Strawberry consumption improves aging-associated impairments, mitochondrial biogenesis and functionality through the AMP-activated protein kinase signaling cascade

Strawberry consumption improves aging-associated impairments, mitochondrial biogenesis and functionality through the AMP-Activated Protein Kinase signaling cascade

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Abstract

Dietary polyphenols have been recently proposed as activators of the AMP-activated protein kinase (AMPK) signaling pathway and this fact might explain the relationship between the consumption of polyphenol-rich foods and the slowdown of the progression of aging. In the present work, the effects of strawberry consumption were evaluated on biomarkers of oxidative damage and on aging-associated reductions in mitochondrial function and biogenesis for 8 weeks in old rats. Strawberry supplementation increased antioxidant enzyme activities, mitochondrial biomass and functionality, and decreased intracellular ROS levels and biomarkers of protein, lipid and DNA damage ($P < 0.05$). Furthermore, a significant ($P < 0.05$) increase in the expression of the AMPK cascade genes, involved in mitochondrial biogenesis and antioxidant defenses, was also detected after strawberry intake. These in vivo results were then verified in vitro on HepG2 cells, confirming the involvement of AMPK in the beneficial effects exerted by strawberry against aging progression.

Keywords: AMPK activation, aging, oxidative stress, mitochondrial functionality, strawberry polyphenols

1. Introduction

It is known that oxidative stress, mitochondrial dysfunction and bioenergetic alterations are the main factors involved in the aging process and in the development of age-related diseases, such as metabolic syndrome, type 2 diabetes and cardiovascular diseases (the “free radical theory of aging” and the “mitochondrial theory of aging” respectively described by Harman in 1956 and Ochoa et al. in 2011). The production of reactive oxygen species (ROS), the major source of cellular damage, mostly occurs in mitochondria and accumulates during aging. Oxidative stress induced by increased ROS production leads in turn to accumulated damage in the mitochondrial DNA (mtDNA) genome, thus perpetuating the increased production of ROS and aberrant electron transfer chain components, and reduced ATP synthesis and impaired mitochondrial function, in a self-perpetuating cycle (Ochoa et al., 2011). Mitochondrial biogenesis is a crucial process for cell viability and survival, since its dysfunction impairs maintenance of energy production and metabolism regulation as well as oxidative stress resistance (Gesing et al., 2011). In eukaryotic cells, mitochondrial biogenesis is prompted through the modulation of the ATP/ADP ratio, activation of AMP-activated protein kinase (AMPK) pathway, and the subsequent expression of peroxisomal proliferator activator receptor γ co-activator 1α (PGC-1α) and nuclear respiratory factor-1 (Nrf1) transcription factors (Hardie, Ross & Hawley, 2012). The AMPK cascade, one of the main systems to ensure the maintenance of energy homoeostasis, is also involved in the cellular response against ROS-induced oxidative stress damage, through increased expression levels of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), Mn superoxide dismutase (MnSOD) and catalase, (Colombo & Moncada, 2009; Cordero et al., 2013; Steinberg & Kemp, 2009). Indeed, activation of the AMPK cascade has been associated with the improvement of glucose and lipid metabolism, with the inhibition of platelet aggregation and thrombi reduction, as well as with neuroprotective and anticancer effects (Takikawa Inoue, Horio, & Tsuda, 2010; Park, Inoue, Horio, & Tsuda,, 2014; Lee, Lee, Kim, & Park., 2010; Zhang, Wang, Wang, Liu, & Xia, 2013). Furthermore, the contribution of aging-

associated reductions of AMPK activity in mitochondrial dysfunction and increased oxidative damage associated with aging has been already advanced (Reznick et al ., 2007).

Dietary polyphenols have been recently proposed as activators of the AMPK signaling pathway, and this fact might explain the relationship between consumption of polyphenol-rich foods, disease prevention, and the slowdown of aging progression (Gasparrini et al., 2015). In spite of the high polyphenolic content of berries, literature data evaluating the *in vivo* anti-aging effects of berry bioactive compounds through the activation of the AMPK cascade are still scarce.

The aim of the present study is to evaluate the protective effect of strawberry consumption against oxidative damage, antioxidant defence and mitochondrial impairment in old rats, paying particular attention to the implication of the AMPK pathway. To do this, a 2-month animal feeding trial with 19-21 old Wistar rats was carried out. Biomarkers of DNA, protein and lipid oxidation damage, antioxidant enzyme activities and other mitochondrial bioenergetic parameters were analyzed. The obtained results were also corroborated in human hepatoma HepG2, by assessing the capacity of the strawberry extract to activate the AMPK signalling pathway and so counteract oxidative stress and improve mitochondrial functionality.

2. Materials & methods

2.1 Reagents

Media and reagents for cell culturing were purchased from Carlo Erba Reagents (Milan, Italy), while Tali™ CellROX® Orange Reagents and ATP kit were obtained from Invitrogen™, Life Technologies (Milan, Italy). All chemicals and solvents were acquired from Sigma-Aldrich Chemical (Milan, Italy), while EPR probes were purchased from Noxygen, Elzach, Germany. Primary and secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, USA).

2.2 Strawberry fruit analysis

Strawberry fruits (*Fragaria* × *ananassa*, Alba cultivar) were hand harvested, detached from the sepals, frozen and lyophilized. The strawberry powder was kept under vacuum, in the dark, at a temperature of -80 °C until compositional analysis and meal preparation. Fruit powder analysis included measuring total antioxidant capacity, total phenol and flavonoid content, vitamin C, and HPLC–DAD/ESI–MS-driven anthocyanin characterisation, as described in our publications (Giampieri et al., 2016, 2017).

