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Anti-inflammatory effect of strawberry extract against LPS-induced stress in RAW 264.7 macrophages

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ABSTRACT

A common denominator in the pathogenesis of most chronic inflammatory diseases is the involvement of oxidative stress, related to ROS production by all aerobic organisms. Dietary antioxidants from plant foods represent an efficient strategy to counteract this condition. The aim of the present study was to evaluate the protective effects of strawberry extracts on inflammatory status induced by *E. Coli* LPS on RAW 264.7 macrophages by measuring the main oxidative and inflammatory biomarkers and investigating the molecular pathways involved. Strawberry pre-treatment efficiently counteracted LPS-induced oxidative stress reducing the amount of ROS and nitrite production, stimulating endogenous antioxidant enzyme activities and enhancing protection against lipid, protein and DNA damage ($P < 0.05$). Strawberry pre-treatment exerted these protective effects primarily through the activation of the Nrf2 pathway, which is markedly AMPK-dependent and also by the modulation of the NF- κ B signalling pathway. Finally, an improvement in mitochondria functionality was also detected. The results obtained in this work highlight the health benefit of strawberries against inflammatory and oxidative stress in LPS-stimulated RAW 264.7 macrophages, investigating for the first time the possible involved molecular mechanisms

Introduction

An increasing number of epidemiological studies highlights a remarkable association between a diet rich in fruits and vegetables

and a lower incidence of different chronic pathologies, such as obesity, infections, cardiovascular and neurodegenerative diseases and cancer (Giampieri et al., 2014a; Tilman and Clark, 2014). A sustained pro-inflammatory state, characterized by excessive reactive oxygen species (ROS) production, is the common denominator in the development, progression, and complication of these diseases (Joseph et al., 2014). ROS in particular represent the major source of cellular damage and the main site of their production is mitochondria: in normal conditions these reactive species are detoxified by specified enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) or catalase

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. When these enzymes cannot transform ROS fast enough, oxidative damage occurs and accumulates in mitochondria, altering their metabolism and functionality (Forbes-Hernandez et al., 2016; Giampieri et al., 2016). Comprehension of the molecular and biochemical mechanisms that underlie the beneficial effects of a plant-based diet has encouraged different basic, clinical and epidemiological researches, also considering the remarkable socio-economic and public health impact of the common chronic inflammatory-related pathologies (Tulipani et al., 2009; Ezzati and Riboli, 2012). In recent decades, individual subgroups of fruits have been taken into account to facilitate the observation and promote their specific health benefits (Giampieri et al., 2012a). Among these, the strawberry represents one of the most consumed berries in Europe and is also an important source of bioactive compounds with antioxidant activity (Forbes-Hernandez et al., 2016). It is a relevant source of folate, is rich in vitamin C and contains various phytochemicals, which may greatly influence the nutritional qualities of this fruit. These compounds confer important antioxidant, anti-inflammatory, anti-cancer, and anti-neurodegenerative biological properties (Giampieri et al., 2014a; Afrin et al., 2016; Amatori et al., 2016; Aqilet al., 2016; Mazzoni et al., 2016). Only in the past few years several *in vitro* and *in vivo* studies considering the effects of strawberries on inflammatory status have been published, which investigate their possible role in the inhibition on pro-inflammatory cytokine production, such as tumor necrosis factor alpha (TNF- α) and interleukin 1-beta (IL-1 β) (Alarcón et al., 2015; Lee et al., 2014).

One common inflammatory agent used in different experimental models (Dong et al., 2015; Liao and Lin, 2015) is the endotoxin lipopolysaccharide (LPS), which represents an outer membrane structure and an important virulence factor of the cell wall of Gram-negative bacteria (Mayer et al., 1985; Giampieri et al., 2015). It induces the release of a large number of inflammatory cytokines, which play an important role in metabolic processes through linkage to the pathogen-sensing system; however their excessive production and secretion may result in septic shock, systemic inflammatory response syndrome, severe tissue damage and multiple organ dysfunction (Bosshart and Heinzlmann, 2007; Ryu et al., 2015). In this context, the aim of this work was to evaluate the protective effects of purified extracts from "Alba" strawberry cultivar on *E. Coli* LPS-induced inflammation in murine RAW 264.7 macrophage cell line. Macrophages play an important role in immune reactions and allergy and are considered to be the cells predominantly involved in the inflammatory response. Once activated they induce the expression of pro-inflammatory factors directly involved in the progression of the inflammatory condition and of its related diseases, representing one of the main *in vitro* models for cell culture studies (Suzuki et al., 2009; Zong et al., 2012). In our work, primarily the phytochemical and nutritional characterization of Alba strawberry extract was performed. In order to assess the effect of strawberry treatment on RAW 264.7 cells, in presence or absence of LPS, ROS intracellular production together with the level of the principal biomarkers related to inflammatory and oxidative stress were estimated (including nitrite and cytokine production). The activity and expression of the main antioxidant enzymes, such as damage to the principal biological macromolecules, were also tested. Finally, protein expressions were analysed to clarify the chief molecular pathways involved in LPS and strawberry mechanisms of action, and the oxygen consumption

rate (OCR) related to mitochondria functionality was studied after strawberry and LPS treatments. Strawberry pre-treatment should efficiently counteract LPS-induced damage reducing ROS and nitrite production, improving antioxidant defense and mitochondria functionality and lowering the main inflammatory markers by modulating specific molecular pathways. To the best of our knowledge this is the first study which investigates the molecular mechanisms that regulates the strawberry effects on LPS-induced damage in RAW 264.7 macrophages cell line.

Material and methods

All chemicals and reagents were bought from Sigma-Aldrich Chemical Company (Sigma-Aldrich, St. Luis, MO).

Strawberry fruit preparation and analysis

Ripe fruits of Alba strawberry cultivar were harvested from plants grown in an open experimental field for strawberry breeding and germplasm collection at the Azienda Agraria Didattico Sperimentale "P. Rosati" in Agugliano (Ancona, Italy). Within 2 h after harvest, whole fruits were stored at -20°C and submitted to methanolic extraction as previously described (Giampieri et al., 2014b). The total phenolic content (TPC) was determined by the Folin-Ciocalteu method (Slinkard and Singleton, 1977), total flavonoid content (TFC) by the aluminium chloride spectrophotometric method (Dewanto et al., 2002), while vitamin C (vit C) and folate content were analysed by the HPLC system (Jastrebova et al., 2003; Patring et al., 2005). Anthocyanins (ACYs) solid-phase extraction and HPLC-MS/MS analysis were performed as previously described (Giusti and Wrolstad, 2001). Total antioxidant capacity (TAC) was determined using Trolox Equivalent Antioxidant Capacity (TEAC) (Re et al., 1999), Ferric Reducing Antioxidant Power (FRAP) assays (Benzie and Strain, 1996) and the 2,2-DiPhenyl-1-Picrylhydrazyl free radical method (DPPH) (Prymont-Przyminska et al., 2014). Results were reported as mean value of three replicates \pm standard deviation (SD).

