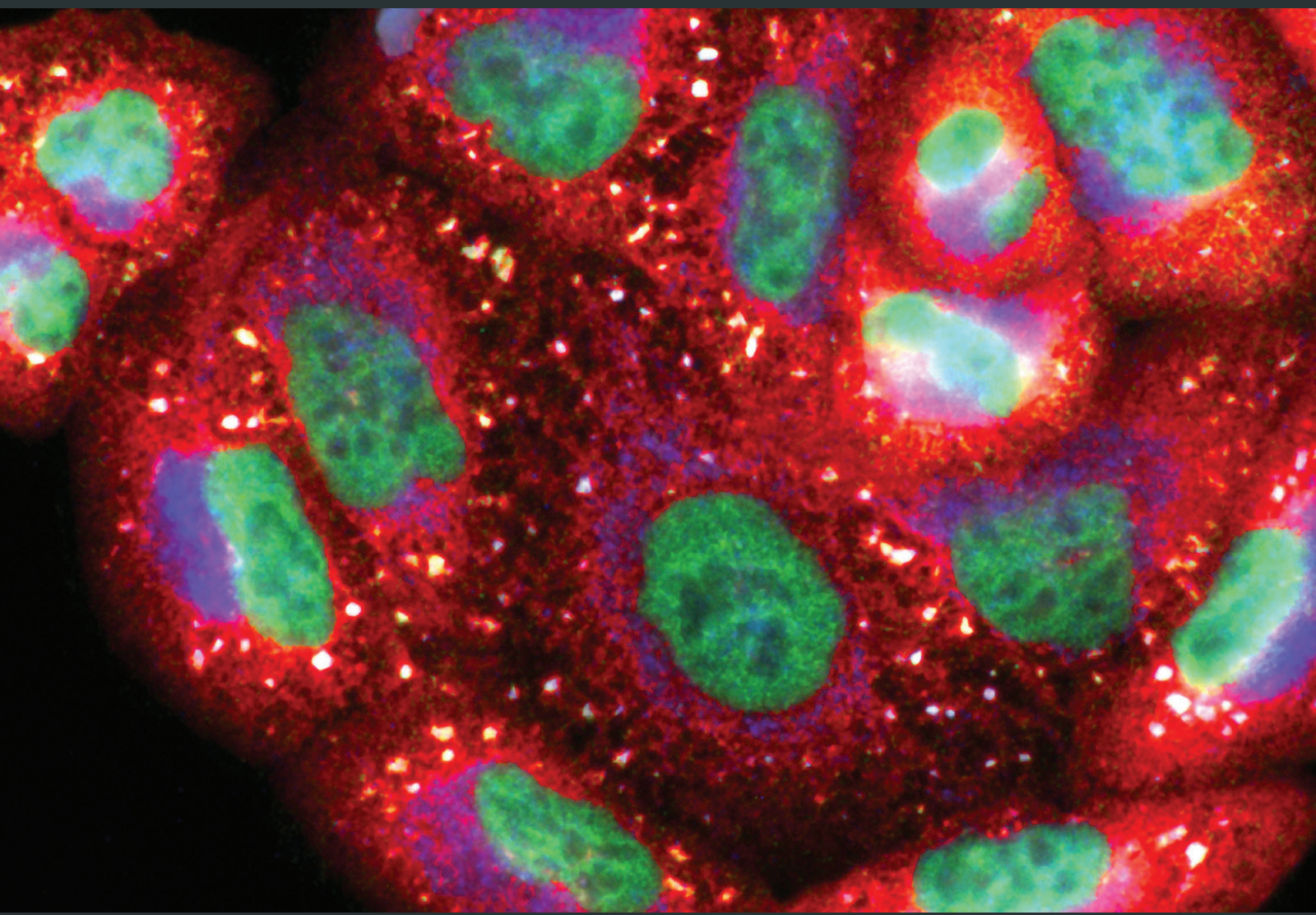
Oxidative Medicine and Cellular Longevity

Biomarkers of Oxidative Stress in Experimental Models and Human Studies with Nutraceuticals: Measurement, Interpretation, and Significance

Guest Editors: Ilaria Peluso, Maura Palmery, Jara Pérez-Jiménez, and Gregor Drummen



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Editorial Board

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 6159810, 2 pages
http://dx.doi.org/10.1155/2016/6159810

Editorial

Biomarkers of Oxidative Stress in Experimental Models and Human Studies with Nutraceuticals: Measurement, Interpretation, and Significance

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Received 24 December 2015; Accepted 24 December 2015

Oxidative stress, the imbalance between reactive oxygen species (ROS) formation and enzymatic and nonenzymatic antioxidants, is involved in the pathogenesis and progression of many malignant, degenerative, and ageing-associated diseases. Most importantly, the chronic low grade inflammation associated with metabolic syndrome is characterized by a chronic oxidative stress condition and increased levels of cytokines. T. Magrone et al. investigated if polyphenol supplementation of farmed sea bass (*Dicentrarchus labrax L.*) would be a viable way to counteract the chronic inflammation present in farmed fish. The authors observed that, in supplemented fish, interleukins 1β and 6 were significantly decreased, whereas splenic interferon γ levels were increased. Concomitantly, a reduction in the number of spleen macrophages compared with control fish was detected. These results open up new perspectives for the use of antioxidants in farmed fish and might contribute to a more sustainable aqua culture.

Inflammation and oxidative stress are also induced by various exogenous agents and are therefore major components that not only determine the toxicity of such agents in somatic cells but also potentially affect the teratogenicity of reproductive cells. S. AE Bashandy et al. reported that increased arsenic levels significantly increased testicular malondialdehyde (MDA), tumour necrosis factor alpha (TNF-α), nitric oxide (NO), and sperm abnormalities and concurrently induced a decrease in testicular superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), and zinc levels. Furthermore, their results suggests that *Spirulina platensis* preparations may represent a potential therapeutic option to protect testicular tissue from arsenic toxicity. In particular, the improved antioxidant status of testicular tissues as a result of *Spirulina platensis* treatment can be deduced from the determined elevated levels of testicular SOD, CAT, zinc, and GSH and from the decrease in MDA in the *Spirulina platensis* + arsenic group as compared to the arsenic group.

In the study by S. A. Sheweita et al., the extracts of the medicinal plant *Alhagi Maurorum* (camel thorn plant) improved glucose levels, the lipid profile, and hepatic function in a rat model of diabetes. Moreover, treatment of diabetic rats with *Alhagi Maurorum* extracts improved the antioxidant status of hepatic tissues by increasing GSH levels and the activities of SOD, glutathione peroxidases (GPx), and glutathione transferase (GST) compared with the control group.

Next to their direct antioxidative properties, polyphenols can act as oxidants in some conditions, inducing redox-sensitive genes such as antioxidant enzymes. J. Marinic and coworkers found in a model of liver resection and intraperitoneal therapy that the higher liver regeneration

induced by olive oil polyphenol extracts was preceded by an initial decrease of GSH and an increase of thiobarbituric acid reactive substances during the first 3 hours after hepatectomy, associated with the induction of GST, SOD, and CAT.

In the defence against ROS, both enzymatic and nonenzymatic antioxidants produced by cells and the low molecular weight molecules absorbed from food stuffs play vital roles in maintaining cellular homeostasis. S. Brundu and collaborators focused their study on the development of a reversed-phase HPLC method for the analysis of cysteine and reduced glutathione in biological samples from mice. Given the established and pivotal role of glutathione in many cellular functions, its determination in the assessment of oxidative stress is quite relevant. In particular, the authors validated their method in samples of diverse tissue origin, including spleen, lymph node, brain, and pancreas according to strict US and European standards.

Despite the fact that an increasing body of evidence suggests that natural functional compounds have bioremedial properties and might consequently be beneficial to human health, clinical evidence remains turbid. Since bioavailability is a key factor that determines if a component is taken up and transported to tissues and cells, not surprisingly, a significant research effort focuses on determining compound bioavailability in humans. X. Guo et al. reported results from a 5-year study that was conducted within the framework of the PREDIMED trial on subjects with high cardiovascular risk. In this study urinary total polyphenol excretion (TPE) was inversely associated with a number of clinical cardiovascular risk factors, namely, plasma glucose and triglycerides concentrations and systolic blood pressure. These results suggest that TPE could be useful as a marker of compliance in intervention studies with foods with high-polyphenol content. On the other hand, polyphenols can also exert their bioactivity without reaching the systemic circulation. Such poorly absorbable antioxidants primarily exert their protective effect against oxidative stress at the interface between the gastrointestinal lumen and gastrointestinal lining. As such, these antioxidants might be attractive therapeutics against oxidative damage-induced gastrointestinal diseases. E. Azzini and collaborators studied the effect of polyphenol-rich extracts from *Cichorium intybus* L. on Caco-2 cells in an *in vitro* monolayers model. Their results show that, at low concentrations, the polyphenol fractions indeed show antioxidative effects, but at higher concentrations they rapidly become cytotoxic. This effect is characterized by increased tight-junction permeability, epithelium abnormalities, and loss of plasma membrane integrity. The authors further confirm that the protective effect by polyphenol extracts from chicories occurs via the interaction with the mucopolysaccharide complexes in the glycocalyx.

Oxidative stress markers are important tools to assess both the redox status of subjects and the health-enhancing effects of antioxidants in humans. However, there is a lack of consensus, analytical validation, standardization, and reproducibility regarding many oxidative stress markers, evaluation models, and antioxidant assays. I. Peluso et al. investigated the relationship between the Peroxidation of Leukocytes Index Ratio (PLIR) and the increasingly studied postprandial metabolic stress. For this, in a pilot clinical trial with a functional cookie containing dark chocolate and pre- and probiotics, they measured PLIR in lymphocyte, monocytes, and granulocytes. The results of the investigation show that the determination of this parameter in healthy subjects may present some limitations during the postprandial period, probably due to the increase in uric acid.

In a study performed with samples of human subcutaneous abdominal adipose depots, C. Carpéné and coworkers illustrated that some phenolic compounds did not clearly block amine oxidases (AO) activities, whereas they impaired the interaction between hydrogen peroxide and the chromogenic mixture in fluorometric assays, which could be misinterpreted as an AO inhibition. Only resveratrol and quercetin partially impaired monoamine oxidases- (MAO-) dependent [^{14}C]-tyramine oxidation, whereas measurements with the radiochemical method using [^{14}C]-benzylamine clearly indicated that resveratrol did not inhibit human semicarbazide-sensitive AO (SSAO). Therefore, when measuring MAO or SSAO inhibition by antioxidants, the verification of the putative interaction of these agents with hydrogen peroxide or chromogenic mixture should carefully accompany testing.

The manuscripts in this special issue show that caution must be taken when interpreting human, animal, or *in vitro* studies that evaluate the effects of natural functional compounds in relation to oxidative stress. Both measurement and interpretation are far from straightforward and there is a desperate need for standardization, especially since decades of research still leave a murky picture without clear definites regarding the bioremedial effects of supplemented antioxidants. This significantly hinders clinical trials and introduction of such compounds in a routine clinical setting. Nonetheless, we sincerely hope that this special issue contributes to shedding some light on the meaning of oxidative stress markers and the bioremedial capacity of natural functional components in experimental models and human studies.

Acknowledgments

The editors thank all authors who submitted their research to this special issue. They also thank the many reviewers for their valuable contribution to this special issue.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 9191407, 13 pages
http://dx.doi.org/10.1155/2016/9191407

Research Article

Preexposure to Olive Oil Polyphenols Extract Increases Oxidative Load and Improves Liver Mass Restoration after Hepatectomy in Mice via Stress-Sensitive Genes

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Received 9 September 2015; Revised 9 December 2015; Accepted 13 December 2015

Academic Editor: Ilaria Peluso

Polyphenols can act as oxidants in some conditions, inducing redox-sensitive genes. We investigated the effect of preexposure to the olive oil polyphenols extract (PFE) on time-dependent changes in the hepatic oxidative state in a model of liver regeneration—a process in which oxidative stress associated with the metabolic overload accounts for the early events that contribute to the onset of liver self-repair. Liver regeneration was induced by one-third hepatectomy in mice. Prior to hepatectomy, mice were intraperitoneally given either PFE (50 mg/kg body weight) or saline for seven consecutive days, while respective controls received vehicle alone. Redox state-regulating enzymes and thiol proteins along with the mRNA levels of Nrf2 gene and its targets γ-glutamylcysteine synthetase and heme oxygenase-1 were determined at different time intervals after hepatectomy. The liver mass restoration was calculated to assess hepatic regeneration. The resulting data demonstrate the effectiveness of preexposure to PFE in stimulating liver regeneration in a model of a small tissue loss which may be ascribed to the transient increase in oxidant load during the first hours after hepatectomy and associated induction of stress response gene-profiles under the control of Nrf2.

1. Introduction

The liver is endowed with the endogenous mechanism of self-repair which restores the functionality of the organ after injury or surgical resection. This repair process, known as liver regeneration, is mainly divided into two distinctive stages. The first stage is the priming phase, during which quiescent hepatocytes sensitized by cytokines and hormonal and nutritional signals acquire the competence to the cell cycle. The second stage is the proliferating phase, during which cells sequentially transit the G1, S, G2, and M phases, dividing until the original liver mass is restored [1, 2].

Liver resection still remains a common practice in the management of patients with hepatocellular carcinoma [3]. Nevertheless, inadequate hepatic regeneration is still an important cause of morbidity and mortality [4]. Consequently, there is a high interest in the possibility of stimulating this hepatic endogenous mechanism of self-repair to increase survival rates and recovery of patients suffering from hepatic injury of various etiologies. Partial hepatectomy in rodents is commonly used model of liver regeneration which enables quantitative study of the influence of various substances on the liver repair [5]. Although dietary vitamins and plant compounds affect the redox state-regulating enzymes and thiol proteins which are necessary for a successful cell cycle progression and normal growth control [6–8], conflicting impact on liver regeneration was established, involving both impaired [9, 10] and reinforced regenerative response [11]. More recently, diverse classes of plant polyphenols, including those inherent to the olive oil, have been shown to regulate repair-related processes in several wound healing models [12–15]. Moreover, recent developments in oncologic surgery suggest the use of intraperitoneal chemotherapy for the treatment of some human cancers [16, 17]. Besides, intraperitoneal administration has been previously used in animal models [18, 19] and quercetin administered intraperitoneally increased the total quercetin level in tumor tissues more than oral quercetin [20]. Taking into account that the reported effects of olive oil feeding and administration by gavage on antioxidant defenses and liver regeneration have

been previously described [21, 22], we aimed to investigate the effect of intraperitoneal administration of polyphenols extract from the olive oil before hepatectomy. Olive oil based emulsions significantly improved the hepatic regeneration and decreased oxidative damage following hepatectomy. Although it is emphasized that this effect is due to the influence of antioxidants present in olive oil, the contribution of polyphenols to the endogenous antioxidant protection system and the healing process has yet to be determined.

The efficacy of polyphenols in wound healing may be ascribed to their purported antioxidant properties, which are assumed to reside in their ability to scavenge different physiologically relevant radical species and to chelate transition metal ions involved in the generation of reactive oxygen species (ROS) [23–25]. However, compiling body of evidence suggests that protective effects of dietary antioxidants from ROS induced injury are not related to their direct antioxidant activity but rather are due to their interaction with specific aspects of the signal transduction network with the ultimate outcome in the modulation of endogenous antioxidant defenses [26–29].

Redox-sensitive transcription factor nuclear factor-erythroid 2-related factor (Nrf2) is central to coordinating expression of genes encoding antioxidant and detoxification enzymes, xenobiotic transporters, and many other proteins involved in the regulation of cell cycle and cell death [30–32]. Studies in Nrf2-deficient mice demonstrated reduced expression of ROS-detoxifying enzymes after hepatectomy, resulting in oxidative stress in hepatocytes, enhanced hepatocyte apoptosis, and a delay in liver regeneration following hepatectomy [33]. In human lung cancer cell lines, growth inhibitory properties of Nrf2 deficiency have been attributed to the induction of cell cycle arrest at G1 phase [34] while, in primary epithelial culture cells, genetic disruption of Nrf2 impaired GSH-induced redox signaling and prevented the progression along the cell cycle, through G2 into M phase [35]. Since cell cycle regulatory machinery induced by hepatectomy is linked to the Nrf2 signaling machinery, there is a possibility that redox-active substances—such as polyphenols—may affect hepatic oxidative state leading to a different course of liver regeneration.

In view of this potential of phenolics, we obtained the polyphenols extract (PFE) from the olive oil and examined its effect on the antioxidant status during the course of liver regeneration following one-third hepatectomy in mice. Although it is well known that antioxidant enzymes are induced by nuclear translocation of Nrf2, [36–38] we evaluated also a possible modulation of its mRNA expression. For that purpose, the changes in the redox state-regulating enzymes, thiol proteins, and Nrf2 gene signatures that coordinate adaptive stress response were determined at 0 (controls), 1, 3, 6, 12, 24, and 48 hours after hepatectomy.

2. Materials and Methods

2.1. Preparations of Olive Oil Polyphenols Extract (PFE). Olive oil polyphenols extract was prepared from the olive oil obtained from a mixture of olive cultivars grown in the Island of Krk and the oil was purchased from an individual producer. The oil was produced by a process of continuous centrifugal extraction in three phases and was kept in dark glass bottles in a cool place until analysis. Polyphenols were isolated from the olive oil by liquid-liquid extraction using a methanol-water mixture (60 : 40; v/v) according to Swain and Hillis [39]. The total polyphenol content was determined with Folin-Ciocalteu's reagent (Fluka, Buchs, Switzerland) according to Gutfinger [40], and sodium molybdate solution (Fluka, Buchs, Switzerland) was used to establish the content of *ortho*-diphenols as described by Mateos et al. [41].

For the animal experiments, the methanol-water phase was evaporated to dryness under reduced pressure in a rotary vacuum evaporator (Büchie R-3000 with Büchie Vacuum Controller B-720, Switzerland) at 40°C, and under a nitrogen stream. The dry residue was then dissolved in a physiological saline solution (NaCl 0.9%). Resulting PFE was stored at −20°C in aliquots and used as per requirement.

2.2. Animal Experiments. Male C57BL/6 mice, 2-3 months old, with weight 24–30 g, were obtained from the breeding colony of the School of Medicine, University of Rijeka. The animals were housed in propylene cages and maintained in a germ-free environment, at 12-hour light/dark cycle, and constant temperature (20 ± 1°C) and humidity (50 ± 5%), with free access to tap water and a standard rodent diet (pellet, type 4RF21 GLP, Mucedola, Italy). All experimental procedures were conducted in compliance with the Declaration of Helsinki principles and approved by the Ethical Committee of the School of Medicine, University of Rijeka.

Experimental animals were randomly assigned into four groups, with six animals per group as follows: PSS + pHx-mice pretreated with physiological saline solution prior to partial hepatectomy, PFE + pHx-mice treated with olive oil polyphenols extract followed by partial hepatectomy, PSS, and PFE-mice receiving vehicle alone (controls), that is, physiological saline solution or olive oil polyphenols extract.

Physiological solution (0.1 mL per 10 grams of body weight) and a dose of 50 milligrams of the PFE per kilogram body weight (50 mg/kg btw) were intraperitoneally (i.p.) injected daily for seven successive days. The dose of olive oil polyphenols extract used in animal experiments was selected on the basis of our previous study [42] and according to the dose levels reported for oleuropein [43] and its derivative hydroxytyrosol [44, 45], which are the most prevalent phenolic compound in olive oil and the most representative olive oil *ortho*-diphenols [46].

On the eighth day animals receiving vehicle alone underwent sacrifice while animals of the first and the second group were subjected to the 1/3 hepatectomy under appropriate ether anesthesia and in compliance with the rules of asepsis. 1/3 hepatectomy was accomplished by removing the median liver lobe through a midabdominal incision, according to the method described by Beer et al. [47]. In order to avoid the potential impact of circadian rhythm, all operations were performed at the same time of the day, between 8.00 a.m. and 9.00 a.m. Experimental animals were allowed to recover on a 37°C warm plate and then underwent sacrifice at 1, 3, 6, 12, 24, and 48 hours after the surgery. Animals in all

experimental groups were sacrificed by cervical dislocation in ether anesthesia.

Before sacrificing, blood was sampled under ether anesthesia by orbital sinus puncture. Whole blood samples were left to stand for two hours at 25°C and then centrifuged at 2.000 ×g over 20 minutes. Obtained serum was stored at −80°C for subsequent analysis of glutathione *S*-transferase alpha.

Upon sacrifice at indicated times, the liver was removed and repeatedly washed in phosphate buffer saline (PBS, Sigma, St. Louis, MQ, USA) to remove blood clots, and total liver weight (controls) and weight of the liver remnant were obtained. Portions of liver tissue masses of up to 100 mg were exempted for RNA extraction while the remaining tissue was used for the analysis of antioxidant enzymes activity, TBARS levels, and the concentration of total glutathione. Tissue samples were immediately weighed, quick-frozen in liquid nitrogen, and stored at −80°C until further processing.

2.3. Serum Glutathione S-Transferase Alpha (α-GST) Level. Serum level of α-GST was measured spectrophotometrically using standard sandwich enzyme-linked immunosorbent assay (ELISA) (Oxford Biomedical Research, Inc., Oxford, MI, USA) on a Bio-Tek ELx808 Ultra Microplate Reader (BioTek Instruments, Winooski, VT, USA) following the manufacturer's instructions. The quantity of an enzyme bound to the immunosorbent was measured at 450 nm, and the results are expressed as nanograms per milliliter of the serum (ng/mL).

2.4. Hepatic Antioxidant Status. Lipid peroxidation was detected in a 25% liver tissue homogenate prepared in PBS by monitoring pink-colored adduct at 534 nm, which is formed in the reaction with thiobarbituric acid (TBA, Sigma, St. Louis, MQ, USA) under high temperature (95°C) and acidic conditions (pH = 3.5) [48]. The hepatic lipid peroxidation was expressed as TBARS content and was calculated as nanomoles per milligram protein (nmol/mg) according to the standard curve prepared from 1,1,3,3-tetraethoxypropane (Sigma, St. Louis, MQ, USA).

Total glutathione concentration was determined in liver tissue lysates, prepared by tissue homogenization in 5% *meta*-phosphoric acid (w/v) (ACS, Sigma-Aldrich GmbH, Taufkirchen, Germany) using a commercially available HT Glutathione Assay Kit (Trevigen, Helgerman Ct, Gaithersburg, USA), according to the manufacturer's instructions. The quantity of glutathione was established by the optimized enzymatic recycling method with glutathione reductase at 405 nm on a Bio-Tek ELx808 Ultra Microplate Reader (BioTek Instruments, Winooski, VT, USA). The results are expressed as nanomoles of GSSG (equivalent to total glutathione) per gram of liver tissue (nmol/g).

Superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR) activities were measured by commercially available kits from Trevigen (Helgerman Ct, Gaithersburg, USA). Cayman Chemical Company kit (Ann Arbor, MI, USA) was used for catalase (CAT) activity determination. Enzyme activities were measured on a Bio-Tek ELx808 Ultra Microplate Reader (BioTek Instruments, Winooski, VT, USA) according to the manufacturer's protocols.

SOD activity (representing the activity of SOD1, SOD2, and SOD3 isoenzymes) was determined in the cytosolic tissue extract from percent inhibition of the rate of formation of tetrazolium salt upon superoxide anion radicals generation in the xanthine/xanthine oxidase system, and it was monitored by measuring the decrease in absorbance values at 450 nm. The results are given in units of SOD per microgram of protein (U/μg), where one unit is defined as the amount of sample that inhibits the rate of increase in absorbance due to the tetrazolium salt formation by 50%.

CAT activity was measured in tissue homogenate by monitoring the decrease in absorbance at 540 nm due to the hydrogen peroxide consumption. The results are given in units of CAT per microgram of protein (U/μg). One unit is defined as the amount of enzyme that will cause the formation of 1.0 nmol of formaldehyde per minute at 25°C.

GPx activity is measured in a tissue homogenate, in a reaction coupled with glutathione reductase by monitoring a decrease in absorbance at 340 nm. The results are expressed in units of GPx per milligram of protein (U/mg). One unit of glutathione peroxidase is defined as the amount of enzyme that will cause the oxidation of 1 nmole of NADPH to $NADP^+$ per minute at 25°C.

GR activity is determined in cytosolic tissue extract by measuring the rate of NADPH oxidation. The accompanying decrease in absorbance was monitored at 340 nm. The results are expressed as milliunits of GPx per milligram of protein (mU/mg). One unit of glutathione reductase oxidizes 1 μmol of NADPH per minute at 25°C, pH 7.5.

Protein content was estimated by Bradford's method, with bovine serum albumin (Sigma, St. Louis, MQ, USA) used as a standard [49].

2.5. RNA Extraction. Total intact hepatic RNA was extracted using TRI Reagent Solution (Applied Biosystems/Ambion, Foster City, CA, USA) and isolated RNA was purified with RNeasy Mini Kit (Qiagen, Germany) as it is described by the manufacturer. The total RNA concentration was measured based on A_{260} value. The purity of the RNA in each sample was verified by determining A_{260}/A_{280} ratio. The integrity of the RNA molecule as well as the separation efficiency was confirmed by 1% agarose-formaldehyde gel electrophoresis with ethidium bromide staining. The samples in which there were clearly visible ribosomal bands, 28S and 18S, with the ratio of upper (28S) and lower (18S) band of about 2 : 1 and in which there was no apparent contamination of genomic DNA were used for further analysis.

2.6. Quantitative Real-Time PCR Assays. Single-strand cDNA was synthesized from 5 μg of total RNA in a 50 μL reaction volume following the protocol of High Capacity cDNA Reverse Transcription Kit with RNAse inhibitor (Applied Biosystems, USA). To preclude contamination with genomic DNA, negative controls in which the reverse transcriptase was replaced with water were used and subjected to analogous procedures and conditions. Quantitative real-time polymerase chain reaction (qPCR) was performed using

the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and optimized oligonucleotide primers. Primers specific for the mouse genes Nrf2, HO-1, GCSc, and 18S RNA were used. Primers are commercially available as QuantiTect Primer Assay (Mm_Nfe2l2_1_SG for Nrf2, Mm_Hmox1_1-SG for HO-1, Mm_Gclc_1_SG for GCSc, and Mm_Rn18s_2_SG for 18S RNA, Qiagen, Germany). Each reaction was carried out using twenty-five times diluted cDNA product in a 25 μL reaction volume. Two replicates of each reaction were performed. PCR amplification was conducted on Real-Time PCR 7300 (Applied Biosystems, Foster City, CA, USA) and the amplification conditions were 50°C for 2 min, 1 cycle; 95°C for 10 min, 1 cycle; 95°C for 15 sec, 40 cycles; and 60°C for 1 min. Amplicon specificity was verified by first-derivative melting curve analysis for each pair of primers. The expression levels of the genes were normalized to that of the housekeeping gene, 18S RNA, and were calculated using the standard $2^{-\Delta\Delta CT}$ method. The amount of transcript expressed at time zero (vehicle controls) was used as calibrator sample.

2.7. Calculation of Liver Mass Restoration. Surgical procedure resulted in removal of approximately 1/3 of the total liver mass while 2/3 of the remaining liver exerted the compensatory growth. The intensity of that regeneration in the group of mice that received saline or polyphenols extract before surgery (experimental groups) was expressed as a percentage of recovery of excised liver weight in relation to the estimated liver weight at the operation and as a function of time after hepatectomy. The calculations of liver mass restoration were made according to following formula: (actual mass of the liver remnant at a given time − expected mass of the liver remnant immediately after the liver resection)/(prehepatectomy liver mass) × 100. Expected mass of the liver remnant immediately after the resection was calculated as [(actual mass of the liver remnant in experimental group)/(body mass in experimental group)]/[(liver mass in control group)/(body mass in control group)]. Prehepatectomy liver mass was quantified as follows: [(liver mass in control group)/(body mass in control group)] × (body mass in experimental group).

2.8. Statistical Analysis. The data were analyzed using StatSoft STATISTICA version 12 software. Normality of data distribution was assessed by the Kolmogorov-Smirnov normality test. The distribution qualified the normality test, so parametric test was applied. For comparison between groups, the unpaired Student's t-test (in case of only two comparisons) or analysis of variance (one-way ANOVA) was used. Fischer post hoc test was applied when variances across the group were equal, and Dunnett's post hoc test was used when variances were not equal. Variance equality was tested by Levene statistical analysis. Results are expressed as mean ± standard deviation (SD). Differences with $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Extract Composition. The total polyphenol content was 326.23 ± 4.27 mg/kg expressed as gallic acid equivalents and the *ortho*-diphenols content was 22.46±0.39 mg/kg expressed as caffeic acid equivalents.

3.2. Hepatic Lipid Peroxidation. The amounts of TBARS were determined to assess lipid peroxidation level. Initially, untreated animals exhibited a significantly higher level of TBARS (Figure 1(a)), but in both experimental groups substantial enhancement of lipid peroxidation was recorded, reaching a peak at 12 hours after the surgery. However, polyphenols induced marked increase in TBARS levels at earlier times (3 hours after hepatectomy). Although the content of TBARS did not regain initial level until the end of the experimental period in treated experimental group, time intervals following 6 hours after hepatectomy were characterized by a small, but significant, attenuation of lipid peroxidation in polyphenols treated and hepatectomized mice versus mice subjected only to hepatic resection.

3.3. Hepatic Glutathione Content. Hepatic resistance towards oxidative injury was determined by assessing the total hepatic glutathione content (comprising reduced GSH and oxidized GSSG form). Partial hepatectomy alone did not induce a significant alteration in total hepatic glutathione content (Figure 1(b)) until 12 hours after the surgery when it showed significant increase with respect to the initial values which is retained for up to 24 hours after hepatectomy. In spite of the initially higher values, PFE treatment promoted a sharp drop in total glutathione at 3 hours after hepatectomy compared to the group of untreated hepatectomized mice and in relation to the initial values, reflecting increased oxidant load. Although the concentration of the total glutathione showed increasing course in subsequent time intervals in PFE treated and hepatectomized mice, the level of this nonenzymatic antioxidant remained significantly lower compared to the untreated group up to 24 hours after hepatectomy.

3.4. Serum α-GST Level. To determine the capacity of detoxification of lipid peroxidation end products and various electrophilic molecules in the regenerating liver, serum α-GST level was assessed. The level of α-GST (Figure 1(c)) was significantly elevated in both experimental groups, although in PFE treated animals it peaked earlier (3 hours after hepatectomy) and was promptly reduced in magnitude but not significantly changed in relation to the untreated animals, except at 12 hours postoperatively, reflecting lower oxidant/electrophilic load in mice receiving PFE compared to the untreated animals. Although in periods after the maximum α-GST level tends to decrease, detoxification demands still remained high in the regenerating liver of both experimental groups, since the level of α-GST was not fully recovered until the end of the experimental period compared to the initial values of respective control groups.

3.5. Activities of the Thiol Modulating Enzymes (GPx, GR) and Antioxidant Enzymes (SOD, CAT). In addition to glutathione, we determined the functionality of the glutathione-dependent enzymes and other antioxidant enzymes that make up the first and second line of defense against the cytotoxicity of ROS and electrophiles.

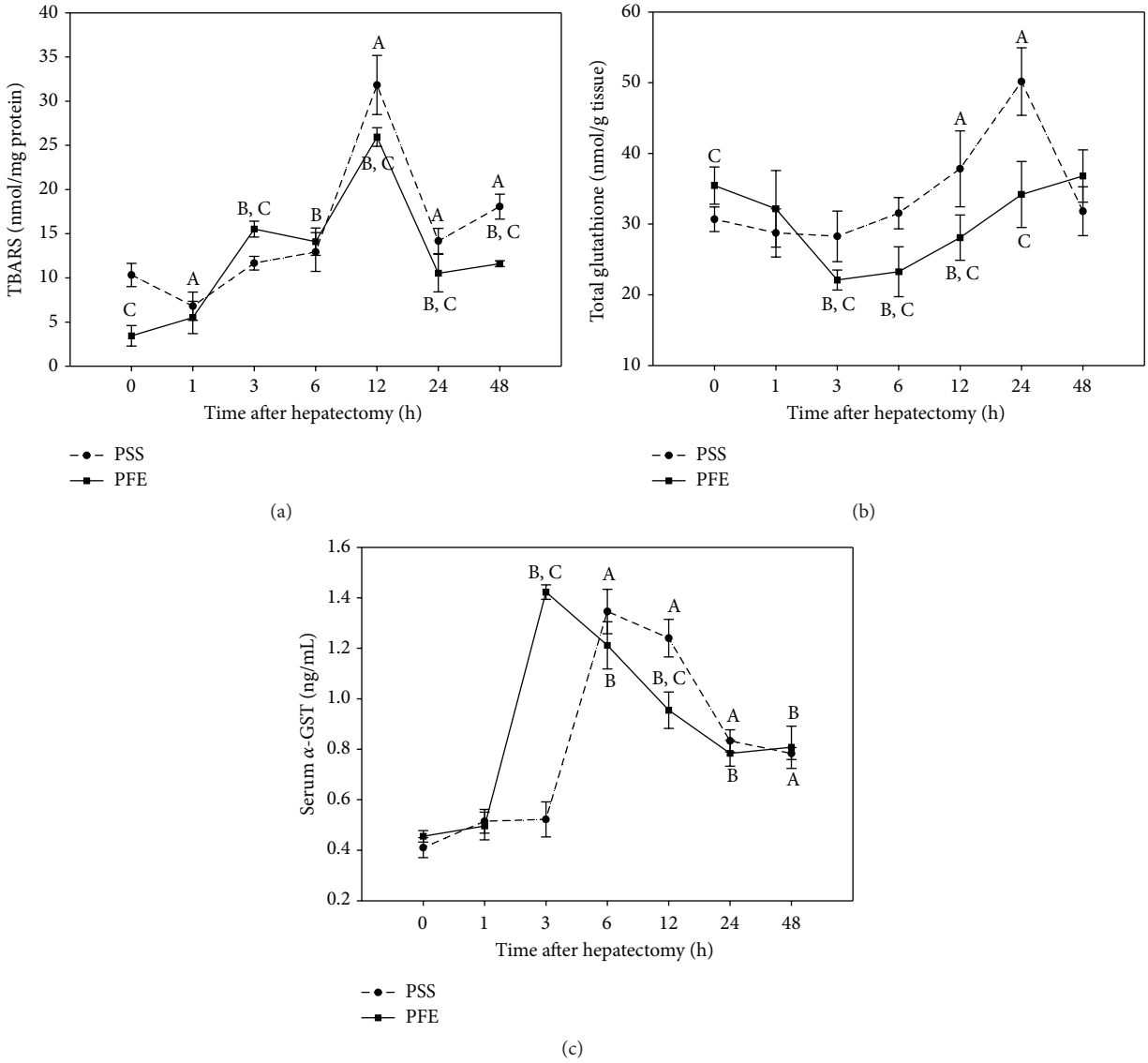

Figure 1: (a) Hepatic TBARS level, (b) total liver glutathione content, and (c) serum glutathione *S*-transferase level in the polyphenols extract treated and untreated hepatectomized mice at different time intervals after the surgery. Time zero correspondents to values from the unhepatectomized, vehicle (PSS or PFE) treated mice. Results are the mean ± SD of six individual determinations for each experimental point, $p < 0.05$; (A) significantly different from the unhepatectomized mice receiving PSS at a given time point; (B) significantly different from the unhepatectomized mice receiving PFE at a given time point; (C) significant difference between PFE treated and untreated hepatectomized mice at a given time point.

Figure 2(a) denotes a gradual increase in SOD activity, in the liver tissue of both experimental groups, reflecting greater demands for the superoxide anions detoxification. However, polyphenols shifted the activity maximum towards the earlier time periods (3 hours after hepatectomy) which was also accompanied by the depletion of cellular glutathione, increased lipid peroxidation products, and electrophiles generation. After a transient decline in SOD activity, which in the treated group starts earlier, the relation of the SOD activities in two experimental groups changes, showing a more pronounced increase in the nontreated hepatectomized group. However, 48 hours after hepatectomy, there was no significant difference in SOD activity in hepatectomized mice receiving PFE and mice receiving vehicle.

Cytotoxicity of hydrogen peroxide, generated during the catalytic cycle of SOD, is counterbalanced by the CAT and GPx activities. These enzymes are characterized by different K_m values for their substrate, and their activity shows an inverse relationship in the treated versus untreated mice. In the group of untreated mice, CAT activity (Figure 2(b)) is close to the initial value until the end of the experimental period. A low CAT activity reflects the lower concentration of hydrogen peroxide. Under these conditions, this nonradical reactive oxygen species is detoxified by the GPx. GPx activity

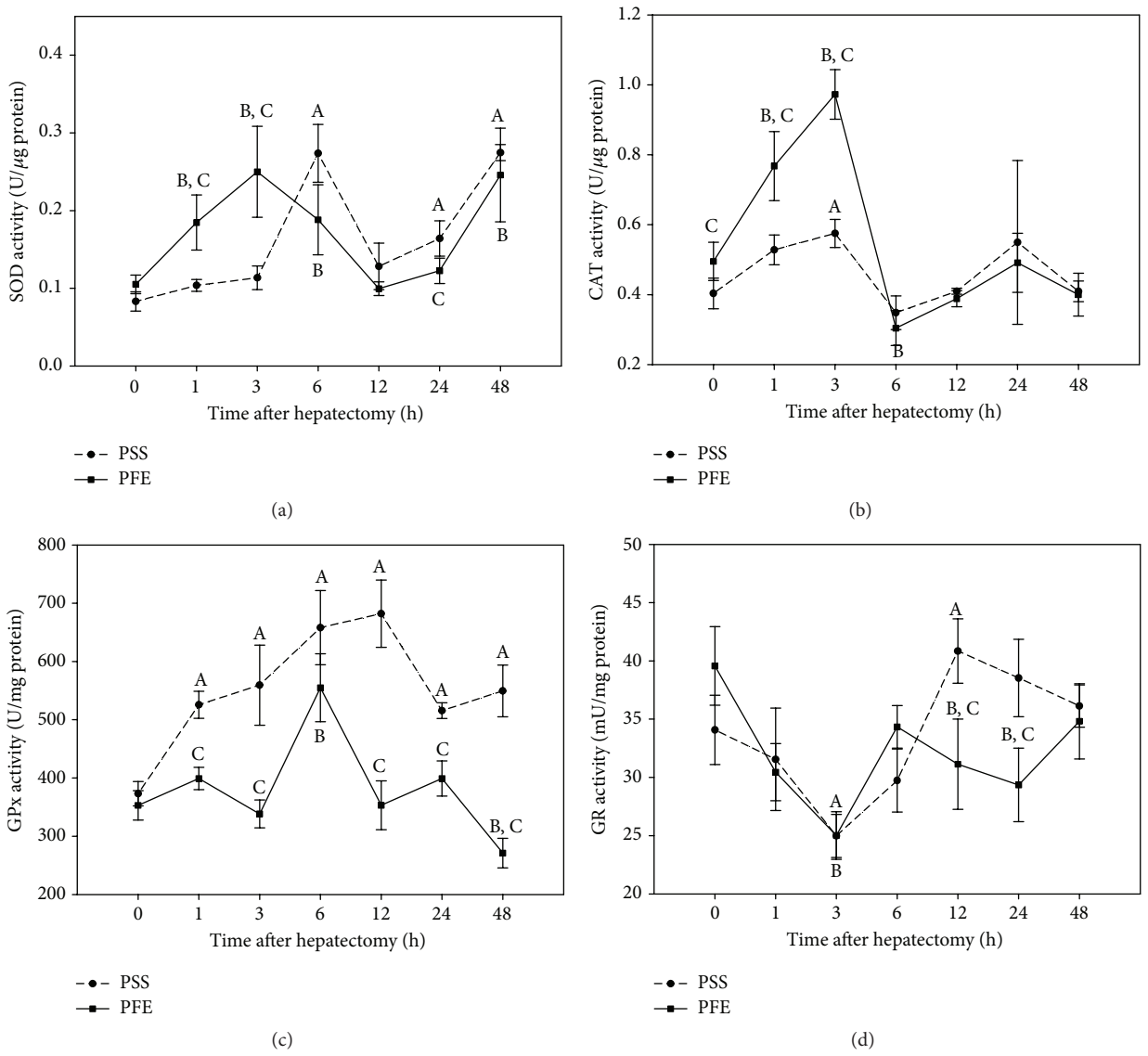

Figure 2: (a) Superoxide dismutase, (b) catalase, (c) glutathione peroxidase, and (d) glutathione reductase activity in the polyphenols extract treated and untreated hepatectomized mice at different time intervals after hepatectomy. Time zero correspondents to values from the unhepatectomized, vehicle (PSS or PFE) treated mice. Results are the mean ± SD of five to six individual determinations for each experimental point, $p < 0.05$; (A) significantly different from the unhepatectomized mice receiving PSS at a given time point; (B) significantly different from the unhepatectomized mice receiving PFE at a given time point; (C) significant difference between PFE treated and untreated hepatectomized mice at a given time point.

(Figure 2(c)) shows a continuous upward trend after hepatectomy in the untreated group and remains elevated until the end of the monitoring period (48 hours after hepatectomy). On the other hand, the polyphenols treatment is reflected in the increase in CAT activity (Figure 2(b)), which peaked at the third hour after hepatectomy. At this time point, SOD activity displays a maximum and glutathione is markedly depleted, indicating the greater extent of oxidative stress in the regenerated liver of treated mice when compared to the untreated mice subjected to the operative procedure. This marked increase is followed by a temporary decline and activity normalization at 12 postoperative hours. In subsequent time intervals SOD activity increases in PFE pretreated mice and displays significantly lower values at 24 hours after hepatectomy in relation to untreated mice. At the same time, GPx activity (Figure 2(c)) was not significantly changed compared to the initial values, except at the 6 hours after hepatectomy, when it shows an increase, most likely to compensate for reduced CAT activity (Figure 2(b)) to counteract peroxides formation, but at the 48 hours GPx activity displays marked activity reduction (Figure 2(c)). Moreover, PFE pretreatment markedly decreased GPx activity in comparison to untreated mice in periods from 12 to 48 hours after hepatectomy.

Processes of glutathione utilization result in the formation of its oxidized GSSG form. To determine the capacity of glutathione recycling back to its reduced form, GR was

assessed. GR activity (Figure 2(d)) was not significantly changed in the regenerating liver tissue of both experimental groups, except at 3 hours postoperatively in both groups, where GR lower activity denotes impaired glutathione recycling in treated and untreated mice. However the hepatic capacity of glutathione regeneration is restored at 12 hours after hepatectomy, but only in the nontreated group, reflecting the increase in glutathione level. Although GR activity showed increasing trend upon polyphenols treatment following 24 hours after hepatectomy, it remained lower compared to the initial values but not significantly different compared to the untreated group. Furthermore, significantly lower GR activity was observed at 12 and 24 hours in the PFE treated hepatectomized versus untreated hepatectomized mice.

3.6. Gene Expression Profile. In order to establish at what stage PFE exert their glutathione increasing activity, mRNA level of the γ-glutamylcysteine synthetase catalytic subunit (GCSc) was analyzed by quantitative PCR. γ-Glutamylcysteine synthetase is the key determinant of the overall glutathione biosynthetic capacity, catalyzing the rate-limiting step of this metabolic reaction. The enzyme is composed of heavy (catalytic) and light (regulatory) subunit, with the former being responsible for all of the catalytic activity, including a feedback inhibition by glutathione [50]. The immediate early period (1 hour) after hepatectomy is marked by the initial rise in GCSc gene transcriptional activity (Figure 3(a)) in both experimental groups. In this period, the level of mRNA GCSc genes in the liver of treated group was approximately two times higher and one and a half times higher in the untreated group when compared with respective controls. After that a gradual decline followed in the level of GCSc transcript, which is detained in the untreated group by the end of the experimental period and, therefore, the established increase in the concentration of glutathione is not a reflection of the increased capacity of its biosynthesis. By contrast, in the treated group at 12 hours postoperatively reinduction of GCSc gene expression was observed that lingers until the end of the experimental period, coinciding with periods in which increase in the concentration of glutathione in this group was found.

Another important cytoprotective gene with the role in cell proliferation is HO-1 [51]. Thus, RT-PCR was employed to establish whether the PFE pretreatment exerts the induction of HO-1 at the transcriptional level. In regenerating liver tissue of PFE treated group, a significant and relatively stable increase in expression of HO-1 gene (Figure 3(b)) in all time intervals after hepatectomy was accomplished, except at 48 hours after the surgery. Transcriptional activity of HO-1 gene in the untreated group was suppressed in all periods, except at 3 and 48 hours after the operation, when approximately two and half times higher level of HO-1 mRNA transcript was accomplished in comparison to the controls.