2.3 Animal study design

Wistar rats (Rattus norvegicus) were chosen for the aged animal model, as previously described (Shi, Liu, Zhang, Xue, Liu, & Chen, 2014). Sixteen old male rats (19-21 months, initial weight of 500-550 g) were provided by the "Istituto Nazionale di Ricovero e Cura per gli Anziani" (INRCA, Ancona, Italy), were housed individually and maintained on a 12 h light/12 h darkness cycle with free access to drinking water. Prior to the feeding trial, the rats were randomly assigned to receive either a standard diet (C group, n =8) or a strawberry-enriched diet (S group, n = 8) for 8 weeks. Both diets were supplied in the form of powder and daily prepared by mixing each individual ingredient using a rotating mixer and kept in the dark at a temperature of 4°C. Compared to the standard diet (AIN93M), the strawberry enriched diet was prepared by substituting 15% of the total calories with freeze-dried strawberry powder, and the amount of macro- and micronutrient adjusted to be identical between the two diets (Giampieri et al 2017).

The animals received their respective food and drink at libitum. The amount of food consumed by each animal was monitored by weighing each day the amount of food present in the feeder before giving the following daily food ration and this was taken into account when calculating the total food consumption per animal. Rats were weighed once a week for the whole experimental period.

At the end of the two months, the rats were anesthetized with 4% isoflurane inhalation at the same time of day to avoid any circadian fluctuation and samples were collected. Blood was

129 collected by intra-cardiac puncture and immediately transferred into heparin-containing tubes.
130 Heparinized plasma was isolated by centrifugation at 1130 *g* for 20 min at 15 °C and stored at -80
131 °C until analyses. After exsanguination, the whole livers were carefully removed, washed with ice-
132 cold 0.9 % NaCl solution, weighed and divided into two portions: one was used for the fresh
133 isolation of mitochondrial fractions as previously described (Pedersen et al., 1978), while the other
134 portion was frozen under liquid nitrogen and stored at -80 °C for biochemical and Western blotting
135 analyses.

136 For biochemical analyses, the livers were homogenized on ice in 5 mM Tris–HCl buffer (pH 7.4)
137 containing 1 mM EDTA (Sigma-Aldrich, Milan, Italy), in a ratio (10 %) of 1 g of wet tissue to 10
138 ml of buffer, using a IKA-Werk (Janke Kunkel, UE) homogenizer. After centrifugation at 12.040 *g*
139 for 20 min at 4 °C, the supernatant was kept and then stored at -80 °C until analysis. Proteins were
140 measured by the Bradford procedure (Bradford, 1976) using BSA as standard.

141 The animals were handled in compliance with all applicable laws and regulations and according
142 to the statements of the European Union (86/609/EEC), concerning the protection of animals used
143 for experimental and other scientific purposes. Experimental protocols were approved by the
144 Institutional Animal Care Committee of the Ministry of Health (Italy) and by the Animal Research
145 Ethics Committee of INRCA.

146

147 *2.4 Cell culture and treatment*

148 Human HepG2 cells were obtained from the American Type Culture Collection (Manassas, Va,
149 USA). Cells were cultured in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, 100
150 µg/ml streptomycin, and 5.5 mmol/l D-glucose and were incubated in a humidified atmosphere of
151 5% CO_2 at 37°C. Strawberry extract was prepared as previously described (Amatori et al., 2016),
152 concentrated under vacuum and resuspended in DMEM to achieve a final concentration of 10
153 µg/ml. This concentration represents the lowest effective dose of strawberry extract, which gave the
154 best results in terms of cell viability and reproducibility according to the MTT assay for cytotoxicity

studies (data not shown). Cells were incubated for 48 h with strawberry extract, while control cells were incubated only with DMEM.

For biochemical analysis, cells were treated with RIPA buffer, incubated on ice for 5 minutes and stored at -80 °C until analyses.

2.5 ROS production

In HepG2, the determination of intracellular ROS levels was performed using the probe CellROX® Orange reagent, as previously described (Gasparrini et al., 2017). Results were expressed as the percentage of cells with increased ROS levels compared with the control.

In animals, Electron Paramagnetic Resonance (EPR) spectroscopy was used to measure the kinetic rate of $O_2^{\bullet-}$ accumulation in plasma, by the hypoxanthine/xanthine oxidase $O_2^{\bullet-}$ generating system in the presence of the hydroxylamine spin probe PPH (1-hydroxy-4-phosphono-oxy-2,2,6,6-tetramethyl-piperidine) following the procedure described by Watanabe et al. (2007). EPR spectra were recorded on a Bruker EMX EPR spectrometer (Bruker, Karlsruhe, Germany) operating at X-Band equipped with an XL microwave frequency counter and a temperature controller, with the following settings: frequency 9.78 GHz, field width 100 G (Gauss), power 20 mW, modulation amplitude 2 G, gain 2 x 10^6, time constant 40.96 ms, scan time 42 s, number of scans 7. As a control, the EPR spectrum obtained from a reaction mixture containing PPH, xanthine and xanthine oxidase (positive control) was used and the peak height of the low field component of the spectrum was compared to those recorded in the presence of plasma. Kinetics were determined by plotting the nitroxide concentration, as determined from a calibration curve for intensity of the signal of 4-hydroxy-2,2,6,6-tetramethyl-pyperidin-1-oxyl at various known concentrations *vs* time (7 min). The results are expressed as concentration of nitroxide radical per minute per mg protein (nM/min/mg prot).