Cell culture and strawberry/LPS treatment

RAW 264.7 murine macrophage cell line was purchased from American Type Culture Collection (ATCC-TIB71). RAW macrophages were plated into T-75 flasks and cultured in Dulbecco's Modified Eagle Medium (DMEM) with high glucose, stable glutamine and sodium pyruvate, supplemented with 10% fetal bovine serum heat-inactivated (65°C for 20 min) and 1% penicillin-streptomycin antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin). Cells were maintained in HeraCell CO₂ incubator at 37°C with 5% CO₂ and the medium was changed every 2-3 days. All the tests and the different pellet preparations were conducted on cells between the 4th and the 6th passage. Strawberry extract was dried under vacuum to eliminate total methanol and was resuspended in DMEM to achieve the final concentration of 100 mg/mL. Cells were treated with (i) DMEM only (ctrl group), (ii) dried extract of Alba cultivar for 24 h (strw group), (iii) LPS (*Escherichia coli* serotype 055:B5) at 1 mg/mL for 24 h (LPS group) or (iv) dried strawberry extract for 24 h and then with LPS at 1 mg/mL for 24 h (strw + LPS group). The combination of dose/time strawberry and LPS treatments was established according to the MTT viability assay for cytotoxicity studies (data not shown).

TALI[®] ROS concentration assay

The determination of intracellular ROS levels was performed using the probe CellROX[®] Orange reagent (Invitrogen[™], Life

Techonoliges, Milan, Italy) according to the manufacturer's instructions. Briefly, on the first day of the assay 1.5×10^5 cells were seeded in a 6-well plate and allowed to adhere for 16 h. The cells were treated, the day after seeding, with different treatments previously indicated (i, ii, iii, iv). At the end of each treatment, the medium was removed and collected, then CellROX[®] Orange Reagent was directly added to 1 mL of complete medium at a 1:500 (v/v) dilution. Samples were incubated for 30 min at 37 °C, centrifuged at 320g once to remove medium and excess dye, and then resuspended in phosphate-buffered saline solution (PBS). After labeling with CellROX[®] Orange Reagent, cells were analysed with the Tali[®] Image-Based cytometer (Invitrogen™, Life Techonoliges, Milan, Italy) collecting 20-fields per sample. Control cells were used to determine baseline levels of intracellular ROS and to set the fluorescence threshold for the Tali[®] instrument. Each treatment was carried out in three replicates and the final results were expressed as fold increase compared to control.

Determination of nitrite production

Nitrite accumulation (NO₂⁻) in cell culture media was determined by the Griess method (Pekarova et al., 2009). Briefly, 1×10^6 cells were seeded in a T75 flask, allowed to adhere overnight, and then treated as previously indicated (i, ii, iii, iv). At the end of the different incubations in the CO₂ incubator at 37 °C with 5% CO₂, the different cell supernatants were collected, the samples (1 mL) were mixed with an equal volume of Griess reagent (1 mL of 1:1 0.1% naphthyl-ethylenediamine and 1% sulfanilamide in 5% phosphoric acid) in a tube, and incubated in the dark for 10 min at room temperature. Then the absorbance of the reaction mixture was measured at 540 nm on a microplate reader (ThermoScientific Multiskan EX). The NO₂⁻ concentration was determined using a sodium nitrite (NaNO₂) standard curve (working range: 0.1e6.25 mM). Each treatment was carried out in three replicates and the final results were expressed as nmol NO₂⁻/mg protein.

Antioxidant enzyme activities and biomarkers of oxidative stress

RAW macrophages, treated as previously indicated (i, ii, iii, iv), were incubated on ice for 5 min with RIPA buffer (Sigma-Aldrich, Milan, Italy) and the obtained lysate stored at -80 °C until analyses (Doktorovová et al., 2014). In cellular lysates the antioxidant enzyme activities of GPx, glutathione Reductase (GR), glutathione Transferase (GST), SOD and catalase were measured spectrophotometrically, by following the specific procedure as previously described, with brief modifications (Giampieri et al., 2016). The results were expressed as nmole NADPH oxidized/mg protein/min for GPx and GR; nmole 1-chloro-2,4-dinitro benzene (CDNB)-reduced glutathione (GSH) conjugate/mg protein/min for GST and as U/mg prot/min for SOD and catalase, where one unit of SOD is defined as the amount of enzyme that gave 50% inhibition of nitroblue tetrazolium reduction, and one unit of catalase was defined as the amount of enzyme that decomposed 1 mmol of hydrogen peroxide (H₂O₂) (Camera et al., 2009). Protein carbonyl content and GSH levels and thiobarbituric acid-reactive substance (TBARS) production assay were quantified as biomarkers of protein and lipid oxidative damage according to standardized methods (Alvarez-Suarez et al., 2016; Giampieri et al., 2016). In the case of carbonyl content and GSH the results were expressed as nmol/mg prot, while for TBARS levels as nmol/100 mg prot. Each sample was analysed in three replicates.

1.1. Immunoblotting analysis for strawberry and LPS related molecular pathways

RAW macrophages were treated as previously described (i, ii, iii, iv), collected, washed with PBS, lysed in 100 mL lysis buffer (120 mmol/L NaCl, 40 mmol/L Tris [pH 8], 0.1% NP40) containing protease and phosphatase inhibitor cocktails (Roche Diagnostics, Mannheim, Germany) and centrifuged at 13 000g for 15 min. Immunoblotting assay was performed as previously described, with brief modifications (Alvarez-Suarez et al., 2016). Proteins from cell supernatants were then charged on a 10e15% dodecyl sulfate-polyacrylamide running gel (Bio-Rad, Hercules, CA, USA). After the electrophoresis run, proteins were transferred from the gel to nitrocellulose membranes, using a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Inc., Hercules, CA) and then membranes were blocked with TBS-T containing 5% non-fat milk for 1 h at room temperature. Membranes were incubated at 4 °C overnight with the primary antibody solution, diluted at 1:500 (v/v), specific for the detection of: nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), phosphorylated nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, alpha (pIkBa), IL-1b, TNF-α, interleukin 6 (IL-6), interleukin 10 (IL-10), inducible nitric oxide synthase (iNOS), nuclear factor E2-related factor 2 (Nrf2), catalase, SOD, heme oxygenase 1 (HO-1), phosphorylated 5^l AMP-activated protein kinase (p-AMPK), sirtuin 1 (SIRT1), peroxisome proliferator-activated receptor coactivator 1, alpha (PGC1α) and 8-Oxoguanine glycosylase (OGG1) (Santa Cruz Biotechnology, Dallas, Texas and Bioss Inc., Woburn, Massachusetts). GAPDH protein was used for the measurement of the amount of protein analysed. Then membranes were probed for 1 h at room temperature with their specific alkaline phosphatase conjugated secondary antibodies (1:80 000 dilution v/v) (Santa Cruz Biotechnology, Dallas, Texas). Immunolabeled proteins were visualized using a chemiluminescence method (C-DiGit Blot Scanner, LI-COR, Bad Homburg, Germany). Quantification of gene expression was carried out using the software provided by the manufacturer of the Blot Scanner (Image Studio 3.1) and data were expressed as fold increase compared to control. The protein was determined by the Bradford method (Bradford, 1976). Each gene expression was performed in three replicates.