Promoter regions of the GCSc and HO-1 gene contain common consensus elements which bind Nrf2 transcriptional factor [52, 53]. Therefore, we next seek to examine the effects of PFE pretreatment on the Nrf2 induction at the transcriptional level. RT-PCR, using Nrf2-specific primers, revealed suppression of the Nrf2 gene transcriptional activity (Figure 3(c)) in all periods after hepatectomy. In contrast, polyphenols treatment induced Nrf2 gene expression at 1 hour after hepatectomy, when approximately two times higher level of Nrf2 gene transcript was accomplished in comparison to the control group. Three hours after the surgery transcriptional activity was slightly suppressed but at 6 hours started to increase and at 12, 24, and 48 hours reached the value of about 2–2.5 times higher than the control.

3.7. Liver Mass Restoration. In both experimental groups, the gradual increase of recovered liver mass was observed throughout the entire experimental period (Figure 4). However, the polyphenols treatment manifested itself in the significantly higher intensity of that regeneration in all time intervals after hepatectomy. At the end of the experimental period, approximately 15% higher regeneration ratio of the remnant liver tissue was recorded in a group of mice receiving PFE compared to those that underwent only hepatectomy.

4. Discussion

Several studies addressing the role of polyphenols in tissue repair attributed wound healing potential of these plants derived compounds to their antioxidant capacity based on the observation that ROS, produced in response to tissue injury, impede or exacerbate the healing process. We investigated the effect of preconditioning with PFE on the course of liver regeneration induced by hepatectomy—a process during which ROS account for the early signals involved in the initiation of tissue mass restoration.

The results of the present study indicate that regenerating liver itself exerts intrinsic oxidative stress. Pretreatment with PFE additionally elevated the overall oxidant load within the first three hours after hepatectomy. This stage corresponds to the priming phase of liver regeneration, which increases the sensitivity of hepatocytes to growth factors leading up to DNA replication and mitosis [1]. Initial oxidative stimuli provoked by polyphenols promoted depletion of total glutathione, one of the major determinants of the hepatic resistance towards oxidative injury, and alterations in processes linked to the glutathione pathway. These processes entailed the impaired glutathione redox cycling due to the decreased GR activity, impaired glutathione biosynthesis as indicated by suppressed GSCc transcriptional activity, and increased glutathione utilization via the GST-mediated reactions. Glutathione depletion coincides with the period in which activity of enzymes that first confront the cytotoxic effects of ROS, that is, SOD and CAT, reached peak values. These antioxidant enzymes afforded only partial protection against hydrogen peroxide, superoxide anion radical, and hydroxyl radical as noted by the simultaneous increase in lipid peroxidation level and a peak in serum α-GST enzyme. It has been demonstrated that α-GST serum levels reflect induction of hepatic α-GST in mice when fed with phase II enzyme inducers [54]. Induction of GSTs has been described for phenols sharing 1,2-diphenol structure [55] and plays important roles in the detoxication of electrophiles generated by phase I enzymes and lipid peroxidation end products. Inducers of GSTs are also substrates for GSTs and,

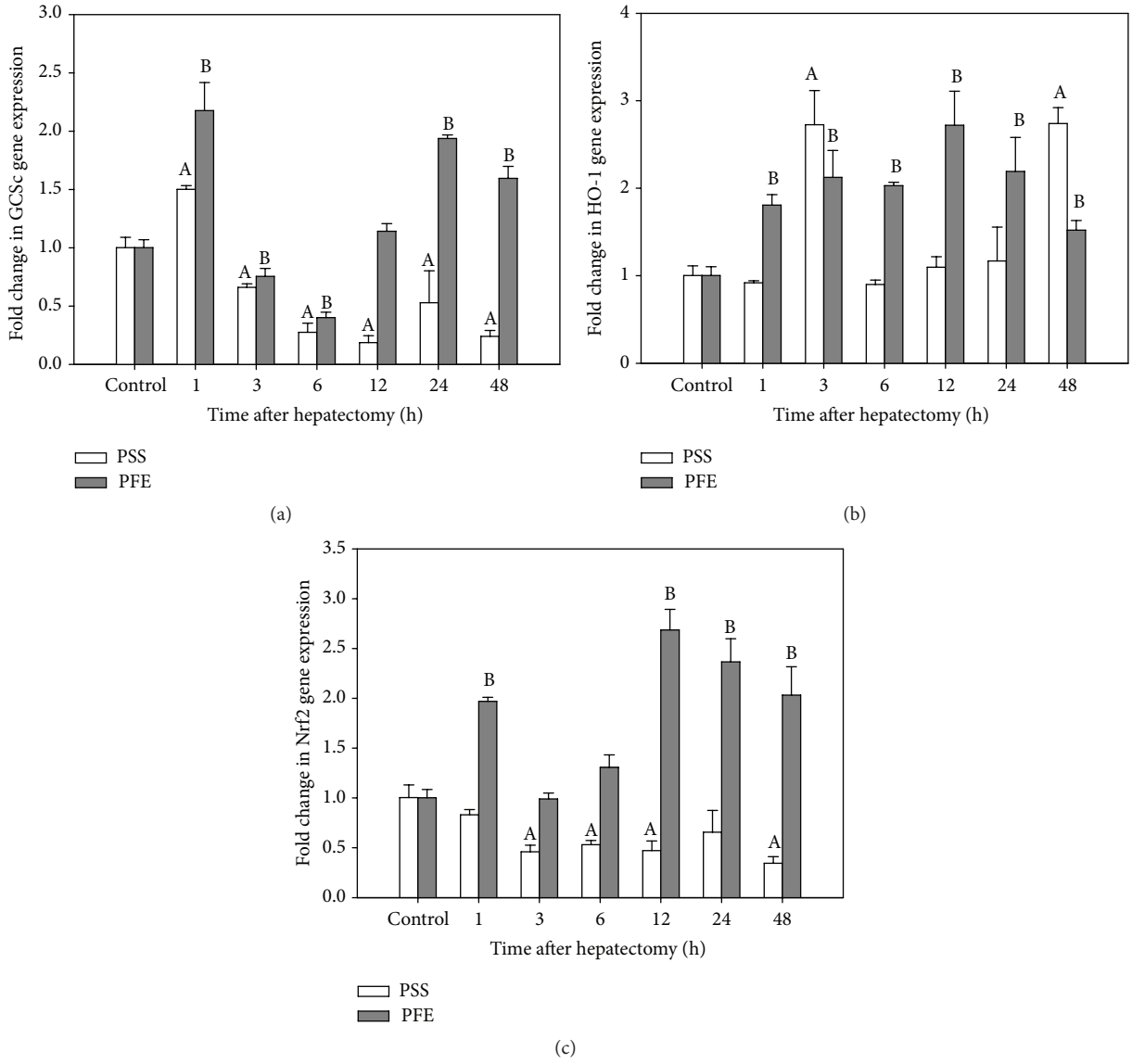

Figure 3: Real-time quantitative PCR analysis of (a) GCSc gene, (b) HO-1 gene, and (c) Nrf2 gene expression levels in a regenerating liver at different time intervals after hepatectomy. Total RNA, isolated from the liver of three animals, was pooled, subjected to a reverse transcription, and the resulting first-strand cDNA was amplified using primers specific for the mouse GCSc, HO-1, and Nrf2 gene. The results are expressed as the relative ratio of each cDNA level over vehicle control (PSS or PFE) after normalization to corresponding 18S RNA levels. Data represent mean ± SD of five to six animals per group ($p < 0.05$), and each determination was conducted in duplicate; (A) significantly different from the PSS vehicle control mice at a given time point; (B) significantly different from the PFE vehicle control mice at a given time point.

in addition, have the ability to elevate tissue glutathione levels [24]. This standpoint is substantiated in our model where a general trend upward was noted in glutathione levels upon PFE pretreatment in periods prior to the onset of DNA synthesis (between 6 and 24 hours after hepatectomy). Concurrently with the increase in glutathione content, α-GST and lipid peroxidation level gradually decline, while the activities of redox state and thiol regulating enzymes return to the basal level which is in accordance with the hypothesis that oxidative stress is reduced before cell division [56]. However, periods coinciding with replicative phase (48 hours after hepatectomy) are characterized by an increase in SOD activity in both experimental groups, which is considered to be a measure of protection against ROS generated due to increased oxidative metabolism in the S phase of the cell cycle [57]. This time point is also characterized by decreased GPx activity in mice receiving PFE, probably due to the inhibition of GPx by nitrogen (II) oxide which rises immediately after hepatectomy and exerts stimulating effects on liver regeneration [58].

Although lipid peroxidation coupled with glutathione depletion can represent one of the mechanisms of cell toxicity, selective enhancement of lipid peroxidation can act as potential mediator of early regenerative capacity by evoking a general cell response involving activation of transcriptional factors [10].

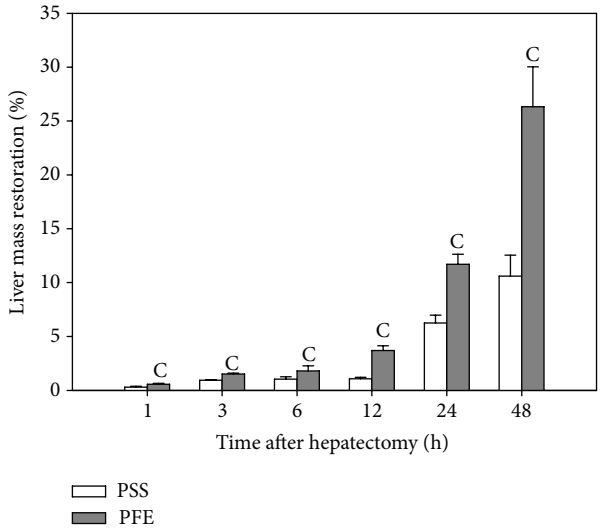

Figure 4: The restoration of the initial liver mass in a function of time after hepatectomy. Regeneration rate of the remnant liver tissue was expressed as the percentage of the restoration of the excised liver mass in relation to the estimated prehepatectomy liver mass at a given time; (C) significantly different from the vehicle control group ($p < 0.05$).

In mammalian cells, low levels of oxidative stress initiate activation of prosurvival transcription factor Nrf2 which is indispensable for a successful cell cycle progression and coordinated induction of battery cytoprotective genes [30, 34, 35], including GCSc and HO-1 [52, 53] which are known to be associated with the control of liver regeneration.

Our results suggest that oxidative stress induced by polyphenols pretreatment elevated mRNA of GCSc and HO-1 genes and increased Nrf2 gene expression.

Based on the observation that decreased expression of Nrf2 target genes results in extensive oxidative stress and in a delay in liver regeneration in $Nrf2^{-/-}$ deficient mice [33, 59], it seems reasonable to conclude that induction of GCSc and HO-1 genes upon polyphenols pretreatment is related to the cellular adaptation to oxidative stress and exerts stimulatory role in liver regeneration.

The ability of polyphenols to induce GCSc and HO-1 mRNA expression has been confirmed in both *in vivo* and *in vitro* models [8, 26, 60], and both of the genes were upregulated in response to PFE pretreatment.

The induction of GCSc gene by PFE is accompanied by the continuous increase in total glutathione concentration, necessary for stimulation of the hepatocytes proliferative response and entry into the S phase of the cell cycle [50]. However, in spite of these changes the concentration of total glutathione remained lower compared to the untreated animals, possibly due to the enhanced biliary glutathione excretion which has been observed to occur as a result of Nrf2-induced GST mRNA expression and enzyme activity [61], as well as the HO-1 induced CO formation [62]. Both HO-1 and its metabolic product CO have a pivotal role in the liver regeneration after resection. Activation of HO-1 in hepatocytes is directly associated with the release of NO, small gas signal transduction molecule which provides protection against apoptosis and favors progression in the cell cycle until the organ size and function are restored after partial hepatectomy [58]. Elevated expression of HO-1 is typically associated with tissue adaptation and activation of survival pathways and improves hepatic graft function and posttransplantation survival, mostly as a consequence of the antiapoptotic and anti-inflammatory effects of CO arising from heme degradation in hepatocytes [63]. Recently, it has been demonstrated that CO increases HGF growth factor secretion from hepatic stellate cells which accelerates early hepatocyte proliferation, possibly through Akt-cyclin-dependent pathway [51].

Although olive oil phenolics are commonly perceived as substances endowed with antioxidant activity, emerging evidences propose that most dietary antioxidants, with the exception of vitamin E, do not have a direct role in reducing intracellular oxidants [64]. In fact, cell culture studies have highlighted that the most representative olive oil phenols, hydroxytyrosol, as well as oleuropein, at high concentration (100 μM) have the ability to produce hydrogen peroxide [65], while tyrosol potentiates hydroperoxides increase irrespective of the concentration used (10–250 μM) [25].

Extensive TNFα signaling and the increased metabolic activity of hepatocytes to maintain homeostasis increase the levels of ROS and oxidative stress [66], shifting intracellular redox status toward a more oxidizing state in the early stages of regeneration [2]. In this context, phenolics, and especially 1,2- and 1,4-diphenols, which are ubiquitously present in the olive oil, can easily undergo (auto)oxidation producing semiquinone or quinone radicals, or they can become radicals themselves during the reaction with free radicals [8, 23], thereby contributing to the overall oxidant/electrophilic load. In most cases, the prooxidant effects of polyphenols are stimulated by the interaction with transition metal ions which catalyze the oxidation of *ortho*- and *para*-hydroquinones to their corresponding quinones and increase superoxide anion radical and hydrogen peroxide formation. The latter can accelerate the generation of highly reactive hydroxyl radicals via Fenton chemistry [67–69]. Certain forms of quinone radicals can act as electrophiles and form conjugates with glutathione thus lowering the cellular glutathione supplies [68]. Recently it has been demonstrated that both hydroxytyrosol and oleuropein at low concentration participate in the initiation process of LDL oxidation induced by Cu^{2+} ions [70]. It may be assumed that interactions with transition metal ions could be important in the regenerating liver since hepatectomy induces early redistribution of iron from the spleen and thymus into the tissue of regenerating liver [71] but also enhances oxygen delivery to the cells, necessary for the oxidative activation of phenolics via the redox cycling mechanism.

Emerging evidence suggests that prooxidant properties and direct interactions with redox-sensing proteins induce antioxidant enzymes and increase their substrates, leading to the maintenance of nucleophilic tone and protection against ROS induced injury under physiological conditions [64]. Central to the regulation of nucleophilic tone is Nrf2 which is under physiological conditions sequestered in the cytoplasm as an inactive complex with its repressor protein Keap1. It has

been proposed that quinone derivatives formed by oxidation of diphenols act as activated Michael acceptors, capable of covalently modifying or oxidizing redox-sensitive cysteine thiols in the sensor protein Keap1. This, in turn, results in the disruption of Nrf2/Keap1 complex and increased stability of Nrf2 and its accumulation in the nucleus where it can transactivate ARE/EpRE-driven target genes [27–29].

Alternatively, by lowering the cellular level of GSH, reactive polyphenol derivatives may temporarily disrupt the redox state of the cell and trigger upstream kinases that phosphorylate Nrf2 aiding in its release from Keap1 [32]. Hydroxytyrosol, principal olive oil diphenol, has been shown to activate Nrf2 through the PI3K/Akt and ERK1/ERK2 pathways in vascular endothelial cells [72]. Similarly, Patel and Maru [27] established that pretreatment of mice with polymeric black tea polyphenols extract increased the level of Nrf2 by posttranslational modifications involving upstream kinases in liver and lung cells, but no significant alterations were observed at the transcriptional level. In this study olive oil extract administration was associated with an increase of Nrf2 mRNA expression level. Those differences may indicate differences in the mode of action of different plant extracts as well as differences in the mechanism of Nrf2 induction under different cellular conditions. One limitation of the present study is that we did not measure Nrf2 activation upon olive oil polyphenols before treatment. The relevance of transcriptional modulation as a regulatory mechanism in the action of Nrf2 will be considered in our future work.

In view of these facts, we propose that increase in oxidant/electrophilic load during the early phase of liver regeneration, mediated via polyphenols *per se* or through glutathione depletion, might represent the signaling mechanism through which the induction of Nrf2 dependent gene signatures could be achieved, resulting in timely and adequate appearance of events associated with liver regeneration. Detailed mechanistic studies are needed to improve the understanding of the antioxidant or prooxidant effects of olive oil phenolics in liver regeneration.

5. Conclusion

To our knowledge, this study is the first to demonstrate that PFE preexposure affects endogenous cellular defense mechanisms via the stress response gene-profiles associated with hepatoprotection in the model of liver regeneration induced by one-third hepatectomy. Our results suggest that treatment with PFE, prior to hepatectomy, evoked transient and early increase in electrophilic and oxidative load, which was followed by the increase in Nrf2 and Nrf2-dependent gene expression, leading to certain regenerative signals that resulted in the efficient liver mass restoration. Since Nrf2 participates in the maintenance of redox-homeostasis and regulation of cell cycle, it is plausible to believe that a possibility of inducing Nrf2 could represent a foundation for molecular events leading to cytoprotection and more rapid recovery. These findings support the hypothesis that hepatectomies less than 40%, which are generally characterized by a slower regenerative response, can be efficiently modulated by increasing metabolic load before the resection [73]. Furthermore, we demonstrate that olive oil polyphenols can act as oxidants in some conditions and activate protective mechanisms under the control of redox-sensitive genes which could have stimulating beneficial health response during stressful conditions. Specifically, the possibility of enhancing liver mass restoration by polyphenols in cases of small tissue deficit holds a promise of affecting the development of liver regeneration in situations of overly small liver and displays a potential of application in the field of regenerative medicine.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

This work was supported by the Croatian Ministry of Science, Education and Sports, under Grant no. 062-0621341-0061.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 5264064, 8 pages
http://dx.doi.org/10.1155/2016/5264064

Research Article

Changes in Oxidative Stress and Antioxidant Enzyme Activities in Streptozotocin-Induced Diabetes Mellitus in Rats: Role of *Alhagi maurorum* Extracts

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Received 14 September 2015; Revised 12 November 2015; Accepted 30 November 2015

Academic Editor: Maura Palmery

Alhagi maurorum (camel thorn plant) is a promising medicinal plant due to the presence of flavonoids and phenolic compounds as major contents of its constituents. No previous study has been conducted before on *A. maurorum extracts* as an antioxidative stress and/or antidiabetic herb in STZ-induced DM in rats. Therefore, four groups of rats were allocated as control (C), STZ-induced DM (D), and STZ-induced DM supplemented with 300 mg/kg BW of either aqueous extract (WE) or ethanolic extract (EE) of *A. maurorum*. The plasma levels of glucose, TG, TC, LDL-C and VLDL-C, MDA, and bilirubin and the activities of transaminases and GR were significantly increased in the diabetic group. Also, diabetic rats showed severe glucose intolerance and histopathological changes in their livers. In addition, levels of insulin, total proteins, GSH, and HDL-C and the activities of SOD, GPx, and GST were significantly decreased in the diabetic rats compared to those of the control group. The ingestion of *A. maurorum* extracts lowered the blood glucose levels during the OGTT compared to the diabetic rats and restored all tested parameters to their normal levels with the exception of insulin level that could not be restored. It is concluded that *A. maurorum* extracts decreased elevated blood glucose levels and hyperlipidemia and suppressed oxidative stress caused by diabetes mellitus in rats.

1. Introduction

The incidence of diabetes mellitus has been increased annually all over the world and the number of diabetic patients will jump from 382 million patients in year 2013 to 592 million in year 2035 [1]. The majority of diabetic patients are non-insulin-dependent and relatively small proportions (7–10%) of diabetic patients have insulin-dependent diabetes (T1D) [2]. Type 1 diabetes (T1D) is a chronic disease that results from an autoimmune destruction of β-cells of the pancreas. Therefore, insulin deficiency and hyperglycemia are the main outcomes of T1D [3]. This may generate an array of disturbances in glucose and lipid homeostasis resulting in hyperglycemia and dyslipidemia [4]. Persistent hyperglycemia in diabetes causes increased production of oxygen free radicals from autoxidation of glucose [5] and glycosylation of protein [6] which lead to oxidative stress which is associated with several health complications including antipathies, cardiovascular disorders, blindness, renal failure, neuropathies, and cancers [3, 7].

Recently, drug formulation from natural herbs, for treatment of diabetes mellitus drugs and other diseases, attracted the attention of many researchers [8]. *Alhagi maurorum* (Leguminosae) also called camel thorn plant or aqool is a favorable food for camels. It is widely distributed in Asia, the Middle East, Europe, and Africa [9]. It has been used as diaphoretic, diuretic, expectorant, and ulcer treatment [10]. Oil from its leaves was used for rheumatoid treatment and as laxative [11, 12]. Water extract of the roots is used to enlarge the ureter and to remove the kidney stones, whereas the methanolic extract is used as an antidiarrheal agent [13] and as herbal cough syrup [12]. *A. maurorum* species contains fatty acids and sterols, flavonoids, coumarins, alkaloids, and vitamins. In addition, six main flavonoid glycosides were isolated from the ethanolic extract of *A. maurorum* [10].

Moreover, *A. maurorum* roots contain lupeol [11], which is used as an antiangiogenic, antioxidative, and anti-inflammatory agent [14, 15].

Streptozotocin-treated rats developed clinical features and signs, which are similar to those found in type 1 diabetes mellitus [16]. To the best of our knowledge, no previous studies have been conducted before to investigate the antidiabetic effects of ethanolic and aqueous extracts of *A. maurorum* through determination of blood glucose level, free radicals, antioxidant enzymes, and lipid profile in STZ-induced diabetic rats. Therefore, the present study was undertaken to investigate the effectiveness of *A. maurorum* extracts in STZ-induced diabetic rats and to evaluate their therapeutic potential for treatment of diabetes mellitus.

2. Methods and Materials

2.1. Preparation of Ethanolic and Aqueous Extracts of Alhagi maurorum. Camel thorn plant (*A. maurorum*) was collected from Wadi El Natrun region (Egypt) after getting the agreement of the Director of Wilderness Areas in El-Beheira Governorate and authentication by Salama El Darer, Professor of Plant Ecology, Botany and Microbiology Department, Faculty of Science, Alexandria University. We confirm that no specific permission was required for collection this plant from Wadi El Natrun region because it is a desert. Moreover, we confirm that this field study did not cause any danger for any plant in this area. The aerial parts of *A. maurorum* were collected, washed three times with tap water and two times with distilled water, dried in the shade, and milled to fine powder by Wiley mill (Model 4-GMI, Germany). Ground plant (100 g) was refluxed with 1 liter of 70% ethanol or with 1 liter of double distilled water for 1 hour. After filtration, solvents were removed under reduced pressure at 40°C using rotary evaporator (Buchi, Model 462, Germany) and freeze dried by lyophilizer to obtain the dried extracts. Aqueous suspensions were prepared from ethanolic or aqueous extracts and administered to rats orally.