2.6 Biomarkers of oxidative stress and antioxidant enzymes

In cellular lysates from HepG2 and in rat plasma and liver homogenates, protein carbonyl content and lipid peroxidation levels were determined as reported in our previous publications (Giampieri et al., 2016). Antioxidant enzyme activities (catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione transferase) were evaluated spectrophotometrically as previously reported (Giampieri et al., 2016)

2.7 Mitochondrial bioenergetic functions

2.7.1 Total mitochondrial ROS production

Total mitochondrial ROS production was measured by EPR spectroscopy in rat liver, as previously described by Panov, et al. (2005). The results are expressed as concentration of nitroxide radical per minute per mg protein (nMl/min/mg prot).

2.7.2 Measurement of citrate synthase and ATP levels

The specific activity of citrate synthase was measured in cellular lysates and liver homogenate at 412 nm minus 360 nm (13.6 mmol/L/cm) by using 5,5-dithio-bis(2-nitrobenzoic acid) to detect free sulfhydryl groups in coenzyme A, as previously described (Bullon, Cordero, Quiles, Morillo, del Carmen Ramirez-Tortosa, & Battino, 2011). Citrate synthase data were expressed as enzyme activity. ATP levels were determined by a bioluminescence assay using an ATP determination kit according to the instructions of the manufacturer.

2.7.3 Determination of mitochondrial respiration rate

Oxygen consumption rate (OCR) in HepG2 cells and rat liver mitochondria was measured in real-time using a XF-24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica MA, USA) as previously reported by Richardson et al. (2012) et Giampieri et al. (2016), respectively. For cells, after an OCR baseline measurement, a profiling of mitochondrial function was performed by

sequential injection of four compounds that affect bioenergetics as follows: 55 µl of oligomycin (2.5 µg/mL) at injection in port A, 61 µl of FCCP (2 µM) at injection in port B, and 68 µl of antimycin/rotenone (10 µM/1µM) at injection in port C. For isolated rat liver mitochondria, the following compounds were used: 50 µl of NADH (final concentration 300 µM final) at injection A, 55 µl of rotenone (final concentration 2 µM) at injection B, 60 µl of succinate (final concentration 10 mM) at injection C and 65 µl antimycin A (final concentration 4 µM) at injection D. Five wells were utilized per condition in any given experiment and data are expressed as *p*mol of O_2 consumed per minute normalized to 1000 cells (*p*mol O_2/1000 cells/min) or as as *p*mol of O_2 consumed per minute for animal analysis.

2.8 Immunoblotting analysis

After treatment, HepG2 were collected, washed with PBS, lysed in 100 µl lysis buffer (120 mmol/L NaCl, 40 mmol/L Tris [pH 8], 0.1% NP40) containing protease and phosphatase inhibitor cocktails and centrifuged at 13000 *g* for 15 minutes.

Proteins (100 µg/ml) from cell supernatants and from liver homogenate were separated on a 10-15% acrylamide SDS/PAGE (Bio-Rad, Hercules, CA, USA). Proteins were transferred onto a nitrocellulose 0.2-µm membrane, using the trans-blot SD semidry electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA) and then membranes were blocked with TBS-T containing 5% non-fat milk for 1 h at room temperature. Phosphorylated AMPK (p-AMPK), SIRT1, PGC-1α, Nrf-2 and OGG-1 antibodies were used to detect proteins by Western blotting. Membranes were incubated at 4°C overnight with the respective primary antibody solution, diluted at 1:500 and then membranes were probed 1 hour at room temperature with their specific alkaline phosphatase conjugated secondary antibodies (1:80000). Immunolabeled proteins were detected by using a chemiluminescence method (C-DiGit Blot Scanner, LI-COR, Bad Homburg, Germany). The protein was determined by the Bradford method (1976).

2.9 Statistical Analysis

Statistical analyses were performed using STATISTICA software package (Statsoft Inc., Tulsa, OK, USA). Plasma, liver and mitochondria data were subjected to the Wilcoxon paired samples test. The mean of three analyses was used and the results reported as mean ± standard error (SE) and as % changes from the control group. Differences at $P < 0.05$ were considered statistically significant.

3. Results

3.1 Effects of strawberry intake on old rats

3.1.1 Body weight

Data on body weight and liver ratios showed no significant variations between groups, indicating that the strawberry supplementation did not interfere with normal animal maintenance (Giampieri et al., 2017). These results were confirmed by plasma biochemical parameters and liver histological analysis, which showed no difference between control group (C-group) and strawberry group (S-group) (Giampieri et al., 2017); no significant changes were observed even for daily food intake.

3.1.2 Biomarkers of oxidative stress and antioxidant defences in plasma and liver

As shown in Table 1, strawberry supplementation resulted in a significant decrease of $O_2^{\bullet-}$ accumulation in plasma (17.8% reduction, $P < 0.05$) compared to the rats fed the standard diet. The consumption of strawberries also led to a significant decrease of circulating biomarkers of protein (47.4% reduction of carbonyls levels, $P < 0.05$) and lipid oxidation (34.2% reduction of TBARS levels, $P < 0.05$) (Table 1). Compared to the control group, an improvement in biomarkers of oxidative stress was also observed in the liver of old rats fed the strawberry enriched diet, showing a reduction of about 62.8% in protein carbonyls ($P < 0.05$) and 57.7% TBARS ($P < 0.05$) levels (Table 1). In agreement with these results, the expression of OGG-1 (8-Oxoguanine glycosylase), an enzyme responsible for the excision of a mutagenic base that occurs as a result of ROS exposure,

showed a significant decrease (56.8%, $P < 0.05$) in S group compared to C group (Fig. 1), highlighting a marked protective effect of strawberry consumption on all the biological macromolecules against oxidative stress. Furthermore, a positive effect of strawberry enriched-diet was observed on liver antioxidant enzymes (Table 1): GPx, GR, GST activities significantly increased in the S group (28.5%, 39.4% and 19.5% respectively, $P < 0.05$) and the same trends were also found for catalase and SOD activities (16.9% and 55.9% respectively, $P < 0.05$) (Table 1).