Determination of mitochondrial respiration rate in cells

OCR in mitochondria of RAW macrophages was measured in real-time using a XF-24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica MA, USA) as previously reported (Richardson et al., 2012). 3×10^4 cells were seeded for 16 h in the XF-24 plate and treated as previously indicated (i, ii, iii, iv). At the end of the treatment the medium was replaced with 450 mL/well of XF-24 running media (Seahorse Bioscience, Billerica MA, USA), supplemented with 25 mM glucose, 2 mM glutamine, 1 mM sodium pyruvate, without serum and pre-incubated at 37 °C for 20 min in the XF Prep Station incubator (Seahorse Bioscience, Billerica MA, USA) in the absence of CO₂. The plate was then transferred to the XF-24 Extracellular Flux Analyzer and after an OCR baseline measurement a profiling of mitochondrial function was performed by sequential injection of four compounds that affect bioenergetics, as follows: 55 mL of oligomycin (Sigma-Aldrich, Milan, Italy) (1 mg/mL), 61 mL 231 of 2,4-Dinitrophenol (2,4 DNP) (1 mM), and 68 mL of antimycin A/rotenone (Sigma-Aldrich, Milan, Italy) (10 mM/1 mM) at injection in port C. Each treatment was carried out in three replicates and the final results were expressed as pmol of O₂ consumed per 10⁵ cells per minute (pmol O₂/10⁵ cells/min). Moreover the Maximal Respiratory Capacity value of each treatment was calculated with the following equation:

Maximal Respiratory Capacity $\frac{1}{4} \times 2 \times 4$ —dinitrophenol OCR value
—antimycin A = rotenone OCR value $\times 6$

Also in this case, the final results of the three replicates were expressed as pmol O_2 /10⁵ cells/min.

Statistical analysis

Statistical analyses were performed using STATISTICA software (Statsoft Inc., Tulsa, OK, USA). Data were subjected to one-way ANOVA analysis of variance for mean comparison, and significant differences among different treatments were calculated according to HSD Tukey's multiple range test. Data are reported as mean \pm SD. Differences at $P < 0.05$ were considered statistically significant.

Results and discussion

Strawberry fruit analysis

As reported in Table 1, Alba strawberry extract possessed a good content of vit C (0.58 mg vit C/g fresh weight (FW)), polyphenols (2.52 mg Gallic Acid Equivalent/g FW) and flavonoids (0.66 mg Catechin Equivalent/g FW). HPLC-MS/MS analysis allowed to detect five anthocyanin pigments, with Pg 3-glucoside (39.74 mg/100 g FW) and Pg 3-malonylglucoside (6.69 mg/100 g FW) being the most representative anthocyanin strawberry components (Table 1). According to the methods applied, strawberry extract showed TAC value of 22.64, 22.85 and 7.71 mmol Trolox Equivalent/g FW for TEAC, FRAP and DPPH, respectively (Table 1). These results were in line with those previously obtained for other strawberry varieties, confirming the TAC values of this cultivar (Ariza et al., 2015; Giampieri et al., 2012b, 2014b). Finally, in the Alba cultivar 0.06 mg of 5-methyltetrahydrofolic acid/g FW and 0.99 mg of folic acid calcium salt hydrate/g FW were quantified (Table 1): these folate compounds represent, together with ascorbic acid, phenols and ellagitannins, the elements responsible for several beneficial actions of strawberries in human health, as previously described by a number of authors (Buendía et al., 2010; Tulipani et al., 2008).

Strawberry treatment reduced ROS and NO₂⁻ accumulation

The interaction of the cellular immune system with endogenous or exogenous inflammatory stimuli determines the generation of ROS, that can result in hyperactivation of inflammatory responses leading to tissue damage and oxidative stress phenomena (Schieber and Chandel, 2014). For this reason the measurement of ROS intracellular production could represent a very useful parameter to quantify oxidative stress induced by LPS. In the present work, the protective effect of strawberry extract on LPS-induced ROS production was demonstrated (Fig. 1a). In RAW macrophages, strawberry treatment showed a reduction of ROS amount compared to the control group. In cells pre-treated with strawberry and stressed with LPS, a significant decrease in the amount of ROS was obtained compared to the LPS group ($P < 0.05$). The results obtained for the first time with strawberries were in line with those found by several authors, which tested the efficacy of different bioactive compounds against LPS-induced damage in macrophage cell models (Choi et al., 2015; Mo et al., 2014). As previously reported, LPS can lead to the release of pro-inflammatory cytokines and in turn activate a second level of inflammatory cascades including cytokines, lipid mediators and adhesion molecules such as nitric oxide (NO) (Zong et al., 2012). NO is an important regulatory and effector molecule with different biological functions and it is

Table 1

Nutrient composition, phytochemical content and antioxidant capacity of Alba strawberry extract. Data are presented as mean value \pm SD.

Parameter	Quantification
vit C (mg vit C/g FW)	0.58 \pm 0.02
TPC (mg GAEq/g FW)	2.52 \pm 0.01
TFC (mg CEq/g FW)	0.66 \pm 0.01
ACYs (mg/100g FW)	
Cy-3-glucoside	3.11 \pm 0.03
Pg 3-glucoside	39.74 \pm 0.13
Pg 3-rutinoside	3.87 \pm 0.16
Pg 3-malonylglucoside	6.69 \pm 0.04
Pg 3-acetylglucoside	0.39 \pm 0.01
TAC (mmol Teq/g FW)	
TEAC	22.64 \pm 0.49
FRAP	22.85 \pm 0.39
DPPH	7.71 \pm 0.32
Folate (mg folate/g FW)	
5-methyltetrahydrofolic acid	0.06 \pm 0.01
folic acid calcium salt hydrate	0.99 \pm 0.09

considered a fundamental component involved in many physiological and pathophysiological processes. As shown in Fig. 1b, strawberry extract was able to reduce the level of NO derivative nitrite production compared to the untreated RAW macrophages. LPS-treatment significantly increased ($P < 0.05$) the NO₂⁻ level, which was efficiently counteracted with strawberry pre-treatment, restoring values similar to the control group. These results were comparable with those previously obtained by other authors on RAW macrophages, which represent the best-studied example of the regulation of NO₂⁻ production (Mo et al., 2014; Park et al., 2005, 2011; Zhang et al., 2012).