2.2. Determination of Total Phenolic and Flavonoid Contents of Alhagi maurorum Extracts. Total phenolic contents of ethanolic and aqueous extracts of *A. maurorum* were determined by the Folin-Ciocalteu method [17]. Gallic acid (0–0.9 mg/dL) was used as standard for phenolic compounds. The data were expressed as milligram gallic acid equivalents/g lyophilized powder. The total flavonoid content was determined using aluminum chloride colorimetric method as described earlier [18]. Rutin (0–20 mg/L) was used as a standard for flavonoid. The results were expressed as milligram rutin equivalents/g lyophilized powder.

2.3. Induction of Diabetes Mellitus by Streptozotocin. Type 1 diabetes mellitus was induced in rats by the intraperitoneal injection of freshly prepared streptozotocin (STZ) at a dose of 45 mg/kg dissolved in 0.1 M citrate buffer solution BW [19]. Three days after the STZ injection, the blood was withdrawn from the tail vein, and the glucose level was determined. Rats were diabetic when their fasting blood glucose levels were more than 200 mg/dL.

2.4. Experimental Design. Forty healthy, mature male Albino rats were provided by the Animal House of the Faculty of Medicine, Alexandria University, Egypt. The average weight of rats was 140 g and maintained in wire-bottomed cages. Rats had free access to food and water and were kept at 25 ± 2°C and 50–60% humidity. The protocol of animals handling was approved by the ethical guidelines prescribed by the MRI, Alexandria University, Alexandria, Egypt. After induction of diabetes, the diabetic rats were randomly allocated to 3 groups (10 rats each): (1) diabetic group (D-group); (2) diabetic group receiving 300 mg/kg BW water extract of *A. maurorum* (WE-group); and (3) diabetic group receiving 300 mg/kg BW ethanolic extract of *A. maurorum* (EE-group). Ten rats received distilled water and were used as control group (C-group). The *A. maurorum* extracts were suspended in distilled water and administered orally as a daily dose for four weeks. Rats were fasted overnight and euthanized by cervical dislocation. Blood samples were collected in EDTA-coated tubes. Livers and pancreases were removed, washed with cold saline, and stored at −80°C.

2.5. Oral Glucose Tolerance Test and Assay of Biochemical Parameters. Briefly, after overnight fasting, rats were intragastrically loaded with glucose (2 g/kg). Blood samples were withdrawn from the tail vein at 0, 30, 60, 90, and 120 minutes and the blood glucose levels were determined using commercial kit as described by Tietz [20]. Nonradioactive assay was used for determination of insulin level in plasma of rats according to the manufacturer's protocol [21]. Triglyceride, total cholesterol, LDL-C and HDL-C, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin were measured in the plasma using commercial kits from Bio-System Company (Egypt). The protein content was determined according to the method of Lowry et al. [22].

2.6. Evaluation of Oxidative Stress Markers. Lipid peroxidation was evaluated by measuring thiobarbituric acid reactive substances (TBARS) according to the method of Draper and Hadley [23]. Reduced glutathione was measured spectrophotometrically as described by Shaikh et al. [24]. Liver superoxide dismutase was estimated according to the method of S. Marklund and G. Marklund [25]. Glutathione peroxidase activity in the liver supernatant was measured according to the method of Flohe and Gunzler [26]. The hepatic activity of glutathione reductase was assayed according to the method of Smith et al. [27]. The glutathione-S-transferase activity was measured according to the method of Habig et al. [28].

2.7. Histological Analysis. Specimens of pancreas and liver tissues of the different groups were immediately fixed in 10% formalin and then treated with conventional grade of alcohol and xylene. For histopathological examination, 6 μM specimens thicknesses of both pancreas and livers were stained with hematoxylin and eosin (H&E) stains[29].

2.8. Statistical Analyses. Statistical analysis was performed using SPSS software package (Version 17.0). The data were analyzed using one-way analysis of variance (ANOVA) and the differences between means of all groups were tested using

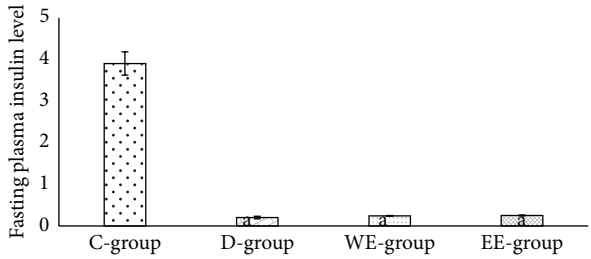


Figure 1: The fasting insulin level in diabetic rats treated with 300 mg/kg BW of either water or ethanolic extract of *Alhagi maurorum*.

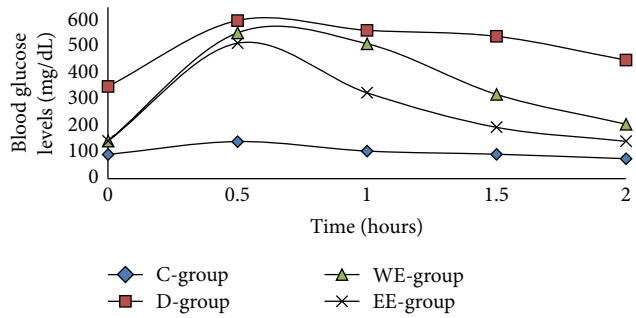


Figure 2: Changes in blood glucose level during oral glucose tolerance test (OGTT).

Least Significant Difference (LSD). Probability value less than 0.05 was considered statistically significant.

3. Results

3.1. The Total Flavonoids and Phenolic Contents of Alhagi Extracts. The results of the present study showed that concentrations of the total phenolic and flavonoid compounds in the aqueous extract of *A. maurorum* were gallic acid (90.87 ± 1.5 mg) and rutin (5.20 ± 0.24 mg) equivalent/100 g dried-weight, respectively, while the contents of the ethanolic extract of *A. maurorum* from these components were gallic acid (105.16 ± 2.6 mg) and rutin (6.16 ± 0.27 mg) equivalent/100 g dried-weight, respectively.

3.2. Alhagi Extracts and Biochemical Parameters. Activities of ALT and AST and the level of the total bilirubin were significantly increased ($P \leq 0.05$) in diabetic rats compared to those of control group. Water and ethanolic extracts of *A. maurorum* exhibited significantly improved hepatic function of the diabetic rats (Table 1). The fasting blood glucose levels of diabetic rats were significantly ($P \leq 0.05$) higher than those of the control group. However, administration of water and/or ethanolic extract of *A. maurorum* to the diabetic group significantly ($P \leq 0.001$) restored their fasting blood glucose levels to the control value. Plasma insulin levels were significantly ($P \leq 0.05$) decreased in the diabetic group compared to the control group. However, WE and EE did not restore the plasma insulin levels in diabetic groups to the normal level (Figure 1).

The time-course changes in the blood glucose levels during the oral glucose tolerance test (0–120 min) of all groups were shown in Figure 2. The blood glucose levels reached the maximum after 30 minutes of administration of 2 g glucose/kg BW and then reduced to initial levels within 2 hours in all groups except diabetic rats that showed severe glucose intolerance throughout the experimental period (0–120 min). However, treatment of diabetic rats with 300 mg/kg BW of either water or ethanolic extracts of *A. maurorum* significantly ($P \leq 0.05$) lowered their blood glucose levels to the normal level (Figure 2).

Plasma levels of triglycerides (TG), total cholesterol (TC), LDL-cholesterol (LDL-C), and VLDL-cholesterol (VLDL-C) were increased in diabetic rats compared to the control rats (Table 1). On the other hand, HDL-cholesterol (HDL-C) level was decreased in the diabetic rats compared to control group. Oral administration of either water or ethanolic extracts of *A. maurorum* resulted in significant ($P \leq 0.05$) decreases in the levels of TG, TC, LDL-C, and VLDL-C compared to the diabetic group and increased HDL-C concentration in both WE- and EE-treated diabetic groups compared to nontreated diabetic group (Table 1).

3.3. Alhagi Extracts and Oxidative Stress. The level of MDA and the activity of GR significantly ($P \leq 0.05$) increased in hepatic tissues of diabetic rats compared to control group (Table 2). However, the hepatic content of GSH and activities of SOD, GPx, and GST were significantly ($P \leq 0.05$) decreased in diabetic rats compared to control group (Table 2). Both WE and EE treatments significantly ($P \leq 0.05$) reduced hepatic MDA level and GR activity compared to the diabetic group. Also, treatments of diabetic rats with either WE or EE improved the antioxidant status of hepatic tissues by increasing GSH level and the activities of SOD, GPx, and GST compared to the diabetic group (Table 2).

3.4. Histopathological Studies

3.4.1. Pancreatic Tissues. Control rats reveal normal pancreatic architecture; the closely packed pancreatic acini were composed of pyramidal shaped cells with rounded nuclei (a), the pale-stained normal islets of Langerhans (IS) scattered between acini with well-preserved cytoplasm, and nucleus normal interlobular connective tissue septa (red arrow), Figure 3(A): Figures 3(B1), 3(B2), and 3(B3) of STZ-diabetic group of rats showing disturbance of the acini pattern structure, pyknotic nuclei of some acini cells (black arrow) with severe damage; dilation, thickening, and congestion of the blood vessels (dotted arrow and red arrow); and vacuolated acini (green arrow). Islets with irregular outline, vacuolated cytoplasm (circle), and degeneration of β-islet cells (green arrow) inflammatory cells infiltrate around the pancreatic duct (p.d) (Figures 3(C) and 3(D)): STZ + WE- and STZ + EE-treated rats showing slight histological alterations of the pancreatic acini only.

3.4.2. Hepatic Tissues. The histological examinations of hepatic tissues are represented in Figure 4. The light micrographs of liver tissues demonstrated normal architecture of hepatic

Table 1: Changes in levels of liver function markers, blood glucose levels, and lipid profile in plasma of diabetic rats treated with either water or ethanolic extract of *Alhagi maurorum*.

Parameters	Animals treatments			
	Control	STZ-group	STZ-WE-group	STZ-EE-group
AST (U/L)	18.67 ± 1.41^{c}	503.46 ± 29.61^{a}	24.33 ± 2.62^{b}	21.57 ± 1.23^{b}
ALT (U/L)	$6.141 \pm .079^{c}$	103.63 ± 12.563^{a}	9.64 ± 1.479^{b}	8.62 ± 1.216^{b}
Bilirubin (mg/dL)	0.51 ± 0.03^{c}	$6.982 \pm .18^{a}$	0.43 ± 0.04^{b}	0.72 ± 0.04^{b}
Cholesterol (mg/dL)	104.76 ± 7.6^{c}	203.14 ± 9.03^{a}	63.92 ± 4.76^{b}	90.47 ± 7.90^{b}
LDL-C (mg/dL)	83.58 ± 7.01^{c}	173.18 ± 8.47^{a}	54.60 ± 6.41^{b}	56.51 ± 4.88^{b}
HDL-C (mg/dL)	88.26 ± 8.15^{c}	40.246 ± 1.774^{a}	73.965 ± 6.927^{b}	63.499 ± 7.549^{b}
LDL/HDL ratio	1.1 ± 0.14^{c}	4.35 ± 0.23^{a}	0.76 ± 0.08^{b}	0.99 ± 0.13^{b}
VLDL-C (mg/dL)	24.37 ± 2.15^{c}	97.52 ± 12.51^{a}	10.14 ± 1.60^{b}	16.70 ± 2.24^{b}
Triglyceride (mg/dL)	121.83 ± 10.7^{c}	487.60 ± 62.57^{a}	50.68 ± 8.02^{c}	83.52 ± 11.21^{b}
Glucose (mg/dL)	90.81 ± 5.36^{c}	437.6 ± 14.10^{a}	106.83 ± 8.57^{b}	82.24 ± 7.09^{b}

Values are expressed as mean ± SE of 10 rats in each group.
^{abcd}Mean values within a row not sharing the same superscript letters were significantly different, $P < 0.05$.

Table 2: Changes in level of free radicals and activities of antioxidant enzymes in liver of diabetic rats treated with either water or ethanolic extract of *Alhagi maurorum*.

Parameters	Animals treatments			
	Control group	STZ-group	STZ-WE-group	STZ-EE-group
MDA (nmoles/g tissue)	551.20 ± 64.29^{a}	1222.40 ± 101.9^{d}	845.00 ± 31.32^{b}	647.80 ± 52.73^{c}
GSH (nmoles/g tissue)	876.35 ± 57.55^{d}	424.15 ± 48.34^{a}	1103.66 ± 105^{b}	1317.82 ± 133.8^{c}
GPx (mU/mg protein)	634.12 ± 61.31^{d}	355.28 ± 41.68^{a}	474.50 ± 25.37^{b}	506.19 ± 21.51^{c}
GR (mU/mg protein)	15.04 ± 0.53^{b}	23.35 ± 1.85^{a}	13.62 ± 1.09^{b}	15.98 ± 0.96^{b}
GST (mU/mg protein)	17.95 ± 1.16^{b}	13.81 ± 0.45^{a}	17.05 ± 1.67^{b}	15.57 ± 1.01^{d}
SOD (U/mg protein)	216.57 ± 19.01^{b}	139.94 ± 7.39^{a}	197.79 ± 32.37^{b}	205.50 ± 16.6^{b}

Values are expressed as mean ± SE of 10 rats in each group.
^{abcd}Mean values within a row not sharing the same superscript letters were significantly different, $P < 0.05$.

cells and central vein and normal blood sinusoids in the control group (Figure 4(a)), while STZ-diabetic rats revealed severe pathological changes including congestion and dilation of hepatic sinusoids. Moreover, portal areas showed hyperplasia in the biliary epithelium and wall thickness of hepatic arteries. Focal aggregations of lymphocytes were also noticed in diabetic rats. The hepatic cells revealed degenerative and necrotic changes. Also, diffuse vacuolar, hydropic degeneration, and hypertrophied Kupffer cells were seen in diabetic rats (Figure 4(b)). However, livers of diabetic rats treated with *A. maurorum* extracts markedly reduced and attenuated the histological changes from severe to moderate alterations (Figures 4(c) and 4(d)).

4. Discussion

From ancient times, diabetic patients have used medicinal plants to maintain blood glucose level [30]. In this regard, the present study is extended to show the influence of *A. maurorum* extracts on blood glucose level, oxidative stress, and lipid profile in STZ-induced diabetic rats [31]. Diabetes mellitus type 1 is caused due to lack of insulin secretion [32]. Consistent with this finding, the present study showed a significant reduction in insulin levels in diabetic rats with no recovery after their treatments with either water or ethanolic extracts of *A. maurorum*. The hypoglycemic effects of *A. maurorum* extract were not attributed to regeneration of β-cells or to increase of insulin secretion. This finding was confirmed by the histological analysis of pancreatic tissue since diabetic rats showed a reduction in numbers of islets and degeneration of β-cells. In agreement with the present study, a selective necrosis of β-cells islets of Langerhans of STZ-treated rats has been found [33]. It has been found a variable changes in nuclei of islets of pancreas in diabetic rats and some of them appeared as pyknotic nuclei due to condensation and shrinkage of the nuclear materials [34], and this is in agreement with the finding of the present study. However, treatment of diabetic rats with *A. maurorum* extracts showed a slight attenuation in pancreatic acini only.

It is known that hyperglycemia in both animals and humans with type 1 diabetes results from the increase in hepatic glucose output and the decrease in peripheral glucose utilization [35]. Because *A. maurorum* extracts do not affect β-cells regeneration or insulin secretion, the hypoglycemic effects of these extracts may be due to the presence of gallic acid and rutin as major constituents of these extracts. Interestingly, gallic acid increased glucose uptake and enhanced the translocation of GLUT4 at concentrations comparable to the amount of gallic acid. The hypoglycemic effects of *A. maurorum* extracts may be due to presence of phenolic

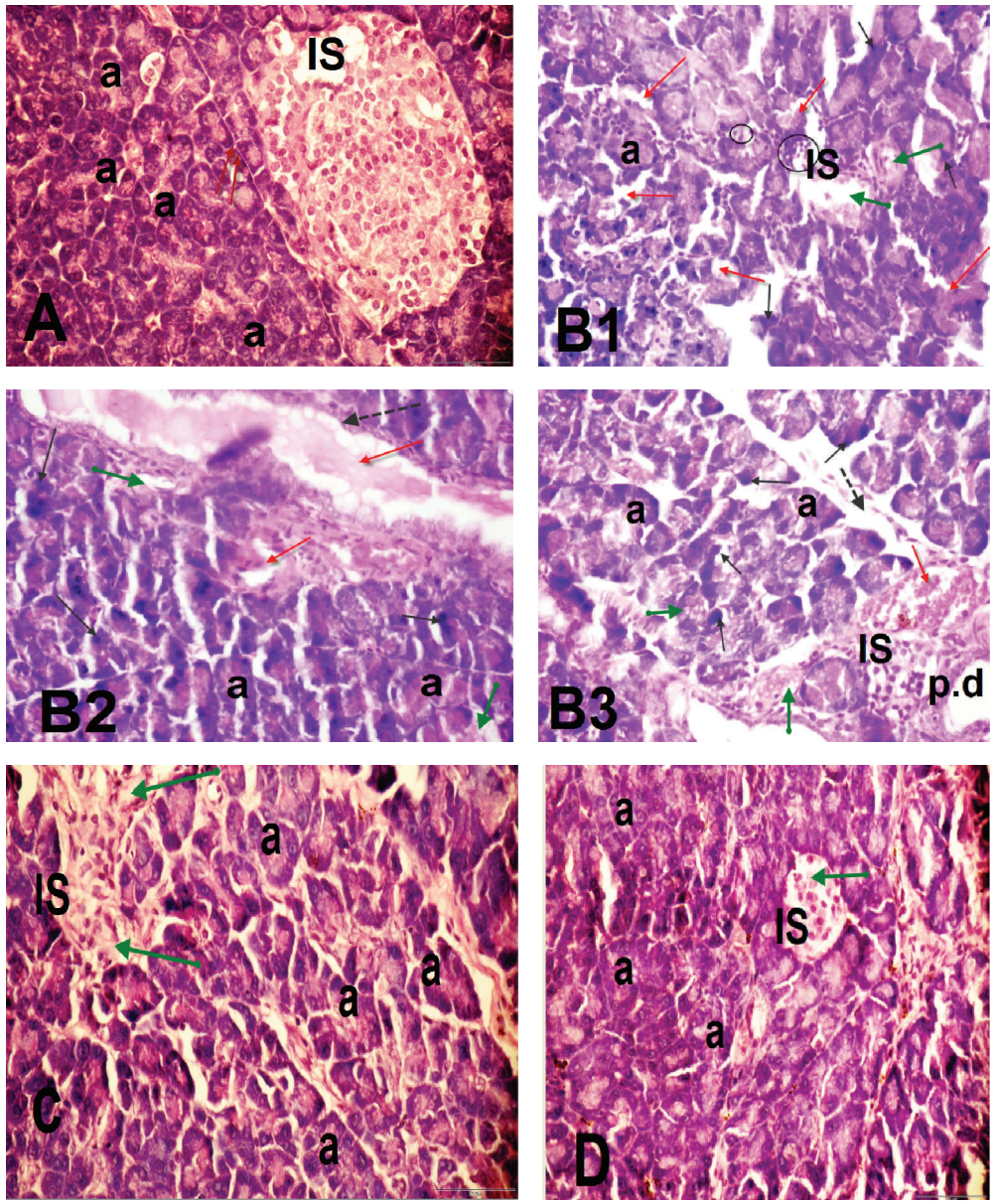
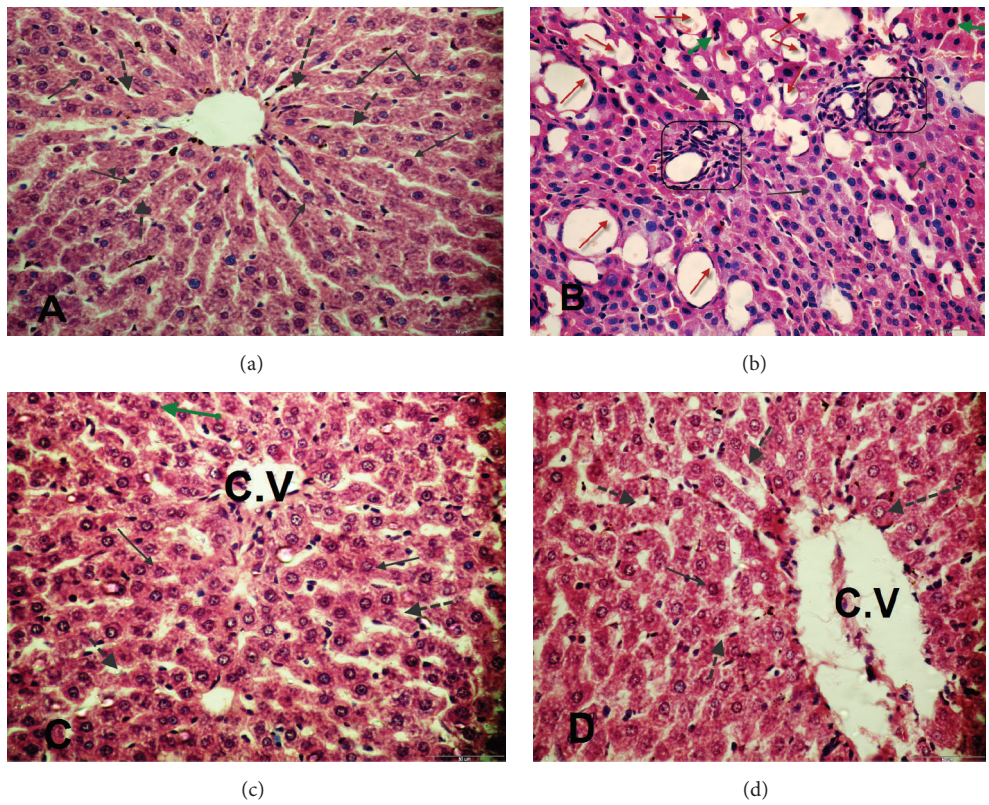


Figure 3: Light micrographs of pancreatic sections of the following. (A) Control rats revealed normal pancreatic architecture; the closely packed pancreatic acini composed of pyramidal shaped cells with rounded nuclei (a), the pale-stained normal islets of Langerhans (IS) scattered in between acini with well-preserved cytoplasm, and nucleus normal interlobular connective tissue septa (red arrow). B1, B2, and B3 represent STZ-diabetic group of rats and showed disturbance of the acinar pattern structure, pyknotic nuclei of some acini cells (black arrow) with severe damage; dilation and thickening of blood vessels (dashed arrow) and congestion of the blood vessels (red arrow) and vacuolated acini (green arrow). Islets with irregular outline, vacuolated cytoplasm (circle), and degeneration of β-islet cells (green arrow). C and D represent STZ + WE- and STZ + EE-treated rats and showed a slight reduction in the histological alterations of the pancreatic acini only. H&E, 400x.

compounds in these extracts. Supporting our finding, it has been found that gallic acid increased glucose uptake via different mechanisms [36]. In addition, it has been reported that quercetin, a flavonoid of *A. maurorum*, increased glucose uptake and increased GLUT4 translocation [37]. In addition, it has been found that rutin was served as a potential agent for glycemic control through enhancement of insulin-dependent receptor kinase activity, thereby inducing the insulin signaling pathway causing increased GLUT4 translocation and increased glucose uptake [38]. In skeletal muscle, rutin significantly increases intracellular calcium concentration which may induce glucose transporter-4 (GLUT-4) translocation with consequent glucose uptake [39].