3.1.3 Mitochondrial biogenesis and functionality

The S group showed a significant reduction of mitochondrial ROS (39.1%, $P < 0.05$), when compared to C group (Table 1). In addition, a significant increase (42.3%, $P < 0.05$) in citrate synthase activity, a marker of mitochondrial mass, was found after two months of strawberry consumption (Table 1). These results were confirmed by the measurement of ATP levels, which were lower in the control group and significantly increased after strawberry supplementation (Table 1).

To confirm the potential improvement of strawberries on mitochondrial function, the OCR was measured in isolated liver mitochondria, exposed sequentially to each of four well-defined modulators of oxidative phoshorylation: NADH, rotenone, succinate and antimycin A (Fig. 2A). Addition of NADH, which is a substrate that transfers electrons to the mitochondrial NADH-ubiquinone oxidoreductase (complex I), caused a significant OCR increase (65.4%, $P < 0.05$) in S-group (Table 1). The subsequent addition of rotenone, an inhibitor of mitochondrial NADH-ubiquinone oxidoreductase, arrested electron flow through the mitochondrial respiratory complexes and caused a dramatic decrease in OCR, in both groups examined, with values close to those of the basal respiratory rate (Fig. 2A). The subsequent addition of succinate, a substrate that transfers electrons to the succinate-ubiquinone reductase (complex II), caused a marked increase of OCR in both experimental groups, with a significant increase (28.7%, $P < 0.05$) in S-group compared to C-group (Table 1). Finally, addition of antimycin A, an inhibitor of ubichinol-cytochrome c

oxidoreductase (complex III), completely stopped OCR in both experimental groups confirming total blocking of mitochondrial activity (Fig. 2A).

3.1.4 Up-regulation of AMPK pathway in vivo

Rats fed with the strawberry enriched diet showed significantly higher levels of p-AMPK, SIRT-1, PGC1- α and Nrf2 ($P < 0.05$) compared to rats fed with the standard diet (Fig. 1), suggesting that strawberry intake is associated with enhanced mitochondrial biogenesis and antioxidant defences through AMPK expression.

3.2 Effects of strawberry treatment on cells

3.2.1 Biomarkers of oxidative stress and antioxidant defences

The protective effect of strawberries in reducing oxidative damage and improving mitochondrial functionality was then confirmed *in vitro,* on HepG2 cells. Treatment with the strawberry extract decreased intracellular ROS concentration (43.9%, $P < 0.05$) and consequently attenuated the levels of carbonyl groups (53.6%, $P < 0.05$), TBARS (35.2%, $P < 0.05$) (Table 2) and OGG-1 (46.3%, $P < 0.05$) (Fig. 3). The strawberry extract also exerted a positive effect on the activities of the principal antioxidant enzymes: in fact, a significant increase in GPx, GR, GST activities (89.2%, 70.8%, 99.4%, respectively, $P < 0.05$) was observed (Table 2). The same favorable results were detected for SOD and catalase activities (77,8% and 61.8%, respectively, $P < 0.05$) (Table 2) as well.

3.2.2 Mitochondrial biogenesis and functionality

Besides the antioxidant capacity, strawberry treatment also stimulated mitochondrial biogenesis and functionality. Indeed, a significant increase in citrate synthase activity (45.5%, $P < 0.05$) and ATP levels (42.1%, $P < 0.05$) was found in cells treated with strawberries (Table 2), highlighting an increase in mitochondrial mass. In addition, the protective capacity of strawberry extract on

311 mitochondrial functionality was confirmed by measuring OCR. Cells were sequentially exposed to
312 different well-defined modulators of oxidative phoshorylation: oligomycin (an inhibitor of F_1F_o-
313 ATPase or complex V), FCCP (an uncoupler of oxidative phosphorylation from the electron
314 transport chain) and antimycin + rotenone (inhibitors of complex I and III, respectively) (Fig. 2B).
315 Basal OCR was markedly increased in cells treated with strawberry extract (41.0%, $P < 0.05$)
316 compared to control, mainly due to the increase in mitochondrial biomass. Addition of oligomycin
317 caused an inhibition of electron flow resulting in marked decreases of OCR in both groups, while
318 the FCCP increased OCR to the maximal mitochondrial respiration the cells could reach; the
319 addition of rotenone + antimycin arrested the electron flow through the mitochondrial respiratory
320 complexes causing a considerable decrease in oxygen consumption, as shown by the drop in OCR
321 levels in all cells.
322
323 *3.2.3 Up-regulation of AMPK pathway in vitro*
324 The capacity of strawberry polyphenols to activate AMPK was confirmed in our *in vitro*
325 experimental model: the expression levels of p-AMPK, SIRT-1 and consequently of PGC-1α were
326 significantly increased ($P < 0.05$) in strawberry pre-treated cells, together with the expression levels
327 of Nfr2 (Fig. 3). As expected, treatment with strawberry extract alone caused an increase in the
328 expression of p-AMPK, SIRT1 and PGC1-α as previously demonstrated, while pretreatment of cells
329 with compound C clearly prevented the strawberry–induced AMPK activation (Fig. 4) and impaired
330 SIRT-1 and PGC1-α stimulation, confirming the capacity of the extract to induce AMPK activation
331 and the functional linkage between AMPK, SIRT1 and PGC1-α.
332
333 **4. Discussion**
334 To the best of our knowledge, this is the first study that analyzes the involvement of AMPK
335 pathway *in vivo* after strawberry consumption and its effect on the aging condition. Previous studies
336 have shown that two months of berry supplementation exerts beneficial effects on cognition, motor