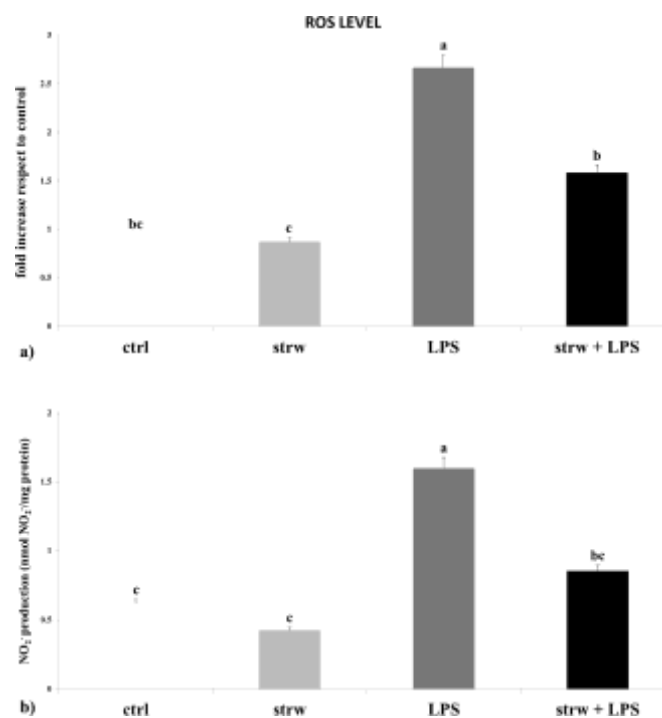


Fig. 1. Cell ROS level (a) and NO₂⁻ levels (b) in RAW macrophages subjected to the different treatments (ctrl, strw, LPS, strw + LPS). Data are expressed as mean values \pm SD. Columns with different superscript letters are significantly different ($P < 0.05$).

Strawberry treatment stimulated the endogenous antioxidant defence system and reduced biomarkers of oxidative stress

Earlier studies indicate that plant bioactive compounds, such as polyphenols, were able to modulate the activity of diverse antioxidant enzymes (GPx, GR, GST, SOD, catalase), reducing the damage induced by LPS and other different stressors, and restoring conditions similar to control levels in RAW macrophages (Park et al., 2005, 2011). In our work strawberry treatment was able to increase the enzymatic activities of GPx, GR and GST compared to the control group, while with LPS treatment, a significant reduction ($P < 0.05$) was registered for all the tested enzymes (Fig. 2a). Enzymatic activities were improved with strawberry pre-treatment and reported values statistically similar to the control group. SOD and catalase activities presented similar results (Fig. 2b). Strawberry treatment increased these enzymatic activities compared to the control group. On the contrary, incubation with LPS determined a significant reduction of both enzyme activities ($P < 0.05$) which were efficiently improved by strawberry pre-treatment. LPS significantly decreased all the enzymatic activities, due to the ROS and NO production which it provoked (Park et al., 2005), while strawberry extract counteracted these effects, suggesting for the first time that the increase of endogenous antioxidant enzyme activities might be one of the important mechanisms of strawberries against oxidative stress damage in LPS-induced stress in RAW 264.7 macrophages. Moreover, the tendency of strawberries to increase and subsequently recover the basal level of these enzymes could be interpreted as the consequence of the initial alteration of cellular steady-state function followed by stress-induction phenomena tending to re-establish the primitive situation (Armeni et al., 2003; Quiles et al., 2001). We also showed that the antioxidant capacity

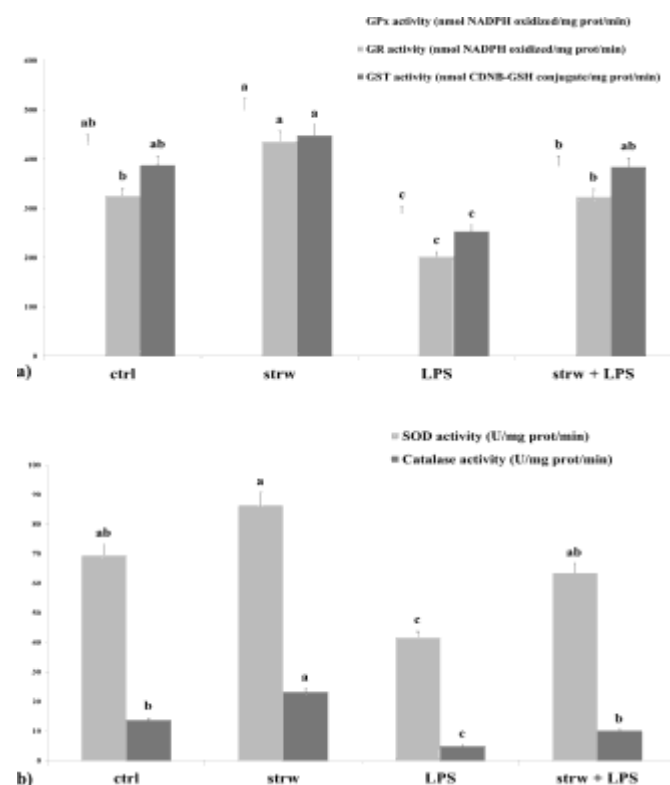


Fig. 2. GPx, GR, GST activity (a) and SOD, catalase activity (b) in RAW macrophages subjected to the different treatments (ctrl, strw, LPS, strw + LPS). Data are expressed as mean values \pm SD. Columns belonging to the same set of data with different superscript letters are significantly different ($P < 0.05$).

provided by strawberries resulted also in enhanced protection against lipid and protein damage induced by LPS-oxidative stress. Lipid peroxidation is a free radical-mediated chain reaction involving several types of free radicals, which could be arrested through enzymatic means or by free radical scavenging by antioxidants (Babujanathan et al., 2011). Protein oxidation, measured as an increase in carbonyl groups, was shown to be an early event in oxidative stress *in vitro* and was used to highlight accumulation of oxidative damage to proteins over the longer term, as in aging studies (Ciolino and Levine, 1997). GSH level is another marker of protein oxidation. It plays an important role in maintaining the normal reduced state of cells, counteracting the harmful effects of oxidative stress and detoxifying xenobiotics (DeLeve and Kaplowitz, 1990). Our results demonstrated that strawberry extracts significantly reduced the TBARS level compared to the control group ($P < 0.05$); on the contrary, LPS-treatment considerably raised TBARS value ($P < 0.05$), which was efficiently counteracted by Alba pre-treatment, restoring values similar to those of the control group (Fig. 3). Analogous results were obtained in relation to protein oxidation markers (Fig. 3). Strawberry treatments ameliorated GSH levels and protein carbonyl content compared to untreated cells while cells treated with LPS showed considerable protein damage ($P < 0.05$), as evidenced by the lowest value of GSH and the highest value of carbonyl content. Pre-treatment with Alba extracts improved the levels of LPS-induced protein damage: values statistically similar to the control groups were obtained, both for GSH and carbonyl groups. The ability of strawberry treatment in efficiently counteracting lipid and protein damage could be related to the capacity of strawberry antioxidant compounds in scavenging free radicals and in activating antioxidant enzymes.