The liver diseases are more prevalent in the diabetic population [40]. The activities of AST and ALT and the level of bilirubin were increased in diabetic rats. However, treatment of STZ-induced diabetes in rats with either water or ethanolic extract of *A. maurorum* reduced AST and ALT



(a) (b) (c) (d)

Figure 4: Paraffin sections stained by hematoxylin and eosin for histopathological examination of hepatocytes of rats: (a) liver tissue of control showing normal structure, central vein (C.V), normal arrangement of hepatic cords, normal blood sinusoids (⇢), and hepatocytes (→); (b) liver tissue of diabetic rats (STZ) showing hepatocyte vacuolization and fatty changes (red arrow), necrosis (green arrow), dilation of hepatic sinusoids (⇢) also, bile duct and portal vein (□), and cell infiltration (○); (c and d) liver tissue of diabetic rats + WE and diabetic rats + EE extracts of *Alhagi* showing normal structure, central vein (C.V), normal arrangement of hepatic cords, normal blood sinusoids and hepatocytes, few necroses, less degenerative changes, and vacuolization. H&E, 400x.

activities and total bilirubin levels compared to diabetic rats which are consistent with the finding of Shaker et al. [41]. The hepatoprotective effect of *A. maurorum* extracts may be due to the presence of flavone structures in the ethanolic extract [42].

Both hypertriglyceridemia and low level of HDL are the most common lipid abnormalities related to diabetes mellitus [43]. In the present study, the diabetic rats exhibited hypertriglyceridemia, hypercholesterolemia, elevated LDL-C, elevated VLDL-C, and reduced HDL-C levels compared to the control group. However, treatment of diabetic rats with either water or ethanolic extracts improved LDL/HDL ratio and lowered TG, TC, and VLDL-C levels. This improvement might be due to the presence of lupeol, a component of *A. maurorum* extract, which plays an important role in normalization of lipid profile [15].

The elevated levels of oxidative stress in diabetic animals are due to autoxidation of glucose, protein glycation, lipid peroxidation, and low activities of antioxidant enzymes [44]. Consistent with this finding, the present study showed that increased MDA level, decreased GSH level, and decreased activities of antioxidant enzymes, such as SOD, GPx, and GST, were seen in livers of the STZ-induced diabetic rats. These results are in agreement with other previous study which showed that glutathione level was decreased in different phases of diabetes [45]. The mechanism of enhancing of oxidative stress might be due to protein glycation and inhibition of antioxidant enzymes activities (superoxide dismutase and glutathione peroxidase) [46]. In addition, it has been found that flaxseed oil diet upregulated expression and induced activities of CAT and SOD and the protein expression of GPx, whereas fish oil diet induced both the activity and expression of CAT in liver of streptozotocin-nicotinamide induced diabetic rats [47]. In the current study, *A. maurorum* extracts alleviated oxidative stress by inducing the activities of antioxidant enzymes (GPx and GST) that were inhibited in diabetic rats. These results are in agreement with other study which reported that the ethanolic extracts of *A. maurorum* ameliorate the oxidative stress by increasing the level of glutathione and decreasing the MDA level [41]. Moreover, the antioxidative effect of *A. maurorum* might be due to the presence of flavonoid compounds [48] such as quercetin, which protects human intestinal cells and hemoglobin against oxidative stress attack [49].

The depletion of GSH level in diabetic rats might be due to its utilization to alleviate the oxidative stress in diabetes [50]. Therefore, the increased activity of GR in the diabetic rats was to compensate the decreased GSH levels through reduction of

the oxidized glutathione (GSSG), which might be increased due to the presence of high levels of free radicals in DM. On the other hand, treatment of diabetic rats with *A. maurorum* extracts elevated the GSH levels and restored the activity of glutathione reductase in diabetic rats to its normal levels. The liver is frequently damaged during diabetes, as a consequence of increased levels of oxidative stress and dysregulation of immune function [16]. The degenerative changes in the histology of liver with abnormal localization and infiltration of hepatocytic nuclei were found in STZ-induced diabetes. On the other hand, livers of the diabetic rats that were treated with *A. maurorum* extracts revealed that most of these changes were attenuated from severe to moderate alterations which are in agreement with the finding of Alqasoumi et al. [51].

In conclusion, *A. maurorum* extracts decreased blood glucose levels and increased antioxidant enzymes activities in diabetic rats. In addition, *A. maurorum* extracts suppressed the level of free radicals and dyslipidemia in diabetic rats and consequently could alleviate complications of TD. Further studies to isolate the active components of *A. maurorum* are needed for treatment of diabetes mellitus.

Abbreviations

DM:	Diabetes mellitus
SOD:	Superoxide dismutase
GPx:	Glutathione peroxidase
GR:	Glutathione reductase
GST:	Glutathione-S-transferase
TG:	Triacylglycerol
TC:	Total cholesterol
LDL-C:	Low density lipoprotein-cholesterol
HDL-C:	High density lipoprotein-cholesterol
VLDL-C:	Very low density lipoprotein-cholesterol
ROS:	Reactive oxygen species
MDA:	Malondialdehyde
STZ:	Streptozotocin
ALT:	Alanine aminotransferase
AST:	Aspartate aminotransferase
OGTT:	Oral glucose tolerance test.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 1746985, 7 pages
http://dx.doi.org/10.1155/2016/1746985

Research Article

Validation of a Reversed-Phase High Performance Liquid Chromatography Method for the Simultaneous Analysis of Cysteine and Reduced Glutathione in Mouse Organs

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Received 4 August 2015; Revised 5 October 2015; Accepted 7 October 2015

Academic Editor: Jara Perez-Jimenez

A depletion of reduced glutathione (GSH) has been observed in pathological conditions and in aging. Measuring GSH in tissues using mouse models is an excellent way to assess GSH depletion and the potential therapeutic efficacy of drugs used to maintain and/or restore cellular redox potential. A high performance liquid chromatography (HPLC) method for the simultaneous determination of GSH and cysteine (Cys) in mouse organs was validated according to USA and European standards. The method was based on separation coupled with ultraviolet detection and precolumn derivatization with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The required validation parameters, that are, selectivity, linearity, lower limit of quantification, precision, accuracy, recovery, and stability, were studied for spleen, lymph nodes, pancreas, and brain. The results showed that the lower limits of quantification were 0.313 μM and 1.25 μM for Cys and GSH, respectively. Intraday and interday precisions were less than 11% and 14%, respectively, for both compounds. The mean extraction recoveries of Cys and GSH from all organs were more than 93% and 86%, respectively. Moreover, the stability of both analytes during sample preparation and storage was demonstrated. The method was accurate, reliable, consistent, and reproducible and it was useful to determine Cys and GSH in the organs of different mouse strains.

1. Introduction

GSH is the prevalent nonprotein thiol in animal cells and the most abundant antioxidant in aerobic cells. It is implicated in many cellular functions, such as degradation and synthesis of proteins and DNA or detoxification of toxins and carcinogens [1].

A depletion or an imbalance of GSH has been observed in several pathological conditions such as neurodegenerative diseases, cystic fibrosis, viral infections, AIDS, diabetes, cancer, and ageing [2, 3]. Moreover, GSH content plays an important role in regulating cellular immune response [4]. Under conditions of moderate oxidative stress, oxidation of Cys residues can lead to the reversible formation of mixed disulfides between protein thiol groups and low-molecular-mass thiols (S-thiolation), particularly with GSH (S-glutathionylation). S-Glutathionylated proteins can be readily reduced to free thiol groups when normal cellular redox status is recovered by reducing agents [5]. Moreover, to restore GSH levels, cells can use Cys causing decrease in this amino acid content [6]. Hence, GSH and Cys could be considered important biomarkers to assess the degree of oxidative damage and the correct redox state replenishment. Development and validation of simple analytical methods to measure GSH and other thiols in biological samples are a prerequisite to obtain an accurate assessment of the

degree of oxidative damage as well as indication of disease progression and consequently evaluation of the effectiveness of antioxidant therapy [5]. In fact, molecules able to augment intracellular GSH levels have been proposed as potential therapeutic tools to combat several diseases and in new immunomodulatory approaches [7]. Hence, determining thiol status is important to understand the basic biochemical response of cells during a pathological condition as well as during ageing and the capacity of drugs to restore cellular glutathione homeostasis.

A lot of methods for measuring thiol species in biological fluids and tissue samples have been evolved [8–15]. However, a few methods have been validated according to US and European standards [9, 10].

Our goal was to validate a simple and rapid method, which allowed the simultaneous determination of the main thiol species, such as GSH and Cys, in different mouse organs by using reversed-phase high performance liquid chromatography (RP-HPLC) with ultraviolet detection. The method was based on precolumn derivatization with Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid), (DTNB)] which reacted with R-SH to form the R-TNB derivative which was separated and quantified. Among the different available derivatization methods, the quantification of thiols by DTNB was selected because it has been described to be particularly simple and useful in the study of thiol redox state and protein glutathionylation [16]. Moreover, RP-HPLC method has the advantage of being accessible to most analytical laboratories since they do not require expensive dedicated instruments.

The method proved good in quality and performance and allowed determining GSH and Cys in the organs of three mouse strains (ICR (CD-1), BALB/cJ, and C57BL/6N mice) commonly used in preclinical studies.

2. Materials and Methods

2.1. Chemicals. Cys, GSH, and DTNB were purchased from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO, USA). Acetonitrile was acquired from Carlo Erba (Carlo Erba Reagenti, Milan, Italy).

2.2. Ethics Statement. Housing and treatment of mice were in compliance with the recommendations in the Guide for the Care and Use of Laboratory Animals by the Health Ministry, law 116, 1992. Experiments were approved by the Committee on the Ethics of Animal Experiments of the University of Urbino "Carlo Bo" and Sapienza University. The animals were suppressed by carbon dioxide. Every effort was made to minimize animal suffering and to limit the number of animals used.

2.3. Animals. Four-week-old female ICR (CD-1) and six-week-old female BALB/cJ mice were purchased from Harlan Nossan (Milan, Italy), while four-week-old female C57BL/6N mice were purchased from Charles River (Lecco, Italy). Throughout the study, the mice were kept at a temperature of 22 ± 1°C and a relative humidity of 60 ± 5%, with a 12 h light/dark cycle and 12 air changes/h.

2.4. High Performance Liquid Chromatography Apparatus. Cys and GSH determination in different organs was performed through HPLC Jasco Model LG-980-02 (Jasco Europe S.R.L., Cremella (LC), Italy). The separation was performed on a Teknokroma Tracer Excel 120 column ODSA 5 μm 15 × 0.46 (Teknokroma Analitica S.A., Barcelona, Spain) protected by a Teknokroma Tracer Excel guard column ODS 10 × 3.2 mm (Teknokroma Analitica S.A., Barcelona, Spain). The mobile phase consisted of KH_2PO_4 solution (10 mM, pH 6.0) (buffer A) and buffer A containing acetonitrile (60% v/v) (buffer B). All buffer solutions after preparation and pH adjustment as well as standards were filtered through 0.22 μm Acrodisc Syringe Filters (Pall Life Sciences, Ann Arbor, MI, USA). The elution conditions were as follows: 10 min 100% buffer A, followed by an increase to 100% buffer B in 15 min; this condition was maintained for 5 min. The gradient was returned to 100% buffer A in 3 min, and the column was regenerated with 100% buffer A for another 4 min before injection of the next sample. The flow rate was 1 mL/min, the injection volume was 50 μL, and detection was at 330 nm. Analyses were performed at 25°C and quantitative measurements were obtained by injection of standards of known concentration.

2.5. Sample Preparation. The organs (spleen, lymph nodes, brain, and pancreas) were quickly excised at the same time of the day (9 a.m.–11 a.m.), 10–20 mgs were immediately put into an Eppendorf microcentrifuge tube containing 500 μL of precipitating solution (100 mL containing 1.67 g of glacial metaphosphoric acid, 0.2 g of disodium EDTA, and 30 g of NaCl). The sample was first homogenized through a grinding pestle and then sonicated at 50 watts for 10 seconds (B. Braun Labsonic U, B. Braun Biotech International); all of these procedures were carried out in ice. The sample was kept in ice for 10 min and then centrifuged at 12,000 ×g for 10 min at 4°C. Fifteen μL of 0.3 M Na_2HPO_4 were added to 60 μL of the acid extract and immediately after 45 μL DTNB were added. DTNB solution was prepared dissolving 20 mg of DTNB in 100 mL of sodium citrate solution (1% w/v). The mixture was stirred for 1 minute at room temperature (RT) and then left at RT for another 5 minutes and finally used for Cys and GSH determination by RP-HPLC.

2.6. Method Validation. The method was validated according to the currently accepted US-FDA Bioanalytical Method Validation Guidance and European Medicines Agency Guideline on Bioanalytical Method Validation with respect to selectivity, linearity, lower limit of quantification (LLOQ), precision and accuracy, recovery, and stability. The method was validated with organs of ICR (CD-1) mice. Selectivity was assessed by comparing chromatograms of standard preparations with those of mouse organs.

Calibration curves for GSH and Cys were obtained by serial dilutions from a stock solution. The standards were diluted either in water or in the precipitating solution used to precipitate organ proteins. The exact concentrations of standard solutions of GSH and Cys were obtained through spectrophotometer readings at 412 nm following the procedure described by Beutler [17]. The linearity of each calibration

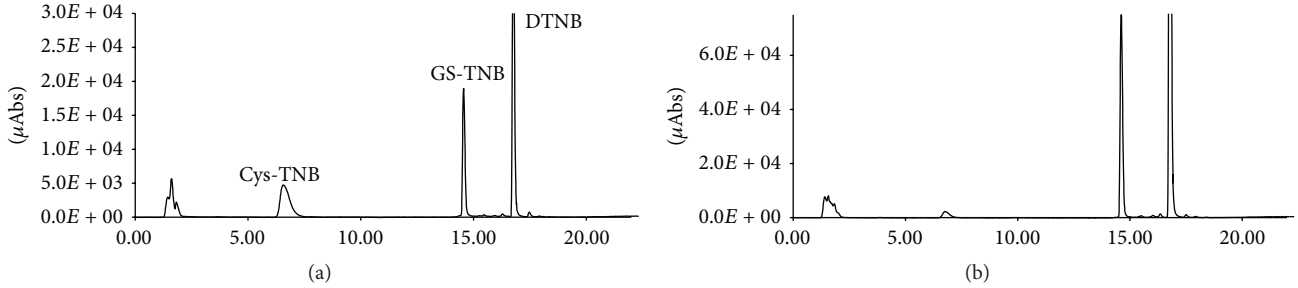

Figure 1: HPLC chromatograms of standards of Cys and GSH (20 μM) (a) and of an extract of mouse spleen (b). The exact concentrations of standard solutions of Cys and GSH were obtained through spectrophotometer readings at 412 nm as described in Section 2.

curve was determined by plotting the peak area (y) versus the corresponding concentration (x). The LLOQ was defined as the lowest calibration standard on the calibration curve with acceptable accuracy within 20% and precision below 20%.

The precision and accuracy of the method were assessed by at least five replicate analyses of organ samples spiked with GSH and Cys ranging from low to high concentrations of the calibration curve. The precision was evaluated in the same analytical run (intraday assay) or in at least five analytical days (interday assay), one of which was in the subsequent week. Precision was defined as the relative standard deviation (RSD%), while accuracy was defined as relative error (RE%), both not exceeding 15%.

Recoveries were calculated by adding known concentrations of GSH and Cys to the organ before submitting it to the processing steps of the method, and the final concentration of each sample represented the mean of five measurements. Results are provided as the difference between the measured and the theoretical values and expressed as percentage of recovery.

2.7. Sample Stability. Sample stability was determined by analyzing organs that were excised, processed, and left for 4 h at room temperature or for 8 h at 4°C or after three freeze-thaw cycles. Sample stability was also evaluated in organs that were excised and frozen in the precipitating solution at −80°C for 3 months. Stability sample results should be within 15% of the analyte concentration encountered in the sample immediately processed.

3. Results and Discussion

Selective, sensitive, and validated analytical methods for the quantitative evaluation of GSH and other thiol species are critical for determination of redox state in experimental models and the successful conduct of preclinical and/or clinical pharmacology studies employing molecules to restore GSH levels that can be altered in pathological conditions [2, 7]. Validating bioanalytical methods demonstrates that a particular method used for quantitative measurement of analytes in a given biological matrix (e.g., mouse organs) is reliable and reproducible. Fundamental parameters for this validation include the following: selectivity, sensitivity, accuracy, precision, reproducibility, and stability. Unfortunately, only a few methods for the determination of GSH and Cys in biological samples have been validated [9, 10]. In this paper, we described validation of a simple, rapid, sensitive, and cost-effective RP-HPLC method for the simultaneous determination of Cys and GSH in different mouse organs according to US and European standards, which can make it of interest to readers who have to measure tissue GSH.

3.1. Selectivity. Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. The retention times of Cys and GSH were 6.7 ± 0.4 and 14.8 ± 0.5 min, respectively. No significant interference from endogenous substances was observed at the retention times of the compounds studied. Figure 1 shows a representative chromatogram of GSH and Cys standards (a) and mouse spleen (b). The chromatograms of other organs were comparable to spleen chromatograms (data not shown).

3.2. Linearity and LLOQ. The standards used for the calibration curve were diluted either in water or in the precipitating solution used to precipitate the organ proteins, obtaining two comparable curves. Figure 2 shows the typical calibration curves and linearity ranges for Cys (a) and GSH (b) diluted in the precipitating solution. The calibration curves were linear in the range of 0.313–50 μM and 1.25–80 μM for Cys and GSH, respectively.

The LLOQ for Cys was 0.313 μM, and the precision and accuracy were less than 8% and within ±5%, respectively. The LLOQ for GSH was 1.25 μM with a precision less than 11% and an accuracy lower than ±9%.

3.3. Sample Stability. Stability of thiol compounds during prolonged storage of the tissues in the protein precipitating buffer or of the deproteinized tissue homogenates is a prerequisite for reliable analysis in experimental setting. Stability was evaluated in samples stored in different conditions as described in Section 2. The results obtained showed that there was no significant difference in the peak areas of Cys and GSH demonstrating the high stability of these thiol species and the validity of the sample preparation protocol in preventing their conversions (Table 1). In the first column values are referred to organs left immersed in the protein precipitating solution. In the second, third, and fourth column values are

Table 1: Stability of Cys and GSH in mouse organs.

Sample	−80°C 3 months	RT 4 h	4°C 8 h	Three freeze-thaw cycles
Cys				
Spleen	101.1 ± 4.2	97.6 ± 3.0	99.3 ± 2.9	98.7 ± 1.0
Lymph nodes	97.8 ± 4.6	98.7 ± 7.8	99.8 ± 1.9	98.4 ± 3.9
Pancreas	97.6 ± 2.2	103.0 ± 3.2	96.9 ± 1.8	98.9 ± 1.4
Brain	99.1 ± 2.3	98.4 ± 1.4	99.0 ± 5.1	98.5 ± 3.9
GSH				
Spleen	99.9 ± 2.4	97.7 ± 1.7	98.9 ± 2.9	99.2 ± 1.9
Lymph nodes	98.9 ± 1.5	97.9 ± 2.3	99.1 ± 6.9	100.1 ± 1.9
Pancreas	99.3 ± 4.0	97.9 ± 2.0	98.0 ± 3.5	98.4 ± 4.1
Brain	99.6 ± 2.4	99.9 ± 7.5	99.1 ± 7.3	98.7 ± 1.4

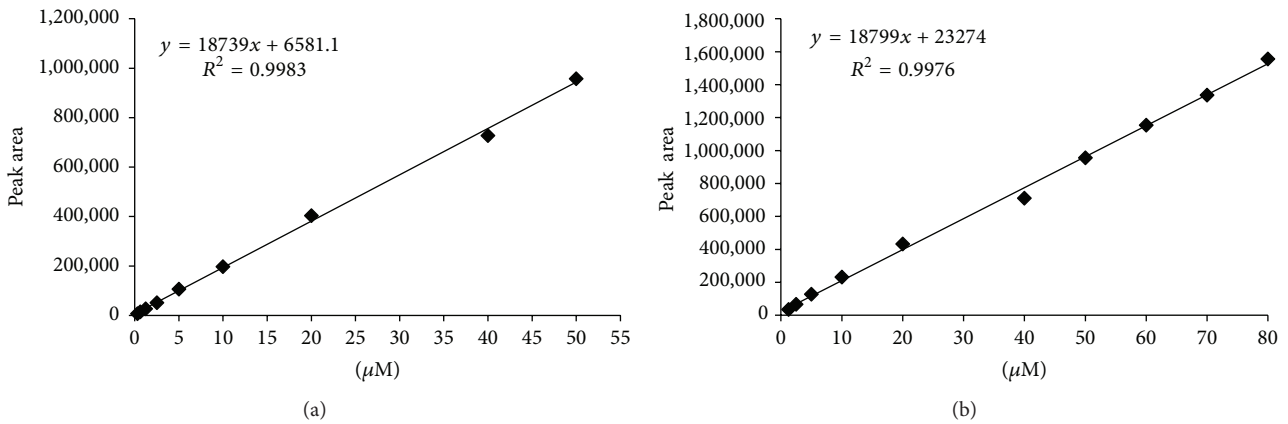

Figure 2: Calibration curves of Cys (a) and GSH (b) diluted in the solution used to precipitate organ proteins. Standards solutions were quantified through spectrophotometer readings at 412 nm as described in Section 2.

referred to as deproteinized organ homogenates (RT: room temperature). Values are the mean ± SD of 5 animals per organ. Stability samples have been compared with the same samples immediately processed and analyzed.

3.4. Precision, Accuracy, and Recovery. The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the actual concentration of the analyte, while the precision describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to a single sample.

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix (mouse organ), compared to the detector response obtained for the true concentration of the analyte in solvent.