behavior and neuronal function as well as on markers of inflammation and oxidative stress in rats aged 19 to 21 months (Poulose, Bielinski, Carey, Schauss & Shukitt-Hale, 2016; Shukitt-Hale, Bielinski, Lau, Willis, Carey, & Joseph, 2015; Malin, et al., 2011). In this study, we aimed to evaluate the effects of two months of strawberry consumption on aging-associated reductions in mitochondrial function and biogenesis and on biomarkers of oxidative damage in old rats, evaluating, in particular, the involvement of the AMPK pathway. The choice of strawberry fruits as feeding material was justified by their commercial relevance in the Mediterranean area, being the most consumed berries in fresh or processed form (such as jams, juices and jellies). Moreover, strawberries provide noteworthy health benefits because of their high nutritional value and content of phenolic compounds, which exert anti-microbial, anti-inflammatory, anti-atherosclerotic and anticarcinogenic effects both *in vitro* and *in vivo* models (Amatori et al., 2016; Giampieri et al., 2012; Park et al., 2016; Basu, Betts, Nguyen, Newman, Fu, & Lyons, 2014). These biological and functional activities are related not only to the antioxidant capacity but also to the modulation of many cellular pathways involved in metabolism, survival, proliferation and antioxidant defenses (Forbes-Hernandez et al., 2016; Giampieri et al., 2014). In addition, among berries, strawberries have received increasing attention in recent years and a growing amount of scientific evidence has demonstrated how short- or long-term intake of strawberries could be beneficial for consumers (Park et al., 2016; Basu et al., 2014; Tulipani et al., 2014; Alvarez-Suarez et al., 2014). The dose of strawberry fruit, used in this study, corresponded to a substitution of 300 calories with strawberry intake (approximately 90 g of dried fruits) in a human 2000 Kcal daily diet.

Traditionally, the best known role of AMPK has been the regulation of energy production from glucose and fatty acids during stress and the inhibition of energy consumption for cholesterol and glycogen synthesis as well as for protein (Hardie, Roos & Hawley, 2012; Steinberg & Kemp, 2009). However, emerging findings show that the role of AMPK is not constrained to energy metabolism maintenance during increased energy consumption, but this kinase can also regulate several biological mechanisms, i.e. oxidative stress, endoplasmatic reticulum stress, autophagocytosis, and

363 inflammation, thus increasing stress resistance in many body tissues (Salminen & Kaarniranta,
364 2012). Beside this, AMPK seems to play a critical role also within the complex signaling network
365 that regulates mitochondrial biogenesis. Mitochondrial biogenesis and functionality decrease with
366 aging, with devastating consequences: indeed, in aged subjects mitochondrial turnover is slower,
367 leading to the further accumulation of modified proteins, lipids and DNA, and exasperating the
368 situation resulting from the insufficient mitochondrial activity (López-Lluch, Irusta, Navas, & de
369 Cabo, 2008). Interestingly, the responsiveness of AMPK activation seems to decline during the
370 aging process and indeed all the above-mentioned processes and conditions are affected during
371 aging: oxidative stress and endoplasmic stress are increased, autophagic capacity and mitochondrial
372 biogenesis are reduced, while low-grade inflammation appears in old subjects (Salminen &
373 Kaarniranta, 2012). Consequently, AMPK seems to be an important key factor in modulating
374 several age-associated processes. The signalling cascade predicts that AMPK activates SIRT1,
375 which, responding to the increase in cellular NAD^+ concentration, is the principal regulator of
376 energy metabolism and survival process, such as proliferation and apoptosis (Gasparrini et al.,
377 2015; Salminen & Kaarniranta, 2012). Once activated, SIRT1 regulates both some stress resistance
378 pathways, including FoxO and NF-KB signalling and downstream targets involved in different
379 biological processes, i.e. PGC1-α. This protein is the crucial factor for the activation of the full
380 program of mitochondriogenesis and acts as a common intracellular mediator during
381 mitochondriogenesis induced by hormones (Hsieh, Yang, Choudhry, Yu, Rue, Bland, & Chaudry,
382 2005). Once activated, PGC1-α coordinates the activities of several transcription factors involved in
383 mitochondrial biogenesis, including nuclear respiratory factor 1, peroxisome proliferator-activated
384 receptor and estrogen-related receptor.

385 Finally, recent studies have demonstrated that AMPK can activate the Nrf2 signaling pathway, a
386 potent inducer of cellular defenses against oxidative stress (Salminen & Kaarniranta, 2012). On the
387 one hand, this pathway seems to be deregulated or inactive during aging and in age-related
388 degenerative pathologies, thus worsening oxidative stress in these conditions. On the other hand, the

activation of Nrf2 signaling pathway has been shown to extend the lifespan of different model organisms (Salminen & Kaarniranta, 2012).