Strawberry treatment positively affected inflammatory and antioxidant related-pathways

The evaluation of the anti-inflammatory and antioxidant effect of strawberries on different molecular pathways, involved in cellular response, was performed through the protein expression analysis. NF- κ B is a key transcriptional regulator of the inflammatory response, and plays an important role in the development of the inflammatory process and cellular injuries. It is activated in response to different extracellular stimuli, including oxidative stress, LPS and cytokines (Park et al., 2005). NF- κ B exists ubiquitously in the cytoplasm as a heterodimer consisting of p50 and p65 as an inactive form by creating a complex with the inhibitor protein

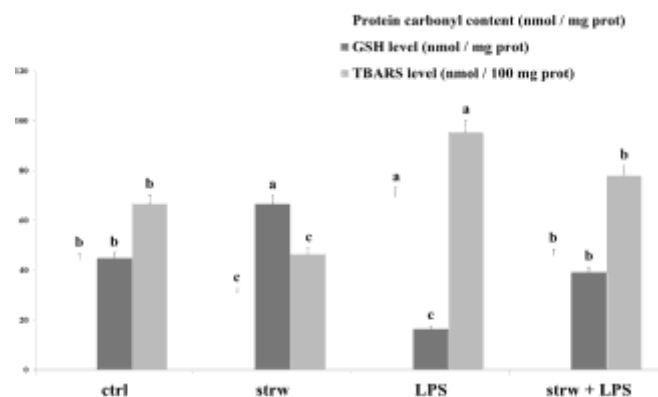


Fig. 3. Protein and lipid oxidation markers (protein carbonyl content, GSH and TBARS levels) in RAW macrophages subjected to the different treatments (ctrl, strw, LPS, strw + LPS). Data are expressed as mean values \pm SD. Columns belonging to the same set of data with different superscript letters are significantly different ($P < 0.05$).

IkBa. In response to inflammatory stimuli IkBa is phosphorylated (pIkBa) and released from NF-kB which, once activated, migrates to the nucleus and up-regulates inflammation-related genes such as iNOS and pro and anti-inflammatory cytokines (Park et al., 2011). Earlier studies investigated the role of different bioactive compounds on LPS-mediated NF-kB response in RAW macrophages (Park et al., 2005, 2011). In our work, strawberry treatments reduced the level of the tested inflammatory markers (NF-kB, pIkBa, TNF- α , IL-1 β , IL-6 and iNOS) (Figs. 4 and 5). The expression of all the inflammatory proteins was considerably increased by the LPS-treatment ($P < 0.05$), while pre-treatment with Alba extracts was able to reduce the inflammatory marker levels ($P < 0.05$), restoring values similar to the control group (Figs. 4 and 5). As expected, pIkBa expression followed the same trend of NF-kB. Moreover, the strong association between NO production and iNOS expression was detected, as shown by other authors (Lee et al., 2014; Mo et al., 2014). A different trend was registered for IL-10: in this case, an increase of the protein expression was observed with strawberry, LPS and strawberry + LPS treatments, with a significant rise ($P < 0.05$), compared to untreated cells (Fig. 5). These results are in line with the data obtained in other studies performed on RAW macrophages, in which the expression of pro and anti-inflammatory cytokines induced by LPS was improved by strawberry (Liu and Lin, 2012, 2013) and other different bioactive compounds (Lee et al., 2014; Mo et al., 2014; Zong et al., 2012).

Nrf2 is a basic leucine zipper transcription factor that binds to the promoter sequence “antioxidant responsive element” (ARE) leading to coordinated up-regulation of ARE driven detoxification and antioxidant genes. Since the expression of a wide array of antioxidant and detoxification genes are positively regulated by the ARE sequence, Nrf2 may serve as a main regulator of the ARE-driven cellular defence system against oxidative stress (Mo et al., 2014). The antioxidant properties of ARE enzymes are related to the inhibition of formation of adhesion molecules, such as NO, and reduction of oxidative stress (Lee et al., 2014). In our work, strawberry extracts increased the level of Nrf2; a similar effect was obtained with LPS and with strawberry + LPS treatments ($P < 0.05$) (Fig. 6). An analogous trend was detected with the other genes investigated. In the case of SOD (Fig. 6), a significant increase compared to untreated cells was obtained with strawberry treatment and with Alba pre-treatment before LPS incubation ($P < 0.05$). Similar results were obtained for catalase and HO-1 (Fig. 6).

Contrarily to the results of enzyme activities, the expression of SOD, catalase and HO-1 were up-regulated not only by strawberry treatment, but also after LPS supplementation, as indicated by different authors (Lee et al., 2014; Mo et al., 2014). These antioxidant enzyme expressions reached the highest value with the concomitant action of strawberry and LPS treatment. This situation, apparently controversial, could be explained if it is taken into account that the oxidative stress induced by LPS treatment stimulated RAW macrophages to protect themselves from this oxidative insult, augmenting the synthesis of their antioxidant defences. For this reason the cells upregulated the expression of genes related to antioxidant defence. The link between Nrf2 and ARE genes in LPS-stimulated cells was highlighted, as indicated in recent works (Lee et al., 2014; Mo et al., 2014).

Finally, the expressions of proteins related to the AMPK pathway were also investigated. The contribution of cellular energy metabolism in inflammatory response has recently become an area of wide interest, although the exact mechanisms are still poorly understood (Mo et al., 2014). AMPK is a sensor of intracellular energy status and represents an attractive target for inflammation control. Emerging evidence shows that AMPK activation can decrease the oxidative stress and inhibit inflammation, serving as a potential target to treat inflammation-related disorders (Mo et al., 2014; Zong et al., 2012). Mechanistic connections between AMPK and inflammation have been limited to links with NF-kB pathway (Mo et al., 2014). It has been shown in fact that chemical activators of AMPK decrease NF-kB-mediated transcription, and that constitutively activate AMPK suppressed NF-kB signalling and fatty acid-induced inflammation in macrophages, although NF-kB subunits are not the direct targets of AMPK (Mo et al., 2014). Consistent with the unequivocal action of Nrf2-pathway on ROS clearance and the suppression of inflammation, the potential for the crosstalk between Nrf2 and AMPK pathways has been also noted (Mo et al., 2014). Indeed, previous works demonstrated that different polyphenols were able to activate ARE sequences through the activation of Nrf2 pathway, positively affecting the expression of antioxidant enzymes (Li et al., 2016; Serra et al., 2016). On the other hand, Nrf2 pathway could also be stimulated indirectly through an AMPK-dependent mechanism. AMPK activation increased the expression of SIRT1 and consequently of PGC1 α , which is directly correlated to the enhancement of Nrf2 protein level (Iwabu et al., 2010). This hypothesis was confirmed in recent studies conducted on different