We evaluated the precision, accuracy, and recovery of the entire method by analyzing tissue samples spiked with 40 μM, 20 μM, or 2.5 μM of Cys or with 60 μM, 20 μM, or 5 μM of GSH. Each concentration was tested five times, and the data of the assay are shown in Table 2. All the results of the tested samples were within 15%, meeting the acceptable criterion. In particular, in all tissue samples, the intraday precision ($n = 5$) was less than 11%, while the interday precision ($n = 5$) was less than 14% for both thiol species.

The accuracies of all the analyzed samples were less than 9% for both thiol species, except for brain spiked with 5 μM of GSH in which case it was less than 14%, indicating that the developed method is accurate and reliable.

For the recovery, the concentration in the spiked samples was expressed as a percentage of the predicted concentration, which was calculated as the sum of the added concentration and the endogenous level of the analyte in the unspiked sample. The mean extraction recoveries ($n = 5$) in all organ samples were more 93% and 90% for Cys and GSH, respectively, except in the brain spiked with 5 μM GSH where the recovery was more than 86%. These results indicate that the recoveries of both analytes were consistent and reproducible and, in comparison to other HPLC-UV methods used for Cys and GSH determination in biological fluids [10], a higher recovery for both thiol species was obtained. Another advantage of the method is represented by the use of DTNB as derivatizing agent requiring shorter derivatization times. Cys and GSH were also quantified in tissue specimens by a chromatographic system equipped with a fluorescent detector [9], but HPLC with a UV detector belongs to the

Table 2: Summary of precision, accuracy, and recovery of the assay for GSH and Cys in mouse spleens, lymph nodes, pancreas, and brains ($n = 5$).

Sample	Concentration (μM)	Intraday (RSD%)	Interday (RSD%)	Accuracy (RE%)	Recovery (%, mean ± SD)
Cys					
Spleen	2.5	4.6	11.7	3.8	98.4 ± 1.3
	20	5.3	8.0	5.8	96.8 ± 2.4
	40	4.6	3.9	2.6	97.7 ± 2.1
Lymph nodes	2.5	6.9	8.9	5.1	97.0 ± 2.3
	20	9.0	12.9	4.8	96.8 ± 2.6
	40	5.0	11.7	6.2	93.9 ± 5.7
Pancreas	2.5	9.1	10.7	4.3	94.2 ± 5.6
	20	7.5	6.8	8.5	94.5 ± 4.2
	40	6.2	9.9	5.7	95.6 ± 2.7
Brain	2.5	9.7	6.7	4.6	95.9 ± 2.6
	20	10.3	10.6	3.4	94.5 ± 4.0
	40	8.7	11.8	6.7	96.4 ± 2.8
GSH					
Spleen	5	3.3	7.5	3.5	92.7 ± 2.2
	20	10.8	9.7	3.3	93.5 ± 4.6
	60	9.0	12.4	1.9	94.7 ± 5.2
Lymph nodes	5	10.8	12.4	8.9	90.4 ± 2.0
	20	1.9	6.2	3.7	96.4 ± 1.5
	60	1.5	9.7	4.5	93.7 ± 4.8
Pancreas	5	9.9	12.8	3.3	91.4 ± 2.8
	20	7.5	9.4	5.0	97.4 ± 3.2
	60	9.3	13.7	6.4	91.1 ± 6.3
Brain	5	2.6	12.6	13.7	86.5 ± 5.0
	20	4.4	10.7	1.2	98.2 ± 3.7
	60	9.0	8.1	5.3	94.3 ± 7.2

standard instrumentation of an analytical laboratory not requiring particular expensive maintenance.

3.5. Determination of Cys and GSH in Organs of Different Mouse Strains. The validated method was successfully applied to determine the concentrations of Cys and GSH in the spleen, lymph nodes, pancreas, and brain of ICR (CD-1), BALB/cJ, and C57BL/6N mice. The data reported in Figure 3 show similar content of GSH (right) in the spleen (a), pancreas (c), and brain (d) of all strains, while a lower content of GSH was found in the lymph nodes (b) of BALB/cJ. The concentration of Cys (left) found in spleen (a) and pancreas (c) was lower in this strain than that in the other ones; moreover the brain was assayed for Cys but it was not detected (d) and in only one lymph node sample (b) it was detectable. This is likely due to the lower concentration of Cys in these two organs of BALB/Cj mice; moreover, we can observe that in this strain Cys levels were lower compared to the other strains, even when measured above LLOQ. The applicability of the proposed method was assessed through the analysis of GSH and Cys in other mouse organs such as liver, kidney, lungs, and heart (Supplementary Table in the Supplementary Material available online at http://dx.doi.org/10.1155/2016/1746985) as well as the analysis of GSH-replenishing molecules containing –SH groups which were identified and clearly distinguished from GSH and Cys [18].

These data provide reliable reference measurements of GSH and Cys in the spleen, lymph nodes, pancreas, and brain of three mouse strains widely used in preclinical studies. Particularly, the data reported are a valuable resource for investigating the role of GSH in modulating several intracellular processes, from oxidative damage to immune responses, as well as the effects of drugs and toxic compounds on glutathione metabolism.

4. Conclusions

Results obtained from validation of the RP-HPLC method herein described show that the method is accurate, reliable, consistent, and reproducible. Moreover, the sample preparation and the extraction procedure developed allow high stability of Cys and GSH in the samples preventing their conversions. The main advantages of the present method are validation, high recovery, simplicity, short derivatization times, and low analytical costs. Therefore, this method is particularly suitable for reliable routine measurement of thiols in mouse organs and it can be used in all of those

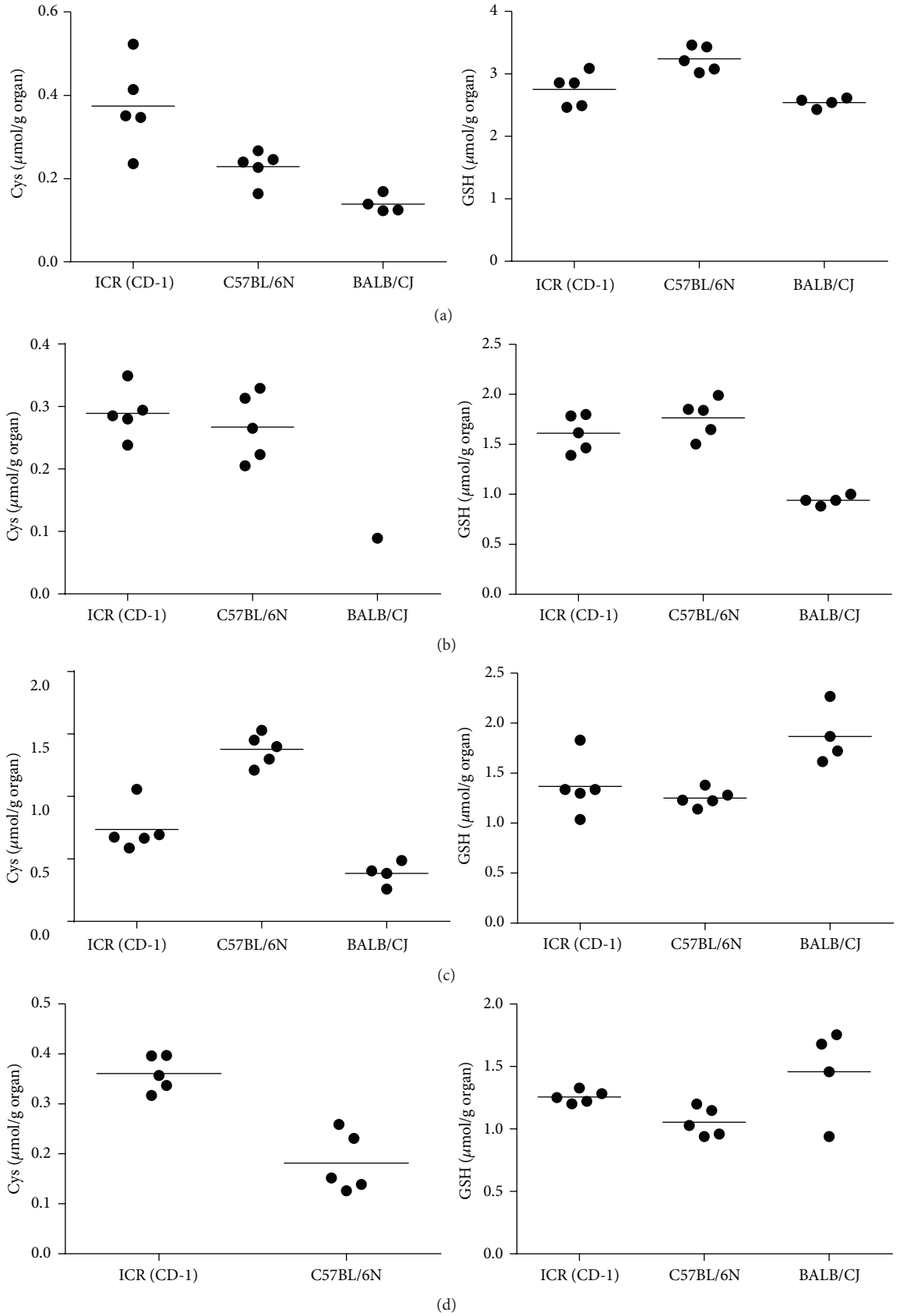

Figure 3: Quantification of Cys (left) and GSH (right) by HPLC in spleens (a), lymph nodes (b), pancreas (c), and brains (d) of ICR (CD-1) ($n = 5$), C57BL/6N ($n = 5$), and BALB/Cj ($n = 4$) mice.

animal models mimicking human diseases characterized by GSH imbalance to both study disease processes and develop therapies including GSH-based antioxidant treatment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by PRIN (Research Projects of National Interest) 2010-2011-prot. 2010PHT9NF_004 granted to A. Fraternale, PRIN (Research Projects of National Interest) 2010-2011-prot. 2010PHT9NF_005 granted to L. Nencioni, and PON 01-01802 granted to A. T. Palamara. The authors wish to thank Professor Timothy C. Bloom for his linguistic revision of the paper.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 7174351, 8 pages
http://dx.doi.org/10.1155/2016/7174351

Research Article

Antioxidant Potential of *Spirulina platensis* Mitigates Oxidative Stress and Reprotoxicity Induced by Sodium Arsenite in Male Rats

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Received 22 October 2015; Revised 7 December 2015; Accepted 15 December 2015

Academic Editor: Ilaria Peluso

The present study aimed to examine the protective role of *Spirulina platensis* (*S. platensis*) against arsenic-induced testicular oxidative damage in rats. Arsenic (in the form of $NaAsO_2$ at a dose of 6.3 mg/kg body weight for 8 weeks) caused a significant accumulation of arsenic in testicular tissues as well as a decrease in the levels of testicular superoxide dismutase (SOD), catalase (CAT), reduced glutathione, and zinc. Moreover, it significantly decreased plasma testosterone, luteinizing hormone (LH), triiodothyronine (T_3), and thyroxine (T_4) levels and reduced sperm motility and sperm count. Arsenic (AS) led to a significant increase in testicular malondialdehyde (MDA), tumour necrosis factor alpha (TNF-α), nitric oxide (NO), and sperm abnormalities. *S. platensis* at a dose of 300 mg/kg was found to attenuate As-induced oxidative stress, testicular damage, and sperm abnormalities by its potent antioxidant activity. *S. platensis* may represent a potential therapeutic option to protect the testicular tissue from arsenic intoxication.

1. Introduction

Arsenic contamination occurs due to its industrial uses in the production of agricultural pesticides, wood preservatives, and glass production and in medicine [1, 2]. Arsenic exposure causes obvious damage in various organs, including the male reproductive function as manifested by decrease of androgenesis, suppression of spermatogenesis, and a reduction in the weight of testes and sex organs [3, 4]. However, emerging evidence supports the role of oxidative stress and inflammation with increased production of proinflammatory cytokines in the pathogenesis of arsenic-induced organ damage [5, 6]. Also, previous studies revealed that several antioxidant agents significantly protected against tissue damage due to arsenic intoxication [6, 7].

The cyanobacterium *Spirulina* is a filamentous blue-green alga belonging to the Oscillatoriaceae family that is generally found in tropical and subtropical regions in warm alkaline water. Spirulina is characterized by high nutritional value where it contains high protein content (60–70% by dry weight), plenty of vitamins, amino acids, gamma-linoleic acid, and minerals [8]. The consumption of *Spirulina* as a diet supplement has health benefits in preventing or managing hypercholesterolemia [9], hyperglycerolemia [10], obesity, inflammation [11], cancer [12], and cardiovascular disease [13]. In addition, *Spirulina* has antidiabetic effect [14]. *Spirulina* provides protection against mercuric chloride-induced oxidative stress and alteration of antioxidant defense mechanism in the liver. These activities were largely related to phycocyanin, an active protein of *Spirulina* [15]. Phycocyanin (Pc) is a biliprotein of the blue-green alga. This protein contains a tetrapyrrole phycocyanobilin, which is responsible for antioxidant properties of Pc [16]. It has been reported that Pc has significant antioxidant and radical scavenging properties, offering protection against oxidative stress [17]. Antioxidants can reduce arsenic toxicity through chelating

it and scavenging free radicals [18]. It was reported that Pc can bind with heavy metals [19]; hence, it can chelate and remove them. In view of the above concerns, the present study was designed to evaluate the antioxidant action of *S. platensis* enriched with phenolic compounds in ameliorating testicular dysfunction and oxidative stress induced by arsenic.

2. Materials and Methods

2.1. Test Chemicals. Sodium arsenite was purchased from Merck, Germany, while *S. platensis* was obtained from Alibaba Comp., China, in the form of powder.

2.2. Animals. Four-month male Wistar albino rats, weighting 180–200 g, were got from the animal house, Faculty of Pharmacy, King Saud University. Animals were housed and fed as previously described [20]. The rats were fed a commercially available rat pellet diet *ad libitum* throughout the experimental period. The rats allowed to adapt to laboratory environment for seven days before the beginning of the experiment. This study was performed in the Zoology Department, Faculty of Science, King Saud University, Saudi Arabia. The care and handling of experimental animals were carried out according to the animal ethical committee of King Saud University, College of Pharmacy.

2.3. Experimental Protocol. The animals were randomly divided into four groups, consisting of eight rats in each, and they were treated for eight weeks as below:

Group I: normal control (rats received only water as vehicle).

Group II: rats received orally arsenic as sodium arsenite, 6.3 mg/kg corresponding to 15% of LD50 (41 mg/Kg) [21].

Group III: rats received orally 300 mg/Kg of *S. platensis* [22] followed by oral administration of arsenic as sodium arsenite 6.3 mg/Kg daily.

Group IV: rats received orally *S. platensis* only as in group III.

All treatments are carried out daily for eight weeks in order to evaluate their effects [23]. The rats were subjected to ether anesthesia using sliding top chamber (Kent Scientific corporation) during sample collection.

2.4. Sample Preparation and Biochemical Analysis. At the end of the experimentation period, blood and organs were collected as previously described [20]. Plasma testosterone, luteinizing hormone (LH), triiodothyronine (T_3), and thyroxine (T_4) concentrations were assayed by enzyme immunoassay using commercial kits from Diagnostic products Co., Los Angeles, CA, USA. Testes, vas deferens, epididymis, prostate gland, and seminal vesicle were isolated from surrounding tissues and placed into tubes. The organs were dried between two sheets of filter paper and their wet weight was determined. The organ weight/body weight ratio × 100 was calculated and expressed as relative organ weight beside absolute weight. Epididymis and testes were processed as previously described in order to perform histological, biochemical, and sperm analysis [20].

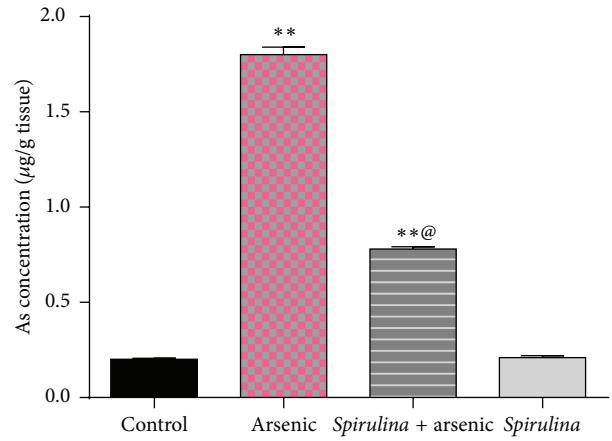


Figure 1: Effect of *Spirulina platensis* on testicular arsenic concentration (μg/g tissue) in arsenic intoxicated rats. All numbers are mean + standard error, $n = 8$. AS: arsenic. **Significantly different from control value, $^{**}P < 0.01$. $^{@}$Significantly different from arsenic group value, $^{@}P < 0.01$.

The supernatant of testicular homogenates was used for determination of malondialdehyde, reduced glutathione, catalase, and superoxide dismutase levels using colorimetric assay kits according to the recommendations of the manufacturer (BioDiagnostic, Egypt). The testicular level of nitric oxide was assayed using colorimetric assay kit following the manufacturer's instructions (Cayman Chemical Company, USA). Also, the level of tumour necrosis factor-α in testicular homogenates was determined by enzyme-linked immunosorbent assay (ELISA) using rat TNF-α immunoassay kit according to the guidance of the manufacturer (R&D Systems, USA). In addition, arsenic and zinc levels in testes were estimated by atomic absorption (Perkin-Elmer, UK).

2.5. Sperm Analysis. Sperm motility, count, and abnormalities were evaluated as previously described [20, 24].

2.6. Statistical Analysis. All values were expressed as mean ± SE. Statistical analysis of data was performed using two-way ANOVA followed by least significant difference (LSD) for comparison of various treatments using the spss 13.0.

3. Results

3.1. Biochemical Analysis. The results demonstrated that supplementation of *Spirulina* to arsenic exposed rats reduced the arsenic content remarkably in the testis (Figure 1). On the other hand, testicular zinc concentration of arsenic treated groups (Figure 2) decreased significantly as compared with control. Testicular zinc concentration in *S. platensis* + arsenic group is significantly higher than those treated with arsenic only. Arsenic treatment without *S. platensis* significantly enhanced the levels of testicular MDA, TNF-α, and nitric oxide concentrations ($P \leq 0.1$), while SOD, catalase, and

Table 1: Effect of *S. platensis* on testicular oxidative stress parameters in arsenic treated rats.

Parameter	Treatment			
	Control	Arsenic	*S. platensis* + arsenic	*S. platensis*
MDA (nmol/mg protein)	10.48 ± 0.36	22.83 ± 0.89**	14.95 ± 0.65$^{**@}$	8.76 ± 0.67*
SOD (unit/mg protein)	24.27 ± 0.65	9.11 ± 0.35**	16.81 ± 0.44$^{**@}$	26.89 ± 0.21**
Catalase (μmol/min/mg protein)	30.28 ± 1.06	15.86 ± 0.35**	22.75 ± 0.74$^{**@}$	32.11 ± 2.10
GSH (nmol/mg protein)	27.25 ± 1.47	14.13 ± 0.89**	21.25 ± 1.14$^{**@}$	39.87 ± 1.86**
TNF-α (pg/100 mg tissue)	11.48 ± 0.21	108.12 ± 3.15**	46.92 ± 2.71$^{**@}$	10.65 ± 0.47
Nitric oxide (nmol/100 mg tissue)	85.20 ± 4.14	216.92 ± 5.78**	130.41 ± 6.37$^{**@}$	87.14 ± 3.60

All numbers are mean + standard error, $n = 8$.
*Significantly different from control value, $^{*}P < 0.05$, $^{**}P < 0.01$.
$^{@}$Significantly different from arsenic group value, $^{@}P < 0.01$.
MDA: malondialdehyde; SOD: superoxide dismutase; GSH: reduced glutathione; TNF-α: tumor necrosis factor-alpha.

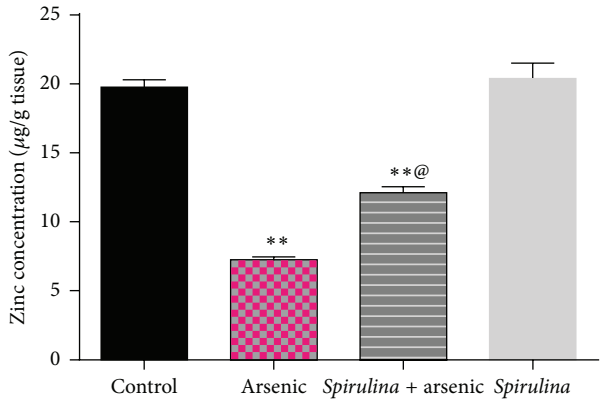


Figure 2: Effect of *Spirulina platensis* on testicular zinc concentration (μg/g tissue) in arsenic intoxicated rats. All numbers are mean + standard error, $n = 8$. **Significantly different from control value, $^{**}P < 0.01$. $^{@}$Significantly different from arsenic group value, $^{@}P < 0.01$.

GSH levels decreased significantly as compared with control (Table 1). The administration of *S. platensis* followed by arsenic intoxication attenuated these effects.

3.2. Reproductive Organ Weights. Sodium arsenite intoxication significantly decreased the testis, vas deferens, epididymis, prostate, and seminal vesicle weights. Treatment with *Spirulina* prior to arsenic administration, however, kept the weight of reproductive organs close to normal (Tables 2(a) and 2(b)).

3.3. Plasma Hormones Level. We observed that sodium arsenite intoxication decreased the levels of testosterone, LH, T_3, and T_4 significantly ($P \leq 0.01$) compared to control values (Figures 3 and 4). Treatment with *S. platensis* was found to be effective in alleviation of alteration in hormone levels induced by the arsenic.

3.4. Sperm Motility, Count, and Abnormalities. Arsenic intoxication decreased the sperm motility and count compared to the normal control (Table 3). In addition, a significant increase of sperm abnormalities was found in rats treated with arsenic. *S. platensis* administration reduced the toxic effects of arsenic on sperms.

3.5. Histopathological Observation. Histological observation of the testes of control animals showed normal spermatogenic cells with normal arrangement (Figure 5(a)). The section of testis of arsenic treated rat showed (Figure 5(b)) thickening of tubules basement membrane, vascular degeneration, marked decrease in spermatogenic cells population, hemorrhage in interstitial tissues, and deformation of Leydig cells. Moreover, the sperm bundles were absent in some tubules. Pretreatment with *S. platensis* could, however, prevent the As-toxicity and maintain the normalcy of the testicular architecture (Figure 5(c)).

