids of chestnut extracts able to affect myotube atrophy, was not an aim of the present work. However, on the basis of the relevance of these compounds for the physiopathology of many tissues, it will be worthy to be investigated.

The here reported protective effect might be mediated by S1P since in our experimental conditions SK inhibitor increases the expression of the atrophy marker Atrogin-1/MAFbx, and decreases the nuclei number and cell diameter (E.M., personal communication), supporting a positive role of SK/S1P axis on the maintenance of a normal differentiated skeletal muscle phenotype. Whether the protective effect of chestnut sweet flour SL extracts is due to the specific bioactive lipid S1P and whether it is related to maintenance of redox homeostasis or to a different mechanism is worth to be further investigated. Indeed, it might help to clarify the nutraceutical properties relevant for skeletal muscle mass regulation and to address an important question on which conditions, in the chestnut flour chain of production (methodological, industrial, etc.) must be kept under control to assure the preservation of these nutraceutical properties.

Moreover, since many studies have suggested that a low vitamin E nutritional status and the level of SLs in plasma are associated with pathological condition[49-51], a diet enriched in natural products containing tocopherol and SL extracts might be advantageous for maintaining a healthy status and wellness.

Conclusion

The present work provide the first evidence that chestnut sweet flour is a source of specific bioactive components, such as tocopherols and SLs, with a relevant role in the prevention of cell degeneration and maintaining of skeletal muscle mass. Therefore, these observations might be useful in designing appropriate nutritional therapeutical approaches in skeletal muscle cell atrophy, consisting in a diet enriched of food naturally rich in SLs or developing products fortified with SLs that may be of help for an aged population that undergo to a physiological decrease of skeletal muscle mass and tone.

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The authors declare that there are no conflicts of interest with any financial organization regarding the results presented in this research manuscript.

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LEGENDS

Fig. 1. Effect of total polyphenol and tocopherol extracts on skeletal muscle cell growth.

A) Cell counting. C2C12 cells were serum-starved for 24 h and, successively, incubated in growth medium in the absence or in the presence of vehicle (control), polyphenols (100 nM) or tocopherols (100 nM) for the indicated time (days). Cells were collected by tripsinization and counted using Burker camera. Values are means ± S.E.M. of at least three independent experiments performed in quadruplicate (*P < 0.05 vs. vehicle).

B) MTS-dye reduction assay. C2C12 cells were treated as above and formazan product was quantified by reading optical density at 490 nm. Values are means ± S.E.M. of three independent experiments performed in quadruplicate (*P < 0.05 vs. vehicle t0, §P<0.05 vs. specific control).

Fig. 2. Effect of polyphenol and tocopherol extracts on skeletal muscle cell atrophy.

A-D) Western blot analysis of MAFbx/atrogin-1 (Atrogin-1) expression. C2C12 myotubes were incubated with vehicle or with polyphenol or tocopherol extracts (100 nM) for 1 hour before the addition of Dexa (A) or serum-starved as indicated (B,C) or treated with γ-tocopherol (24 nM) as indicated in (D). Thirty six hours later, cells were harvested and lysed as described in Methods. Total lysates (20 μg) were used to immunodetect the level of MAFbx/atrogin-1 with specific antibodies and revealed by enhanced chemoluminescence. Equally loaded protein was checked by expression of the non-muscle specific β isoform of actin. The band intensity is reported in the graphics as percentage relative to the intensity of the band corresponding to the respective unchallenged control set as 100. Values are means ± S.E.M. of three independent experiments (*P < 0.05 vs control (vehicle) in 3A and 3D or serum starvation (-FCS) in 3B and 3C, §P<0.05 vs. Dexa).

Fig. 3. Effect of polyphenols and tocopherols on skeletal muscle cell autophagy.

A) Images of a representative field were obtained by phase contrast microscopy. C2C12 myotubes (MT) were treated in the presence or absence of total polyphenol or tocopherol extracts before the addition of Dexa. Note in the magnification the typical autophagic vacuoles.

B) Western Blotting analysis. C2C12 differentiated cells were treated as in A). Twenty-four hours later, myotubes were harvested and lysated as described in Methods. Specific antibodies were used

to immunodetect Beclin-1 (Beclin), revealed by enhanced chemoluminescence. Equally loaded protein was checked by evaluating the expression level of β-actin. Band intensity is reported in the graphic as percentage relative to the intensity of the band corresponding to the respective unchallenged control set as 100. Values are means ± S.E.M. of at least three independent experiments (Student's *t* test *P < 0.05 vs. Dexa).

Fig. 4. Involvement of ERK1/2 on γ-tocopherol action.

Western blot analysis of phospho-ERK1/2 expression was performed using C2C12 myotubes incubated with vehicle or with γ-tocopherol (γ-Toco) for 1 hour before the addition of Dexa as indicated. Thirty six hour later, cells were harvested, lysed and total lysates (20 μg) immunoblotted to detect the level of the active form of ERK1/2 (phospho-ERK1/2, p-ERK1/2). Band intensity corresponding to p-ERK was normalized on the value of β-actin and it is reported in the graphic as percentage relative control set as 100. Values are means ± S.E.M. of at least three independent experiments (Student's *t* test, *p< 0.05 vs. control (vehicle).

Fig. 5 Effect of sphingolipid extract on skeletal muscle cell atrophy.

A) Western blot analysis of MAFbx/atrogin-1 (Atrogin-1) expression. C2C12 myotubes were incubated with vehicle or with total SLs extract for 1 hour before the addition of Dexa or treated with γ-tocopherol (24 nM) as indicated. Thirty six hours later, cells were harvested and lysed as described in Methods. Total lysates (20 μg) were used to immunodetect the level of MAFbx/atrogin-1 (Atrogin-1).

B) Western blot analysis of MAFbx/atrogin-1 (Atrogin-1) expression. C2C12 myotubes were incubated with vehicle or with sphingosine kinase inhibitor (iSK) for 1 hour before the addition of Dexa or serum-starved as indicated. Thirty six hour later cells were lysed and MAFbx/atrogin-1 immunodetected. Equally loaded protein was checked by expression of the non-muscle specific protein (β-actin or β-tubulin). Band intensity is reported in the graphics as percentage relative to the intensity of the band corresponding to the respective unchallenged control set as 100. Student's *t* test, *P< 0.05 vs. control; $^{\#}$P< 0.05 vs. SLs alone (in A) or serum-starved control cells in (B).