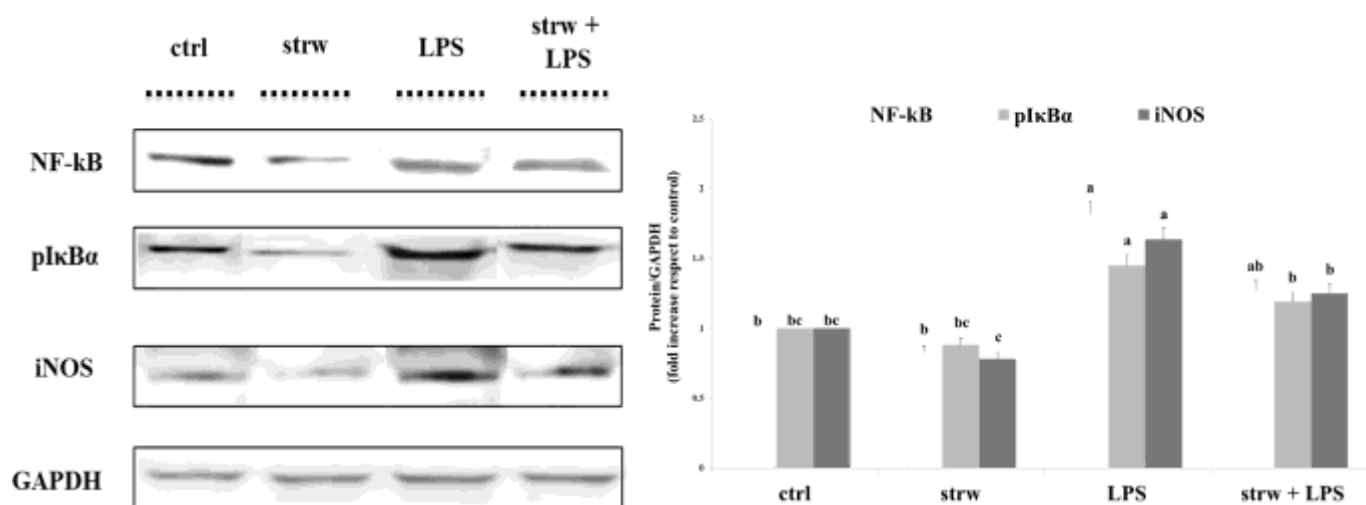


Fig. 4. Levels of inflammatory markers (NF-kB, pIkBa and iNOS) in RAW macrophages subjected to the different treatments (ctrl, strw, LPS, strw + LPS). Data are expressed as mean values \pm SD. Columns belonging to the same set of data with different superscript letters are significantly different ($P < 0.05$).

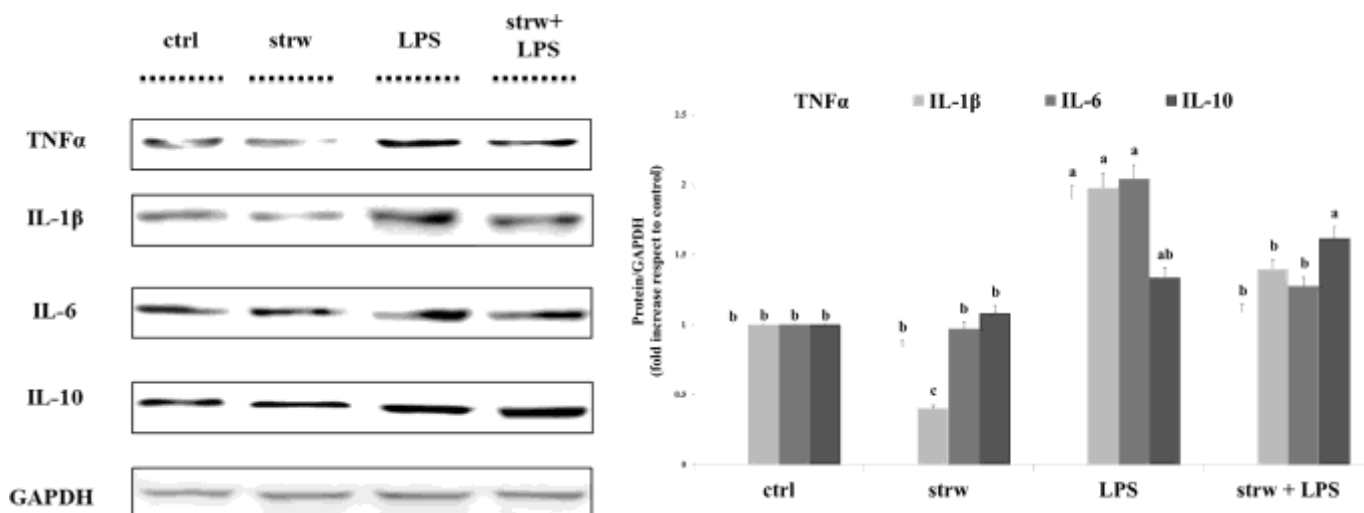


Fig. 5. Levels of inflammatory markers (TNF- α , IL-1 β , IL-6 and IL-10) in RAW macrophages subjected to the different treatments (ctrl, strw, LPS, strw + LPS). Data are expressed as mean values \pm SD. Columns belonging to the same set of data with different superscript letters are significantly different ($P < 0.05$).