4. Discussion

The response of male rats to the protective effects of *S. platensis* against arsenic-induced oxidative stress and reprotoxicity was examined in this study. Our results proposed that the increase of testicular MDA level may result from arsenic accumulation in the testis suggesting oxidative stress following free radical generation. Enhancement of lipid peroxidation and inhibition of the antioxidant enzymes in the testes are important mechanisms for arsenic pathogenesis [25]. The testicular tissue is provided with an antioxidant defense system including several enzymes functioning in a collective manner for the removing free radicals generated within the cell. SOD and catalase are major enzymes that get rid of reactive oxygen species (ROS) [26]. In the present study, the animals treated with arsenic showed decreased activities of testicular antioxidant enzymes, SOD, and CAT that may indicate the antioxidant imbalance induced by arsenic. A decrease in the activity of SOD can be referred to as an enhanced superoxide production during arsenic metabolism. SOD catalyzes the dismutation of superoxide anions and prevents the subsequent formation of hydroxyl radicals [27]. The observed decreased testicular SOD might be responsible for increased lipid peroxidation following arsenic treatment [28]. The superoxide radical also reduced the activity of catalase [29]. Moreover, exposure to arsenic reduces the

Table 2: (a) Absolute reproductive organ weights (g) of arsenic intoxicated rats treated with *S. platensis*. (b) Effect of *S. platensis* on reproductive organ weights (g) relative to body weight in arsenic intoxified rats.

(a)

Parameter	Treatment			
	Control	Arsenic	*S. platensis* + arsenic	*S. platensis*
Left testis	1.76 ± 0.05	1.40 ± 0.07**	1.67 ± 0.05$^{@}$	1.79 ± 0.08
Vas deferens	0.21 ± 0.01	0.13 ± 0.01**	0.17 ± 0.007**	0.19 ± 0.006
Epididymis	0.86 ± 0.03	0.57 ± 0.02**	0.73 ± 0.03*	0.81 ± 0.04
Prostate	0.80 ± 0.02	0.37 ± 0.03**	0.65 ± 0.05$^{**@}$	0.77 ± 0.05
Seminal vesicle	1.51 ± 0.08	0.86 ± 0.06**	1.18 ± 0.06$^{**@}$	1.46 ± 0.07

(b)

Parameter	Treatment			
	Control	Arsenic	*S. platensis* + arsenic	*S. platensis*
Left testis	0.62 ± 0.02	0.51 ± 0.02**	0.59 ± 0.01$^{@}$	0.60 ± 0.02
Vas deferens	0.07 ± 0.003	0.05 ± 0.002**	0.05 ± 0.002**	0.06 ± 0.005
Epididymis	0.25 ± 0.002	0.21 ± 0.006**	0.23 ± 0.007*	0.24 ± 0.008
Prostate	0.31 ± 0.02	0.13 ± 0.01**	0.20 ± 0.009$^{**@}$	0.29 ± 0.01
Seminal vesicle	0.52 ± 0.01	0.31 ± 0.01**	0.47 ± 0.02$^{*@}$	0.50 ± 0.04

All numbers are mean + standard error, $n = 8$.
*Significantly different from control value, $^{*}P < 0.05$, $^{**}P < 0.01$.
$^{@}$Significantly different from arsenic group value, $^{@}P < 0.01$.

Table 3: Effect of *S. platensis* on sperm morphological parameters in experimental arsenic exposed rats.

Parameter	Treatment			
	Control	Arsenic	*S. platensis* + arsenic	*S. platensis*
Sperm motility (%)	83.56 ± 1.18	72.29 ± 2.00**	84.56 ± 0.67$^{@}$	90.42 ± 2.10*
Sperm count per epididymis (million/epididymis)	17.47 ± 1.06	7.09 ± 0.41**	12.68 ± 0.85$^{**@}$	25.5 ± 1.15**
Abnormal sperm rate (%)				
Head	2.16 ± 0.13	8.92 ± 0.56**	4.94 ± 0.19$^{**@}$	1.90 ± 0.07
Tail	1.83 ± 0.11	2.88 ± 0.14**	2.14 ± 0.18$^{@}$	2.11 ± 0.12
Total	3.99 ± 0.14	11.34 ± 0.51**	6.92 ± 0.32$^{**@}$	4.00 ± 0.16

All numbers are mean + standard error, $n = 8$.
*Significantly different from control value, $^{*}P < 0.05$, $^{**}P < 0.01$.
$^{@}$Significantly different from arsenic group value, $^{@}P < 0.01$.

testicular GSH content of the present rats as previously found [30, 31].

The improved antioxidant status of testicular tissues by *S. platensis* can be deduced from elevated levels of testicular SOD, CAT, zinc, and GSH and a decrease of MDA and arsenic concentrations of *S. platensis* + arsenic group as compared to arsenic group. The antioxidant properties of *S. platensis* may be attributed to the presence of potent antioxidant components as β-carotene, vitamin C, vitamin E, selenium, and manganese [32–37]. Moreover, phycocyanin of *S. platensis* significantly inhibited peroxyl radical induced lipid peroxidation [16] and it may chelate arsenic as it binds with heavy metals [38].

Free radicals are able to induce cytokine production from various cell types [39]. The decreased antioxidant enzyme activities with elevated lipid peroxidation, TNF-α, and NO levels indicated impaired antioxidative defense mechanisms with an oxidative injury in the testes of arsenic group. It was reported that there was a link between TNF-α or NO and oxidative stress. Both TNF-α and NO can increase the production of reactive oxygen species and oxidative stress [40, 41]. It was found that both NO and TNF-α inhibited testosterone synthesis pathways [42, 43]. The significant decrease in the plasma level of testosterone in the present rats treated with arsenic may be due to its direct effect on the testis or suppression of luteinizing hormone secretion. *S. platensis* represses proinflammatory cytokine expression and secretion through suppression of nuclear factor kappa (NF-κB). Activation of NF-κB pathway is a major pathway for the development of inflammatory diseases [44]. The antioxidants

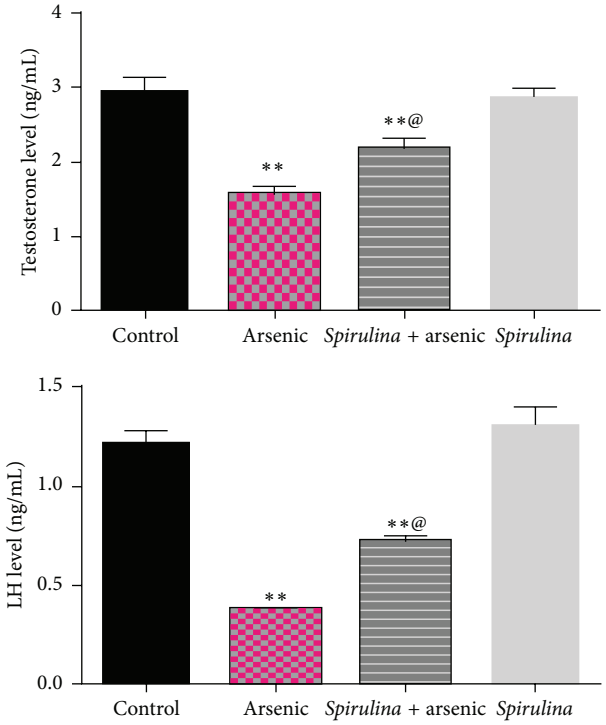


Figure 3: Effect of *S. platensis* on plasma testosterone and luteinizing hormone (LH) levels in arsenic intoxicated rats. All numbers are mean + standard error, $n = 8$. **Significantly different from control value, $^{**}P < 0.01$. $^{@}$Significantly different from arsenic group value, $^{@}P < 0.01$.

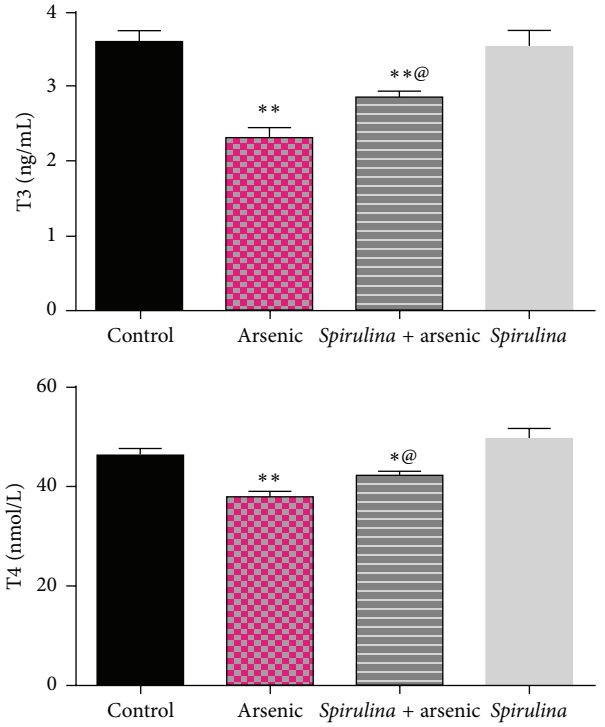


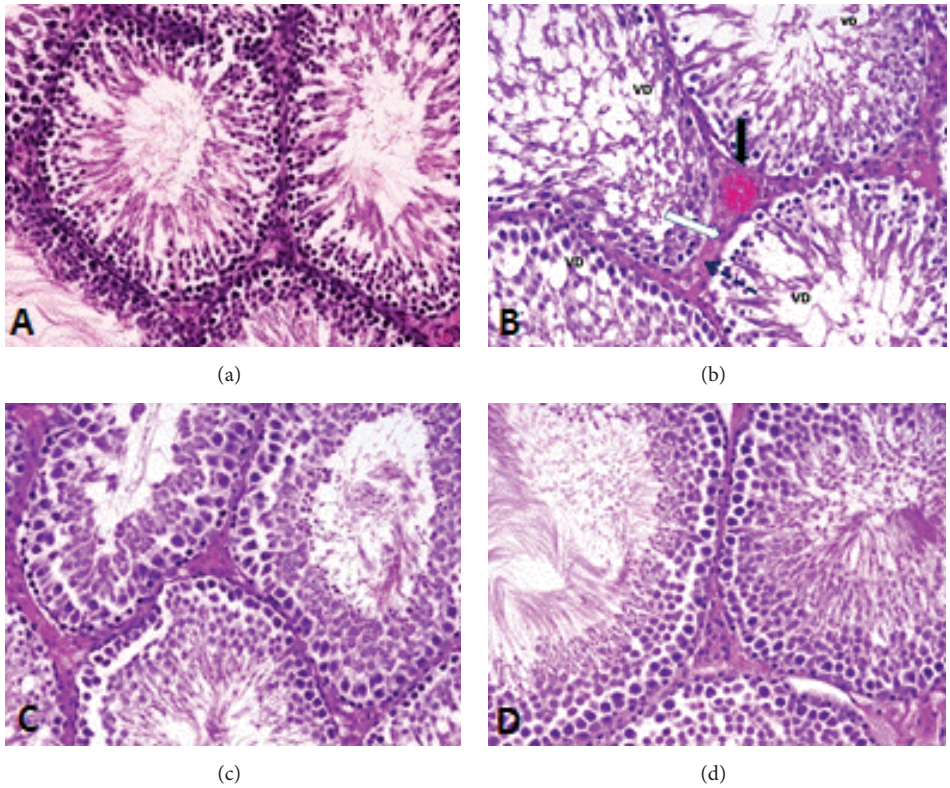
Figure 4: Effect of *S. platensis* on T3 and T4 concentrations in arsenic intoxicated rats. All numbers are mean + standard error, $n = 8$. *Significantly different from control value, $^{*}P < 0.05$, $^{**}P < 0.01$. $^{@}$Significantly different from arsenic group value, $^{@}P < 0.01$. T3: triiodothyronine; T4: thyroxine.

found in *S. platensis* maintain the endogenous antioxidants and inhibit elevation of testicular NO and TNF-α, thus reducing oxidative stress and relieving the pathological changes induced by arsenic in testis which may lead to improvement of testosterone level.

A significant decrease in the weights of testis and accessory sex organs was observed in arsenic exposed rats, which may be due to the inhibition of spermatogenesis and decreased steroidogenesis. It is well known that the testosterone stimulates normal growth and function of male reproductive system [45]. The weight of the testis is also largely dependent on the mass of the differentiated spermatogenic cells and reduction in the testicular weight indicates germ cell loss [3]. Our results showed that *S. platensis* alleviated the reduction in T_3 and T_4 levels induced by arsenic. It is well known that thyroid hormones affect spermatogenesis [46]. In addition, the number of sperm production by testes was decreased significantly in hypothyroid rats and increased in hyperthyroid rats in comparison with the control group rats. It was shown that thyroid hormone receptor expresses in the germ cells from spermatogonia to primary spermatocytes [47].

A higher ROS production or a decreased antioxidant capacity is responsible for stimulation of lipid peroxidation production which affects sperm motility [48]. The observed decrease in the number of sperm count and motility and increase of sperm morphological abnormalities may result from less production of androgen in arsenic exposed rats or from increased level of testicular lipid peroxidation. Spermatozoa are particularly liable to ROS-induced damage because their plasma membranes have large quantities of polyunsaturated fatty acids and their cytoplasm comprises low concentration of the scavenging enzymes [49]. It is documented that ROS generation can induce abnormal sperm morphology [50]. It appeared that *S. platensis,* containing potent antioxidants, significantly reversed the deleterious effects of arsenic on sperms. Thus, the antioxidative properties of *S. platensis* may play a positive role in the defense against oxidative stress induced by arsenic. Our previous findings clearly highlight the role of *S. platensis* as a protective modulator of mercuric chloride-induced testicular injuries and oxidative stress [20]. Here, *S. platensis* significantly lessen the increase in arsenic concentration, and the reduction in zinc concentration of testicular tissue resulted from sodium arsenite administration. Zinc acts as a cofactor for superoxide dismutase, preserves the reduced glutathione, and induces metallothionein which has antioxidant and metal-chelating properties [51]. Zinc acts as an effective anti-inflammatory and antioxidant agent [52]. It can be speculated that *S. platensis* through its antioxidant activity decreased the arsenic burden in testicular tissue and restored the depleted zinc which results in an additional protective effect against arsenic-mediated testicular toxicity.

The present investigation showed that the treatment of the rats with *S. platensis* improves sperm characteristics as



(a) (b)

(c) (d)

Figure 5: (a) Photomicrograph of the testis of control rat showing normal structure of seminiferous tubules containing different types of spermatogenic cells (H&E, ×400). (b) Photomicrograph of the testis of rat that received arsenic showing deformed Leydig cells (white arrow), vacuolated spermatogenic cells (VD), thickened basement membrane (dotted arrow), and congestion of blood vessel (black arrow) (H&E, ×400). (c) Photomicrograph of the testis of rat treated with *S. platensis* + As showing normal spermatogenesis and cell arrangement (H&E, ×400). (d) Photomicrograph of the testis of rat treated with *S. platensis* showing normal structure (H&E, ×400).

manifested by increase of sperm motility and count. The improvement of sperm parameters may be due to antioxidant components of *S. platensis* [53, 54].

In conclusion, the protective actions of *S. platensis* against arsenic are believed to originate from its free radical scavenging, antioxidant activities, maintenance of antioxidant enzymes, and a decrease in the production of inflammatory mediators that are implicated in the pathogenesis of arsenic-induced testicular injury. Therefore, *S. platensis* represents a potential agent to prevent testicular injury and dysfunction induced by arsenic exposure.

Conflict of Interests

There is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors would like to thank Dr. Ibrahim EL-Hazza Scientific Group, for their guidance and helping during the experiment period. The authors would also like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for funding this Research Group no. RGP-1435-093.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 2427618, 15 pages
http://dx.doi.org/10.1155/2016/2427618

Research Article

Dietary Phenolic Compounds Interfere with the Fate of Hydrogen Peroxide in Human Adipose Tissue but Do Not Directly Inhibit Primary Amine Oxidase Activity

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Received 2 October 2015; Accepted 12 November 2015

Academic Editor: Ilaria Peluso

Resveratrol has been reported to inhibit monoamine oxidases (MAO). Many substrates or inhibitors of neuronal MAO interact also with other amine oxidases (AO) in peripheral organs, such as semicarbazide-sensitive AO (SSAO), known as primary amine oxidase, absent in neurones, but abundant in adipocytes. We asked whether phenolic compounds (resveratrol, pterostilbene, quercetin, and caffeic acid) behave as MAO and SSAO inhibitors. AO activity was determined in human adipose tissue. Computational docking and glucose uptake assays were performed in 3D models of human AO proteins and in adipocytes, respectively. Phenolic compounds fully inhibited the fluorescent detection of H_2O_2 generated during MAO and SSAO activation by tyramine and benzylamine. They also quenched H_2O_2-induced fluorescence in absence of biological material and were unable to abolish the oxidation of radiolabelled tyramine and benzylamine. Thus, phenolic compounds hampered H_2O_2 detection but did not block AO activity. Only resveratrol and quercetin partially impaired MAO-dependent [^{14}C]-tyramine oxidation and behaved as MAO inhibitors. Phenolic compounds counteracted the H_2O_2-dependent benzylamine-stimulated glucose transport. This indicates that various phenolic compounds block downstream effects of H_2O_2 produced by biogenic or exogenous amine oxidation without directly inhibiting AO. Phenolic compounds remain of interest regarding their capacity to limit oxidative stress rather than inhibiting AO.

1. Introduction

Resveratrol is a well-known nonenzymatic antioxidant molecule and it has been reported to exert neuroprotective actions for more than a decade [1]. Recently, *trans*-resveratrol and *cis*-resveratrol have been described as inhibitors of recombinant human monoamine oxidases, MAO-A and MAO-B, the two forms of amine oxidases (AO) involved in neurotransmitter metabolism and in the scavenging of endogenous or exogenous amines [2]. Later on, *trans*-resveratrol has been evidenced to dose-dependently inhibit MAO-A in mouse brain in a manner that may participate to its antidepressant effects, while it was less active towards MAO-B [3], a finding which is in agreement with

the pioneering observations of Zhou et al. [4]. Such resveratrol/MAO interaction has attracted great interest since MAOs can be considered as targets of therapeutic approaches for neurodegenerative and psychiatric disorders. However, none of these former studies were performed with the native forms of the human MAO flavoproteins naturally expressed in brain or peripheral tissues. In addition, at least a couple of questions were elicited by this incompletely deciphered interaction between resveratrol and MAO. (1) Does resveratrol interact only with brain MAO or with other members of the AO family? (2) Can phenolic antioxidants other than resveratrol interact with AOs? This context prompted us to further study the interplay between phenolic compounds and AOs.

The AO family encompasses FAD-dependent enzymes (essentially MAO and polyamine oxidase) and copper-containing amine oxidases, having for predominant members the products of the genes AOC1, 2, and 3, the latter being named semicarbazide-sensitive amine oxidase (SSAO) or primary amine oxidase and also currently known as vascular adhesion protein-1 (SSAO/VAP-1) [5, 6]. Many molecules that interact with MAO, even in an inhibitory manner, may also interact with other AOs [7]. To give only a mere example, it can be mentioned that phenelzine, a well-recognized MAO inhibitor used as an antidepressant drug, also inhibits SSAO/VAP-1 [8, 9] and possesses neuroprotective properties [10, 11]. While the different members of the AO family are encoded by distinct genes and exert various biological functions, they have only few specific substrates and inhibitors, since numerous endogenous or exogenous amines exhibit poor selectivity towards the diverse AOs (for an exhaustive review, see the book: [12]). Irrespective of its nature, any enzyme of the AO family oxidizing an amine in the presence of water and dioxygen is releasing the corresponding aldehyde, ammonia, and hydrogen peroxide [13]. Even though being not a radical, the latter end-product, H_2O_2, is a member of the reactive oxygen species (ROS) that readily participate in oxidative stress. Thus, AOs can be included in the cellular ROS-generating systems [13]. We therefore investigated the putative interplay between phenolic compounds (natural ingredients endowed with antioxidant properties) and AO enzymes, supposed to act in an opposite manner on oxidative stress.

Indeed, considering that various so-called MAO inhibitors can also interact with other AOs, we asked whether resveratrol, known to exert antioxidant activities, known to activate sirtuins [14, 15], and recently described to inhibit MAO [2, 16], was able to interact with SSAO, too. Another line of observations also prompted us to perform such verification. It is now established that phenolic compounds exhibit anti-inflammatory effects [17, 18]. Taking into account the fact that many inhibitors of SSAO/VAP-1 (engineered antibodies or soluble small molecules) are also endowed with potent anti-inflammatory actions in various experimental models [19–21], it was of relevant interest to determine whether resveratrol might also inhibit SSAO/VAP-1 and whether such putative inhibition was contributing to its anti-inflammatory actions. All these considerations led us to test whether several phenolic compounds could inhibit human MAO and SSAO/VAP-1 and whether such inhibition may account for a portion of their multiple effects.

Therefore, the objective of our work was focused on testing the capacity, if any, of selected phenolic compounds on the activity of these enzymes under their native form in man, since interspecies differences have been reported regarding substrate and inhibitor selectivity for MAOs and SSAO [22, 23]. Among the stilbenes, we compared *trans*-resveratrol (the most abundant form in foods and beverages) to pterostilbene, one of its hydroxylated derivatives, since both of them have been reported to repress adipogenesis in fat cells [24–26] (see below). Caffeic acid was chosen as representative of the phenolic acids since it has also been demonstrated to be inhibitory on adipogenesis [27]. We also included quercetin in our comparative study, since it is quantitatively the most important phenolic compound present in foodstuffs (as indicated by the phenolic compounds database: http://www.phenol-explorer.eu/ [28]). The following results obtained on human adipose tissue are not only relevant for obesity research but also valuable for oxidative stress aspects because AOs are ROS-generating enzymes and because phenolic compounds, though being among the most important low molecular weight molecules absorbed from foods capable of eliciting nonenzymatic antioxidant actions in consumers, are scarcely described to impair MAO [2–4].

2. Materials and Methods

2.1. Subjects and Adipose Tissue Sampling. Samples of subcutaneous abdominal adipose tissue were obtained from a total of 39 overweight women undergoing reconstructive surgery at Rangueil Hospital, Toulouse, France (mean age: 39 ± 2 years, range: 18–66, and mean body mass index: 25.36 ± 0.62 kg/m^2). The removed pieces of human adipose tissue (hAT), considered as surgical waste, were transferred in less than one hour to the laboratory, under the agreement of INSERM guidelines and ethic committee. These hAT pieces were frozen at −80°C and dispatched for further analyses. Moreover, part of the adipose samples was immediately subjected to liberase digestion at 37°C to obtain freshly isolated adipose cells for glucose transport assays as detailed below.