In the present work, we have reported for the first time that strawberry consumption is associated with an *in vivo* up-regulation of AMPK during aging, explaining, in part, its beneficial effects on health. We found that rats fed with a strawberry enriched diet for two months presented higher levels ($P < 0.05$) of p-AMPK and of the proteins related to this pathway (SIRT-1, PGC1- α and Nfr2) compared to rats fed with standard diet. In addition, our results link, for the first time, strawberry AMPK activation with an improvement of oxidative stress and bioenergetic status *in vivo*. Indeed, alterations induced by oxidative stress generally affect all biological macromolecules and are the basis of the free radical and mitochondrial theory of aging as well as the development of several degenerative diseases. We have shown that strawberry consumption exerted favourable effects against oxidative stress, increasing total antioxidant capacity and decreasing radical levels in plasma, liver and mitochondria of old rats. Even if many ROS, such as hydroxyl radical, are highly reactive and can significantly contribute to cellular oxidative stress, in the present work our attention mainly addressed $O_2^{\bullet -}$, since, in most cases, it is the first radical that is produced by cellular oxidase and during mitochondrial respiration (Shang-U & Frederick, 2012; Turrens, 2003). As a consequence all biomarkers of oxidative stress, such as protein carbonyls, TBARS and OGG-1, decreased with a concomitant stimulation of antioxidant enzymes, like GPx, GR, GST, SOD and catalase. At the same time, we found a relevant increase in mitochondrial mass and bioenergetic status, indicated by the increase in citrate synthase activity and ATP levels, respectively, and a marked improvement in mitochondrial functionality, shown by the improvement in OCR, after strawberry consumption. Interestingly, these results are closely similar to those we obtained on young rats fed with the same strawberry cultivar, in the same amount, for the same period; these findings show that strawberry consumption may lead, in old rats, to a reversion of oxidative stress and mitochondrial functionality comparable to those found for young animals (Giampieri et al., 2016).

5. Conclusion

In conclusion, according to these data, strawberry consumption improves the aging condition through AMPK activation, reinforcing once again the importance of a correct diet in health maintenance, even in the elderly. The reduction of oxidative damage and improvement of mitochondrial functionality after strawberry consumption could, in fact, be an important protective approach to ameliorate the "aging phenotype" and delay the onset of aging-related metabolic diseases. For these reasons, we propose AMPK as a novel way to understand and treat aging and age-associated conditions. Further analyses involving different animal models or a longer period of supplementation are strongly required to confirm these observations. In addition, these effects need to be evaluated also in adult animals, in order to verify if strawberry consumption could delay aging and prevent the occurrence of age-related diseases.

Information from these studies is necessary to promote the use of dietary compounds that directly affect the AMPK pathway for the next generation of functional foods and nutraceuticals, such as strategic tools to expand longevity and improve aging.

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Conflicts of interest

The authors declare no conflicts of interest.

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Figure captions

Fig. 1. Strawberry supplementation increased expression levels of proteins related to mitochondrial biogenesis and cellular antioxidant defence in liver of old rats. The levels of phosphorylated AMPK-α, SIRT1, PGC1-α, Nrf2 and OGG1 in rat livers were determined by using Western blotting (representative subset is shown). Mean values belonging to the same set of data with different superscript letters are significantly different ($P < 0.05$). C group: standard diet; S group: strawberry diet.

Fig. 2. Strawberries improved oxygen consumption rate. (A) In isolated mitochondria of rats fed with standard or strawberry enriched diet and (B) in HepG2, treated with DMEM or strawberry extract, mitochondria oxygen consumption was monitored after sequential injection of different compounds that affect bioenergetics at the indicated time points into each well, after baseline rate measurement. Values are means ± SE. Mean values with different superscript letters are significantly different ($P < 0.05$). C group: standard diet; S group: strawberry diet.

Fig. 3. Strawberry treatment increased expression levels of proteins related to mitochondrial biogenesis and cellular antioxidant defence on HepG2 cells. The levels of phosphorylated AMPK-α, SIRT1, PGC1-α, Nrf2 and OGG1 in cells were determined by using Western blotting (representative subset is shown). Mean values belonging to the same set of data with different superscript letters are significantly different ($P < 0.05$).

Fig. 4. Blockage of AMPK signalling abolished strawberry effects in HepG2. Cells were pre-treated with or without compound C (10 µM) and with strawberry (10 µg/ml). Whole cell lysates were subjected to Western Blot analysis with antibodies against p-AMPK, SIRT1 and PGC1-α.

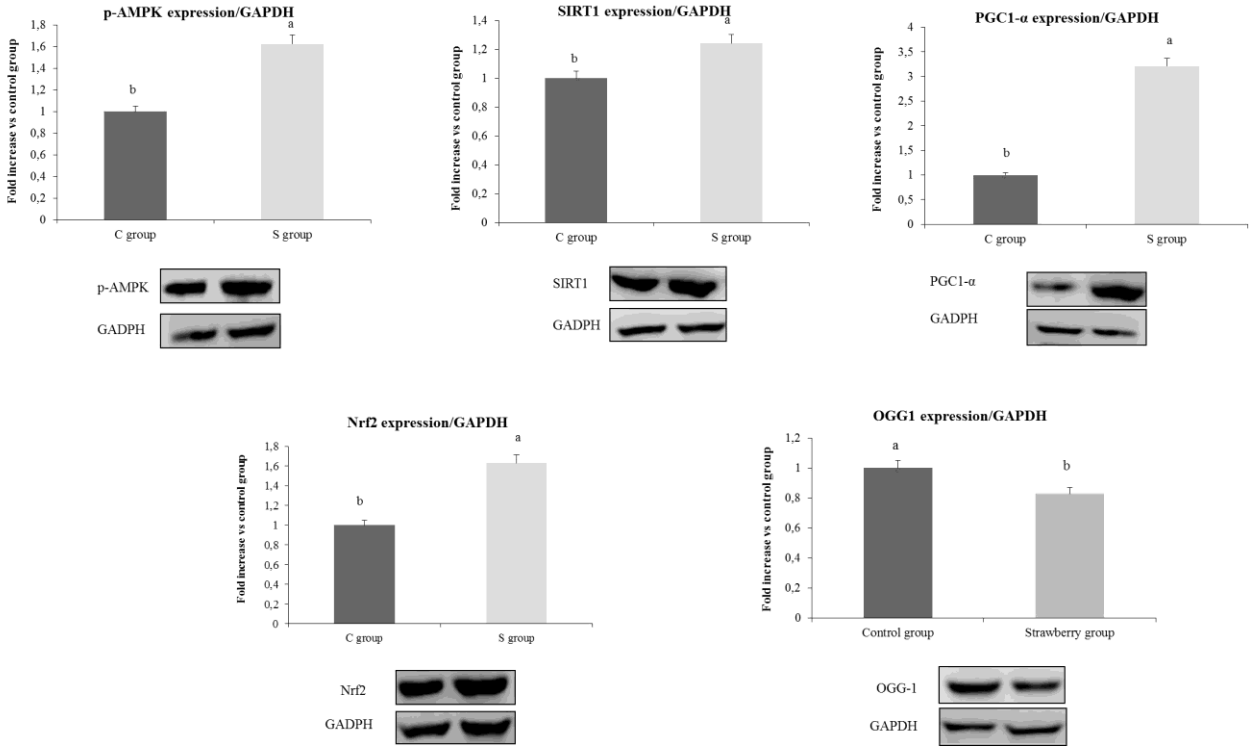
Table 1. Biomarkers of oxidative stress and antioxidant status in plasma, liver and liver mitochondria of old rats. Data are presented as means ± SE; mean values belonging to the same set of data with different superscript letters are significantly different ($P < 0.05$).