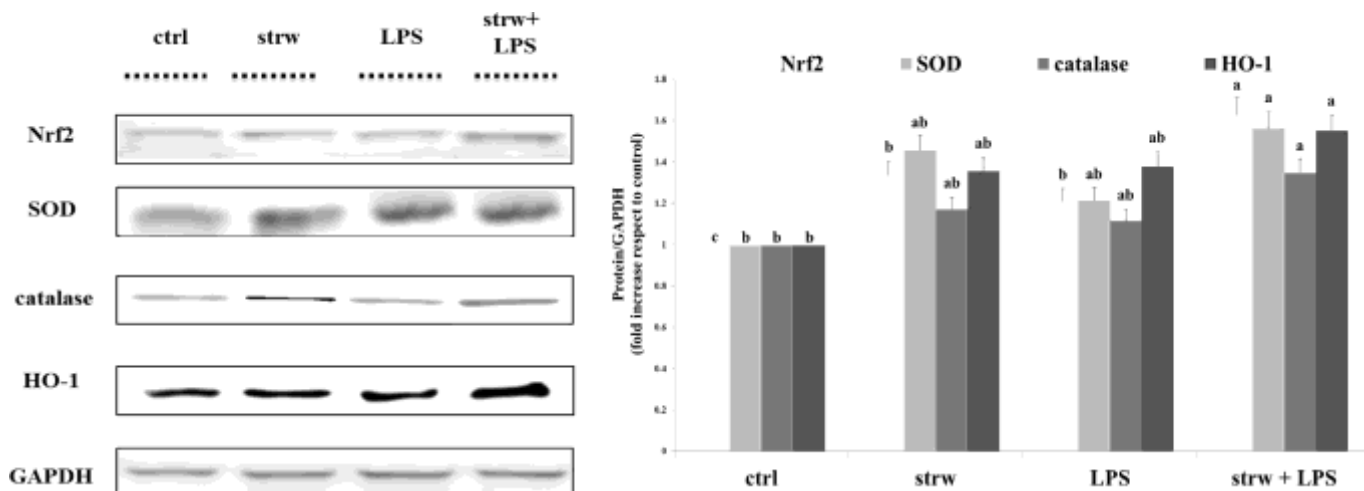


Fig. 6. Level of proteins related to antioxidant response (Nrf2, SOD, catalase, HO-1) in RAW macrophages subjected to the different treatments (ctrl, strw, LPS, strw + LPS). Data are expressed as mean values \pm SD. Columns belonging to the same set of data with different superscript letters are significantly different ($P < 0.05$).

dietary polyphenols (Bruckbauer and Zemel, 2014; Yun et al., 2014) even if to the best of our knowledge there are very few studies on berries (Ou et al., 2011; Eid et al., 2014) and none on strawberries. For this reason we decided to investigate the potential role of a functional interaction between Nrf2 and AMPK pathways. LPS and strawberry treatments increased the levels of pAMPK, the activated form of AMPK (Fig. 7): in this case strawberry extracts significantly increased this protein expression before the LPS-treatment ($P < 0.05$). These results are of great importance and innovation because they demonstrate a direct effect of strawberry, and especially LPS, treatments on pAMPK expression and are also in agreement with the few previous findings (Mo et al., 2014; Zong et al., 2012). The results obtained with SIRT1 were similar to pAMPK (Fig. 7): strawberry pre-treatment remarkably increased protein expression alone and in combination with LPS ($P < 0.05$). Taking into account PGC1 α level (Fig. 7), a significant increase of gene expression compared to the untreated group was obtained with strawberry extracts also before LPS-treatment ($P < 0.05$). Our findings are in accordance with previous results obtained on different cellular models (Han et al., 2012; Rayamajhi et al., 2013).

In cellular compartments, mitochondrion and nucleus, two major targets of oxidative stress, contain a variety of enzymes to repair oxidant-induced DNA modifications. Basically, DNA damage most likely occurs when the endogenous antioxidant network and DNA repair systems are flooded (Häcker et al., 2006). The expression level of 8-Oxo-7,8-dihydro-2-deoxyguanosine in DNA reflects its rate of generation and repair by OGG1 activity, which could represent a sensitive marker of DNA damage. Strawberry extracts reduced OGG1 protein expression; on the contrary LPS-treatment remarkably increased the level of DNA damage ($P < 0.05$), which was efficiently counteracted by pre-incubation with strawberries (Fig. 8).

All the collected evidence underlines, for the first time, that strawberry extracts were able to counteract the LPS-mediated inflammatory response, acting on the AMPK related pathways. The increment of pAMPK expression improved the Nrf2 cascade signalling, also stimulated by LPS-mediated ROS production, which leads to the increase of ARE-antioxidant enzyme expression. The augmentation of antioxidant defence efficiently reduced the inflammatory damage created by endotoxin LPS, through the

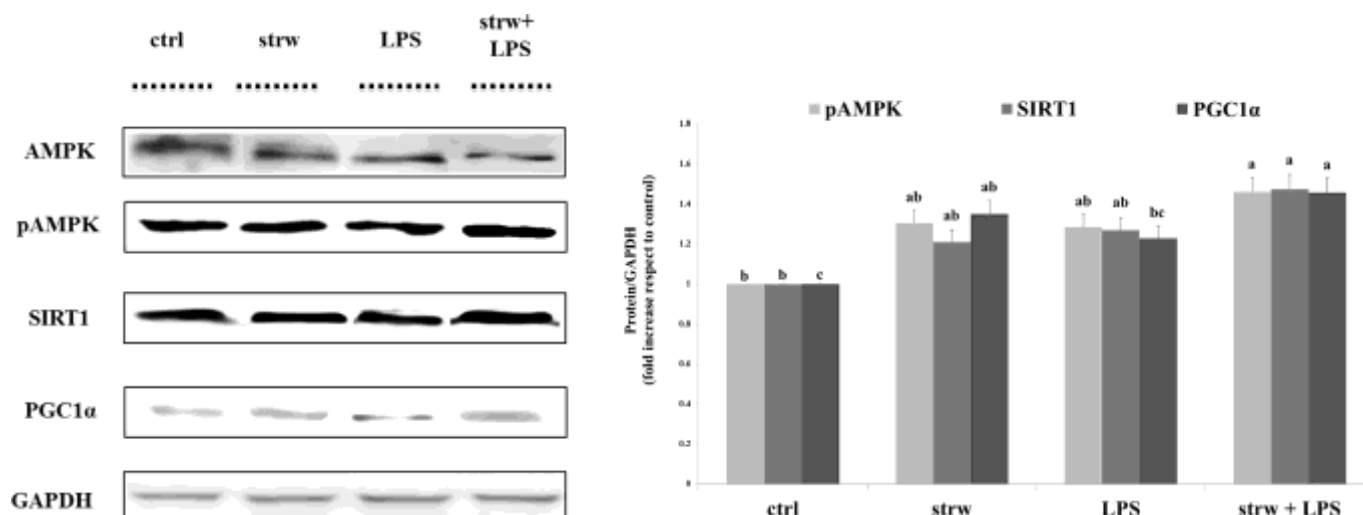


Fig. 7. Level of proteins related to AMPK pathway (p-AMPK, SIRT1, PGC1α) in RAW macrophages subjected to the different treatments (ctrl, strw, LPS, strw + LPS). Data are expressed as mean values \pm SD. Columns belonging to the same set of data with different superscript letters are significantly different ($P < 0.05$).

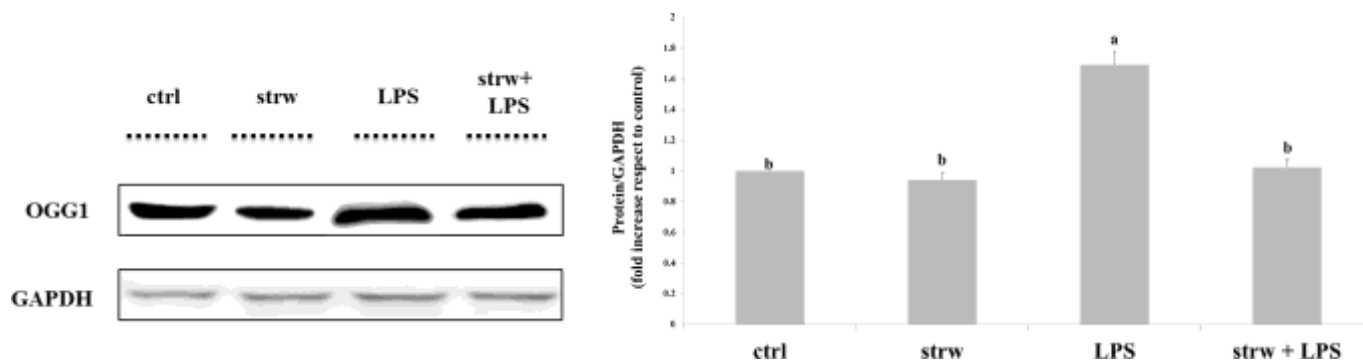


Fig. 8. Level of proteins related to DNA damage level (OGG1) in RAW macrophages subjected to the different treatments (ctrl, strw, LPS, strw + LPS). Data are expressed as mean values \pm SD. Columns with different superscript letters are significantly different ($P < 0.05$).

modulation of NF- κ B pathways and the reduction of NO $_2$ and inflammatory cytokine production.

Effect of strawberry treatment on mitochondrial respiration rate

The most important site of ROS production is represented by the electron transport chain (ETC) in the mitochondria (Forbes-Hernández et al., 2014). In this process, in fact, a small percentage of electrons directly reacts with oxygen, determining the formation of ROS as secondary ETC products (Forbes-Hernández et al., 2014). In this context we investigated the implication of mitochondria dysfunction in RAW macrophages, after strawberry and LPS-treatment, through OCR measurement. Basal respiration is predominantly controlled by the parallel re-entry pathways through the ATP synthase and proton leak. Addition of oligomycin blocks the ATP synthase and residual respiration is due to the proton leak. The decrease compared to basal respiration provides the coupling efficiency. The addition of a carefully calibrated concentration of the protonophore 2,4 DNP introduces a high artificial proton conductance into the membrane. This maximal respiration is now controlled by electron transport chain activity and/or substrate delivery. The increased respiratory capacity above basal respiration provides the maximal respiratory capacity. Finally, ETC inhibitors are added: antimycin A/rotenone that block complex III and I

respectively; in this way any residual respiration is non-mitochondrial and needs to be subtracted from the other rates. Fig. 9a shows the trend of the different groups, in function of the different inhibitors applied. Starting from the baseline values of OCR, LPS treatment increased oxygen consumption compared to the control group. Similarly, the strawberry treatment improved mitochondrial respiration increasing the OCR level, compared to untreated cells. Moreover, strawberry extracts were able to enhance the positive effect exerted by LPS, increasing the OCR value. With regard to the maximal respiratory capacity, untreated RAW macrophages showed the lowest values, while strawberry treatments significantly increased this rate ($P < 0.05$). Also LPS-incubation determined a remarkable improvement of maximal respiratory capacity ($P < 0.05$), this result was further incremented by pre-treatment with strawberries (Fig. 9b). These data appear to be contradictory with the ROS values which were high in the LPS group: in this case the LPS-treatment in fact determined an increase of oxygen consumption which was further enhanced by strawberry extracts. To our knowledge, there are no published studies that underline the effect of LPS-treatment on mitochondrial functionality of RAW macrophages, with particular attention to the OCR results. One possible explanation could be related to the stimulatory effect exerted by this endotoxin on RAW macrophages (Zhang et al., 2012), which could produce an increase of oxygen consumption, despite the ROS results. For this reason further

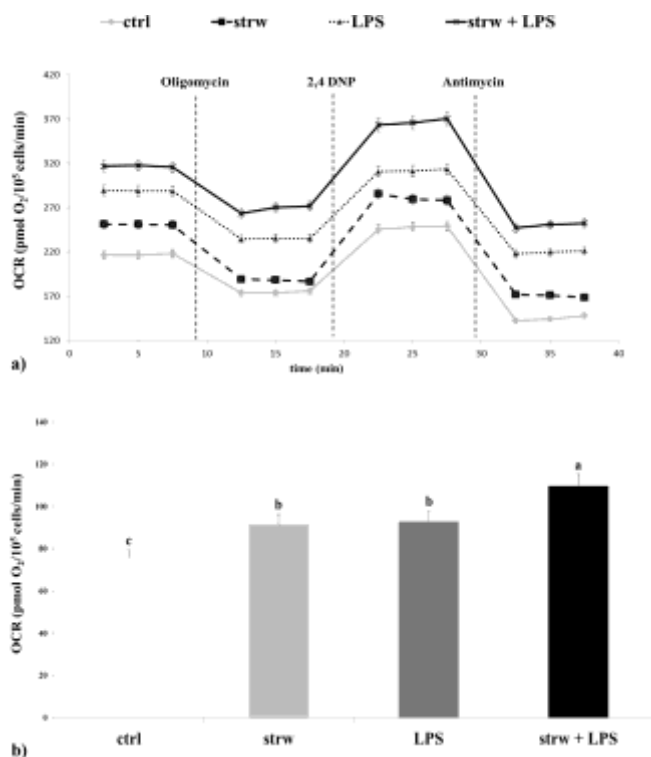


Fig. 9. OCR value (a) and maximal respiratory capacity values (b) of RAW macrophages subjected to the different treatments (ctrl, strw, LPS, strw + LPS). Mitochondria oxygen consumption was monitored with sequential injection of oligomycin, 2,4-DNP and antimycin A/rotenone at the indicated time points into each well, after baseline rate measurement. Data are expressed as mean values \pm SD. Columns with different superscript letters are significantly different ($P < 0.05$).

studies are required to clarify this apparently contradictory aspect.

2. Conclusions

Based on the innovative results of this study, we propose that the anti-inflammatory role of strawberries is primarily involved in the activation of the Nrf2-AMPK pathway, which increases the antioxidant defences and protects against oxidative stress damage. However, as most cell culture studies our work presents some limitations. Factors such as test sample concentrations, stability and/or potential transformation of phenolic compounds, cell line specificity, duration of cell exposure to treatment samples, generation of artefacts by phenolic compounds in cell culture media, as well as other potential reactions should be considered when evaluating cell culture experimental results. Nevertheless, our studies provide preliminary data related to the ability of strawberry extract to counteract the stress induced by LPS in RAW macrophages. Because extrapolations cannot be made between cell culture studies to humans, future animal and human studies should be designed to (i) investigate the potential of this berry for the prevention and treatment of inflammatory conditions, (ii) characterize the bioactive compounds which play a fundamental role against oxidative stress and (iii) completely understand the molecular pathways involved in the strawberry-mediated anti-inflammatory response.

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