	Control group	**Strawberry group**
Plasma:		
Total ROS (nM nitroxide/min/mg prot)	174.10±0.33^{a}	143.00±1.37^{b}
Protein carbonyl content (nmol/mg)	0.78±0.01^{a}	0.41±0.01^{b}
TBARS (nmol/mg)	0.35±0.01^{a}	0.23±0.01^{b}
Liver:		
Protein carbonyl content (nmol/mg)	9.01±0.02^{a}	3.35±0.26^{b}
TBARS (nmol/mg)	0.26±0.06^{a}	0.11±0.04^{b}
GPx (nmol/min/mg)	230.31± 7.79^{b}	295.90±7.57^{a}
GR (nmol/min/mg)	119.94±2.11^{b}	167.25±5.72^{a}
GST (nmol/min/mg)	420.39±3.36^{b}	502.61±4.25^{a}
SOD (IU/mg)	104.80±1.73^{b}	163.45±1.41^{a}
Catalase (IU/min/mg)	21.79±0.11^{b}	25.48±0.52^{a}
Liver mitochondria:		
Total ROS (nM nitroxide/min/mg prot)	1.38±0.06^{a}	0.84±0.04^{b}
ATP (nM/mg prot)	36.05±14.90^{b}	82.75±17.20^{a}
Citrate synthase (specific activity)	179.77±20.72^{b}	255.90±29.41^{a}
OCR (pmol/min)		
Complex I	93.00±2.34^{b}	153.80±2.82^{a}
Complex II	98.10±0.58^{b}	126.30±3.25^{a}

Table 2. Biomarkers of oxidative stress, antioxidant status and mitochondrial functionality in HepG2. Data are presented as means ± SE; mean values belonging to the same set of data with different superscript letters are significantly different ($P < 0.05$).

Parameters	**Control group**	**Strawberry group**
Cellular biomarkers		
Total ROS (nM nitroxide/min/mg prot)	82%	46%
Protein carbonyl content (nmol/mg)	29.41 ± 0.10^{a}	13.63 ± 0.2^{b}
TBARS (nmol/mg)	1.05 ± 0.02^{a}	0.68 ± 0.01^{b}
GPx (µmol/min/mg)	1.86 ± 0.03^{b}	3.52 ± 0.02^{a}
GR (µmol/min/mg)	1.37 ± 0.01^{b}	2.34 ± 0.02^{a}
GST (µmol/min/mg)	3.55 ± 0.09^{b}	7.08 ± 0.07^{a}
SOD (IU/mg)	14.38 ± 0.03^{b}	25.57 ± 0.01^{a}
Catalase (IU/min/mg)	56.94 ± 0.11^{b}	92.15 ± 0.52^{a}
Mitochondrial biomarkers		
ATP (nM/mg prot)	19.40 ± 4.90^{b}	33.51 ± 2.10^{a}
Citrate synthase (specific activity)	176.27 ± 12.07^{b}	256.57 ± 12.08^{a}

Figure 1


p-AMPK expression/GAPDH
Fold increase vs control group
C group
S group
p-AMPK
GADPH
SIRT1 expression/GAPDH
SIRT1
GADPH
PGC1-α expression/GAPDH
PGC1-α
GADPH
Nrf2 expression/GAPDH
Nrf2
GADPH
OGG1 expression/GAPDH
Control group
Strawberry group
OGG-1
GAPDH

679 Figure 2

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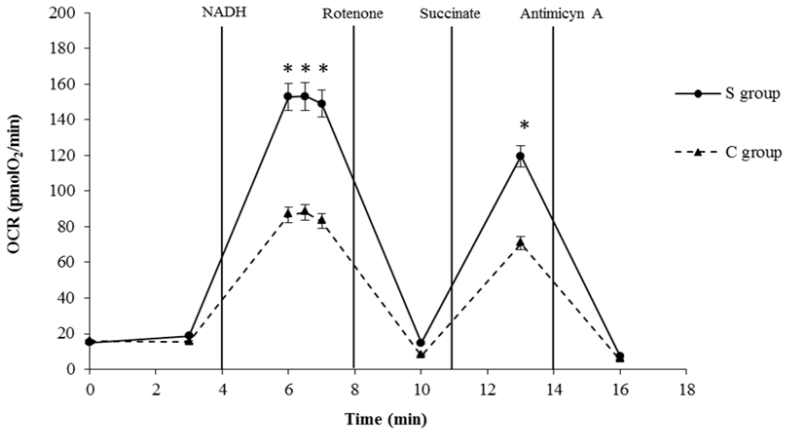
A



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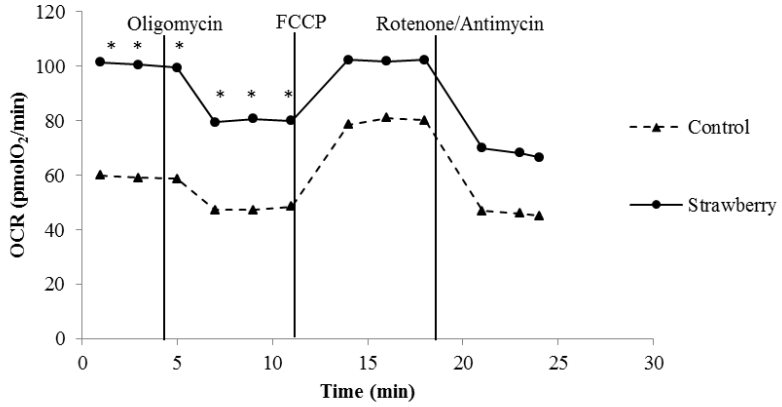

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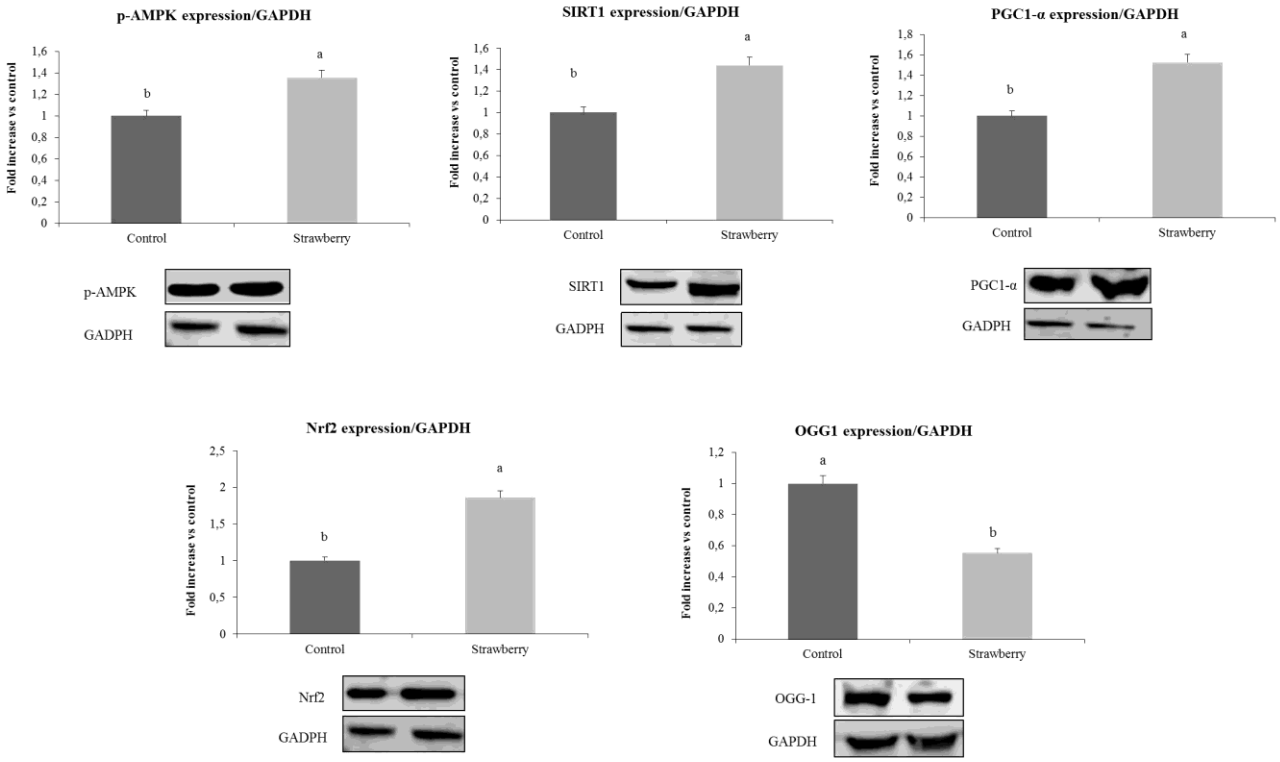
Figure 3


p-AMPK expression/GAPDH
Fold increase vs control
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Control
Strawberry
p-AMPK
GADPH
SIRT1 expression/GAPDH
Fold increase vs control
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Control
Strawberry
SIRT1
GADPH
PGC1-α expression/GAPDH
Fold increase vs control
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Nrf2 expression/GAPDH
Fold increase vs control
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OGG1 expression/GAPDH
Fold increase vs control
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734 Figure 4

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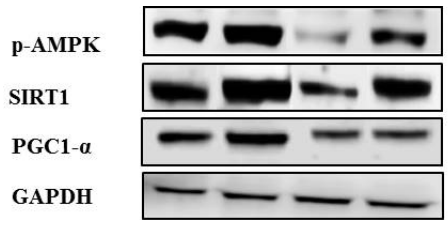

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