2.2. Amine Oxidase Activity Measurement by Fluorometric Determination of Hydrogen Peroxide. Oxidase activity was measured using the fluorescent probe Amplex Red (10-acetyl-3,7-dihydrophenoxazine) designed for the detection of hydrogen peroxide in biological milieu. Assays were performed accordingly to our previous descriptions [29, 30], which corresponded to slight modifications from the adaptation of such fluorometric method for MAO activity determination [31]. Briefly, hydrogen peroxide release was quantified owing to a chromogenic mixture containing 40 μM Amplex Red and 4 U/mL horseradish peroxidase and to the parallel use of hydrogen peroxide standard solutions ranging from 0.05 to 5 μM (final concentrations) [32]. Thawed hAT samples were homogenized in 200 mM phosphate buffer (pH 7.4) during 30 sec with a homogenizer Tissue Master-125

(Omni International, Kennesaw, GA, USA) just prior to the determination of their amine oxidase activity, as previously reported [32], except that no antiprotease cocktail was added. Homogenates were distributed in 96-well dark microplates (at 50 ± 5 μg protein/well) and incubated at 37°C in the dark for at least 30 min in 200 μL final volume after a 30 min preincubation without (control) or with 1 mM pargyline or semicarbazide to inhibit MAO or SSAO activity, respectively, or even with both reference inhibitors to abolish all AO activity. All phenolic compounds tested for their inhibitory properties were also added during this preincubation step. Tyramine or benzylamine was added to the medium in order to obtain 1 mM concentration and to serve as substrates for AO activities, as already stated [29]. The DMSO vehicle used for phenolic compounds solubilization was present at the final higher concentration of 1% w/v and inhibited amine-induced signal by less than 5%.

Spontaneous hydrogen peroxide formation by homogenates of thawed material was also quantified without any addition of exogenous amine or inhibitor and defined as basal release. The influence of chemical compounds or biochemical agents on the fluorescence readouts generated by the hydrogen peroxide standard curve was also performed without any presence of hAT in a set of experiments called "without biological material." In all cases, fluorescence data (ex/em: 540/590 nm) were collected on a Fluoroskan Ascent plate reader (ThermoLabsystems, Finland).

2.3. Radiochemical Assay of Amine Oxidase Activity. Samples of adipose tissue were homogenized as described above. 50 μL of crude homogenates were then incubated for 30 min at 37°C in 200 μL of 200 mM phosphate buffer in the presence of radiolabelled amine after 30 min preincubation without or with inhibitor(s). MAO-dependent oxidation was defined as sensitive to inhibition by 1 mM pargyline, whereas SSAO-dependent oxidation was abolished by 1 mM semicarbazide. Assays were started by addition of the labelled substrate and stopped by adding 50 μL of 4 M HCl. Reaction products of amine oxidation were extracted by 1 mL of organic solvent (toluene/ethyl acetate, 1/1 v/v), according to Tipton's method [23]. Then, 0.7 mL aliquots of the organic phase were counted for radioactivity. Maximal velocity and optimal conditions for determination of amine oxidase activities have been already detailed for human adipocytes, cultured preadipose cells, or hAT homogenates [33–35]. Thus, inhibition studies were performed only under conditions reaching maximal oxidation velocity, that is, corresponding to the use of tyramine or benzylamine isotopic dilutions giving 1 mM final concentration and reaching approximately 640000 dpm/50 μL for [^{14}C]-tyramine (provided either by PerkinElmer, Evry, France, or by Sigma-Aldrich) or 200000 dpm/50 μL for [^{14}C]-benzylamine (NEC 835050UC purchased from PerkinElmer). The radioactivity extracted in the organic phase at time 0 ($t0$) represented less than 0.5% of the total radioactivity/tube of each labelled amine and was subtracted to all counts with tyramine and benzylamine oxidation without inhibitor averaging 14160 ± 5620 dpm (n = 16) and 15800 ± 1770 dpm (n = 12), respectively. These absolute values, which varied substantially from one individual to another, were set as 100% reference in each subject for the calculation of percentages of inhibition.

2.4. Hexose Uptake in Adipocytes. To determine glucose transport activity, hAT was grossly minced and digested at 37°C under shaking in 20 mL of Krebs-Ringer medium containing 0.015 mg/mL liberase (type TM, Roche Diagnostics), 15 mM sodium bicarbonate, 10 mM HEPES, and 3.5% bovine serum albumin. Buoyant adipocytes were separated by filtration through nylon screen and carefully washed in the same medium at pH 7.4 without liberase to obtain adipocyte suspensions as already described [36]. Freshly isolated adipocytes were incubated for 45 min with the tested agents just before [^{3}H]-2-deoxy-glucose uptake assays (PerkinElmer) performed in 10 min at 37°C in plasticware as already described [37].

2.5. Chemicals. Tyramine hydrochloride, benzylamine hydrochloride, amine oxidase inhibitors, quercetin, *trans*-resveratrol, and other reagents were obtained from Sigma-Aldrich (Saint-Quentin-Fallavier, France), except otherwise specified.

2.6. Computational Studies of Molecular Docking in the Active Site of Amine Oxidases. The structure of *trans*-resveratrol (https://pubchem.ncbi.nlm.nih.gov/compound/445154) was used for computational docking in the structure of MAO-A cocrystallized with harmine at 2.2 Å resolution (http://www.rcsb.org/pdb/explore.do?structureId=2Z5X), MAO-B co-crystallized with coumarin-analogue (https://pubchem.ncbi.nlm.nih.gov/compound/11616886) at 1.7 Å resolution (http://www.rcsb.org/pdb/explore/explore.do?pdbId=2V61), and SSAO/VAP-1 cocrystallized with a pyridazinone analogue at 2.8 Å resolution (http://www.rcsb.org/pdb/explore/explore.do?pdbId=4BTW). Flexible ligand approach with standard precision (SP) was performed with Schrödinger's software package (release 2015-2), using Glide software version 6.7 (http://www.schrodinger.com/Glide/). The phenolic compounds were treated as noncovalent ligands to dock them into the substrate pocket: next to FAD in MAO-A (about 4 Å distance) and MAO-B (about 5.5 Å distance) and next to the topaquinone residue (about 8.5 Å distance) in SSAO/VAP-1. Docking boxes were positioned on the cocrystallized ligands, the size of the boxes was 20 Å in every direction, and no constrains were used.

2.7. Statistical Analysis. Results are given as means ± standard error of the means (SEM). Statistical significance was assessed by use of Student's t-test. Significance level was set at $P < 0.05$. IC_{50} values were calculated by nonlinear regression using GraphPad Prism (CA, USA).

3. Results

3.1. ROS Release by Human Subcutaneous Adipose Depots in Response to Amines. Spontaneous and amine-stimulated hydrogen peroxide production by hAT preparations was measured on 30 min incubation (Figure 1). When prolonging

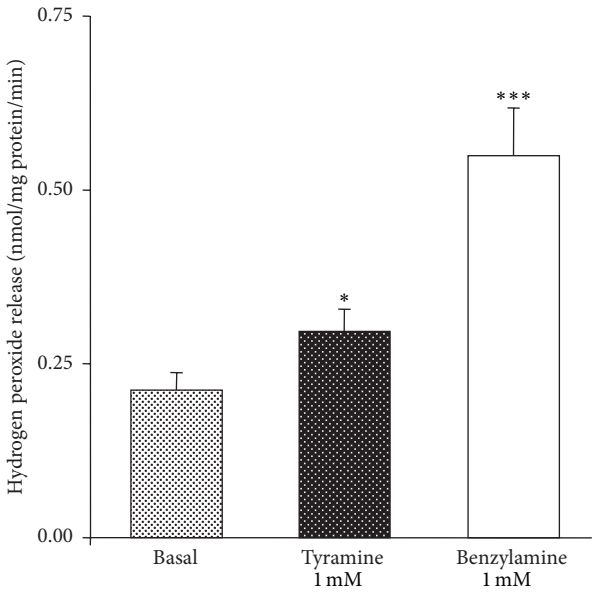

Figure 1: Hydrogen peroxide release by human subcutaneous adipose tissue: influence of amine substrates. Amplex Red-based fluorescence detection of hydrogen peroxide was performed in homogenates during 30 min incubation without any added amine (basal) or with 1 mM of the indicated amine. Results are expressed as velocity of hydrogen peroxide production per mg of protein per minute. Mean ± SEM of 22 homogenates. Different from basal at $^{*}P < 0.05$ and $^{***}P < 0.001$.

incubation conditions it was observed that such ROS release was linear with time for at least one hour (not shown). Benzylamine, and tyramine to a lesser extent, significantly increased the amount of detected hydrogen peroxide. Since the chromogenic mixture was already present in the incubation medium at time 0 when the amines were added and since the net fluorescent intensity was calculated as the difference between $t30$ and $t0$ signal, the observed increase in amount of hydrogen peroxide corresponded to a real time-dependent H_2O_2 production by hAT preparations and its subsequent release into the medium. Such widely recognized method of AO activity determination consisted in monitoring the release of only one of the oxidative deamination end-products, hydrogen peroxide. On the basis of the stoichiometry of the oxidative deamination, it was supposed that one molecule of H_2O_2 corresponded to one molecule of amine oxidized by AO. In the absence of amine, there was a spontaneous hydrogen peroxide release: this basal activity represented approximately one-third of the maximal response to benzylamine.

The amine-induced hydrogen peroxide production was then used to test a putative interaction of phenolic compounds with human MAO and SSAO.

3.2. Interactions between Phenolic Compounds and MAO- or SSAO-Induced Hydrogen Peroxide Production. It was tested whether the response to tyramine was sensitive to reference inhibitors: pargyline (MAO-selective) and semicarbazide (SSAO-selective). The former inhibited dose-dependently tyramine action, while the latter was totally inefficient (Figure 2(a)). Of note, the combination of both inhibitors did not inhibit more than pargyline alone, leaving unaltered approximately 30% of the production found in the presence of tyramine. This confirmed that, in human fat stores, tyramine was mainly oxidized by MAO. When phenolic compounds were studied in identical conditions, all the four tested molecules (see Figure 7 for chemical structures) reached at 1 mM the same maximal inhibition of tyramine-induced H_2O_2 release (Figure 2(b)). At the 1 μM dose, all were almost unable to modify the response to 1 mM tyramine, as it was the case for the classical AO inhibitors. The rank order of affinity was: quercetin > resveratrol > caffeic acid > pterostilbene, with respective IC_{50} values being 30, 62, 100, and 107 μM. From these observations, it could be deduced that resveratrol and other phenolic compounds were able to limit the tyramine-induced H_2O_2 release, that is, to inhibit MAO activity, thus confirming previous reports on resveratrol and expanding to other phenolic compounds the capacity to inhibit MAO.

Figure 3(a) shows that benzylamine-induced hydrogen peroxide release was inhibited by 1 mM semicarbazide while being resistant to pargyline. Again, the combination of pargyline and semicarbazide did not inhibit further than the SSAO inhibitor semicarbazide alone, leaving unaltered 20% proportion of the signal. These data confirmed that benzylamine is predominantly oxidized by SSAO in hAT, as previously reported [34].

Phenolic compounds dose-dependently inhibited benzylamine-induced H_2O_2 release (Figure 3(b)), indicating that they apparently behave as SSAO inhibitors. In this case, the IC_{50} values (μM) for caffeic acid, resveratrol, quercetin, and pterostilbene were 36, 45, 69, and 136, respectively. However, resveratrol and caffeic acid abolished benzylamine-induced signal at 1 mM, almost to a deeper extent than the reference inhibitor semicarbazide, while quercetin and pterostilbene impaired partially the benzylamine-induced signal.

This dissimilarity prompted us to further verify whether the phenolic compounds were impairing the generation of hydrogen peroxide or whether they blunted its detection by the chromogenic mixture used for fluorometric detection.

3.3. Phenolic Compounds Reduce Hydrogen Peroxide Induced Fluorescence of Amplex Red-Based Chromogenic Mixture. At this stage, it appeared necessary to determine whether the detection of hydrogen peroxide could be directly influenced by phenolic compounds irrespective of its source: production by AOs, generation by other enzymatic systems, or even exogenous addition by the experimenter. Without any human biological material in the assays, the classical substrates and inhibitors of MAO and SSAO did not alter the fluorescence found in the presence of 5 μM hydrogen peroxide (signal set at 100%) even when present at 0.1–1 mM (Figure 4). DMSO was also inactive at the dose used as a vehicle for polyphenol solutions (1%, not shown). On the opposite side, a strong interaction with fluorescence readouts was found with the four tested phenolic compounds in the range of 1 μM–1 mM.

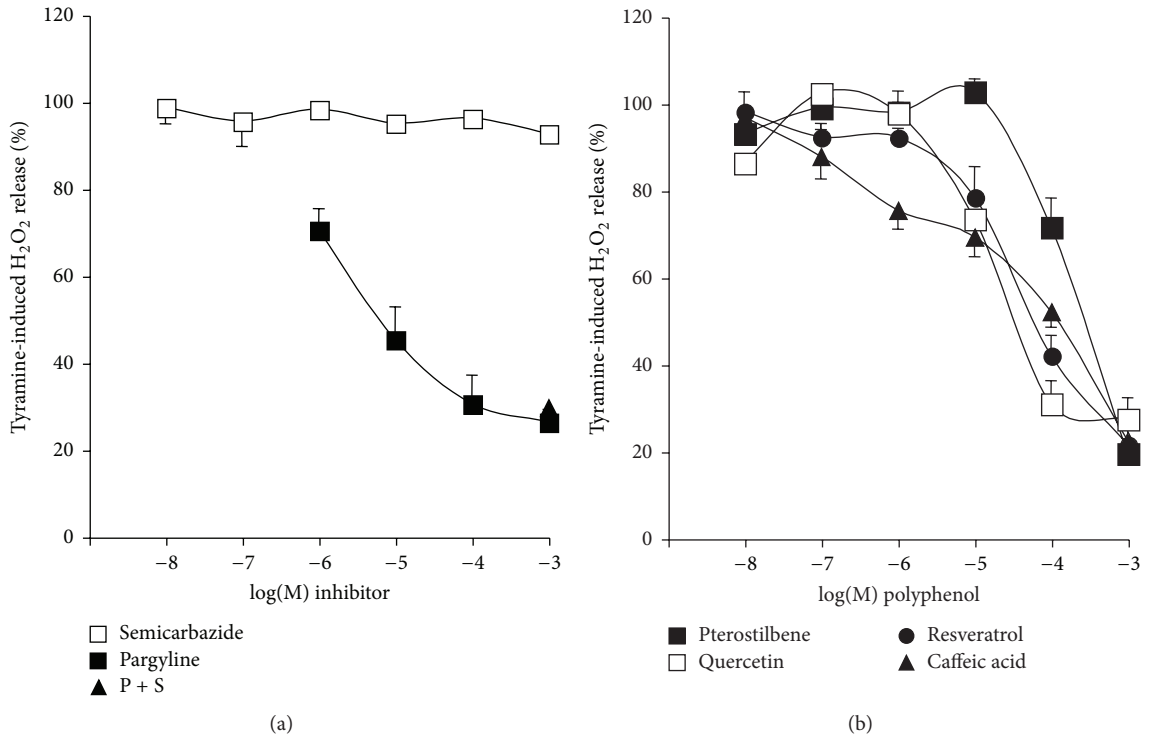

Figure 2: Inhibition of tyramine-induced hydrogen peroxide production in human subcutaneous adipose tissue homogenates by reference MAO and SSAO inhibitors and by phenolic compounds. Fluorescence readouts after 30 min incubation were substracted from corresponding values at *t*0 and net increase was set at 100% for 1 mM tyramine without any inhibitor. (a) The MAO (pargyline, dark squares) or SSAO inhibitor (semicarbazide, open squares) was present during the 15 min preincubation and the 30 min incubation at 0.001–1 mM or at 0.01 μM–1 mM, respectively. The addition of pargyline 1 mM + semicarbazide 1 mM (P + S, black triangle) was used to abolish all amine oxidase activity. Mean ± SEM of 6 to 22 homogenates. When invisible, SEM bars lie within the symbols. (b) The phenolic compounds were tested at the indicated final doses during preincubation and incubation: resveratrol (dark circles), pterostilbene (dark squares), caffeic acid (black triangles), or quercetin (open squares). Mean ± SEM of 7 to 10 homogenates.

Figure 4 clearly shows that all the tested phenolic compounds dose-dependently lowered with almost the same potency the fluorescent signal elicited by 5 μM hydrogen peroxide. Corresponding EC_{50} values were as follows in μM: quercetin 2.0, caffeic acid 3.5, resveratrol 5.0, and pterostilbene 5.2. With 1 mM phenolic compounds, there were even unexplained low readouts that were lower than the blank made with phosphate buffer and chromogenic mixture only, leading to percentages lower than 0%. All this indicated that the phenolic compounds prevented detection of hydrogen peroxide under optimal conditions since they hindered signal generation, likely by preventing the interaction between H_2O_2 and chromogenic mixture components (Amplex Red, peroxidase, or resorufin). Such interaction likely relies on the antioxidant properties of the tested phenolic compounds and deserves further investigation. However, our objective was to assess whether MAO and/or SSAO activities were really altered by resveratrol and related molecules. We therefore used another method to measure more directly amine oxidation, based on the use of radiolabelled substrates.

3.4. Influence of Phenolic Compounds on [^{14}C]-Tyramine Oxidation. Figure 5 shows that pargyline inhibited the MAO-dependent [^{14}C]-tyramine oxidation by hAT. As expected, and accordingly with the above-used method, semicarbazide was virtually unable to abolish this activity. Surprisingly, phenolic compounds did not inhibit MAO activity totally. Only resveratrol and quercetin exhibited partial MAO inhibitor properties, since, at 0.1–1 mM, they inhibited 25 to 60% of the activity. Contrasting with the fluorescent method, pterostilbene and caffeic acid did not abolish enzyme activity when taking into account the production of labelled oxidation products of [^{14}C]-tyramine (Figure 5).

3.5. Interaction of Phenolic Compounds with SSAO-Dependent Oxidation of [^{14}C]-Benzylamine. As expected, benzylamine oxidation by hAT was sensitive to semicarbazide. The addition of both pargyline and semicarbazide could not inhibit further than semicarbazide alone, which eradicated the generation of labelled benzaldehyde. The SSAO-mediated [^{14}C]-benzylamine oxidation remained unchanged with low or high doses of phenolic compounds (Figure 6). Taken as a whole, these data indicated that SSAO/VAP-1 was the unique catalyst participating in benzylamine oxidation and that pargyline as well as polyphenols could not prevent this type of amine metabolism.

Therefore, phenolic compounds, which inhibited benzylamine-induced signal in fluorometry, were likely

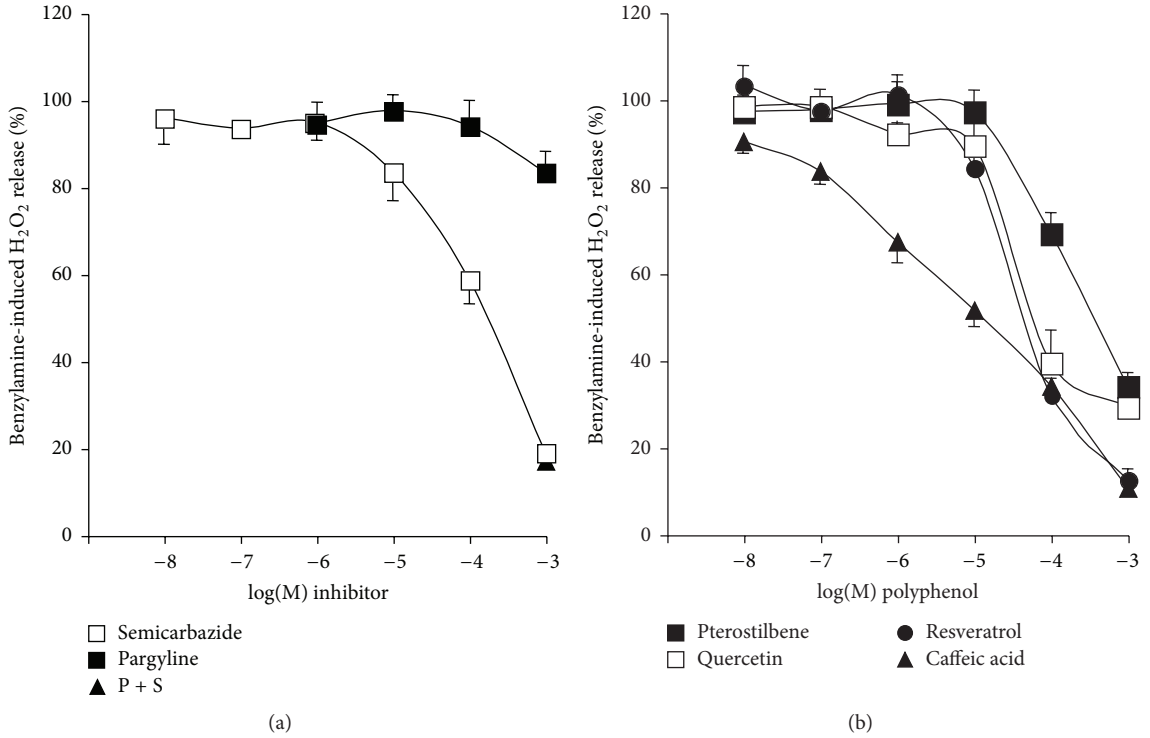

Figure 3: Inhibition of benzylamine-induced hydrogen peroxide release in human subcutaneous adipose tissue by reference MAO and SSAO inhibitors and by phenolic compounds. Fluorescence readouts after 30 min incubation were substracted from corresponding values at *t*0 and net increase was set at 100% for 1 mM benzylamine alone. (a) Pargyline (MAO, dark squares) or semicarbazide (SSAO inhibitor, open squares) was present at indicated final concentrations, while their combination was tested at 1 mM (P + S, black triangle). Mean ± SEM of at least 6 homogenates. (b) The phenolic compounds were tested in parallel at the indicated final concentrations. Means ± SEM of 8 to 12 homogenates.

preventing H_2O_2 from reacting with chromogenic mixture (as this was the case without biological material) and lowered fluorescent readouts rather than really inhibiting benzylamine oxidation and subsequent H_2O_2 release. In addition, it could be mentioned briefly here that SSAO-mediated oxidation of [^{14}C]-benzylamine was not altered by Amplex Red, peroxidase, or DMSO at the doses used for the fluorometric method or by catalase or even by H_2O_2 (not shown).

To summarize, resveratrol, quercetin, pterostilbene, and caffeic acid actually impaired the detection of the produced hydrogen peroxide without preventing benzylamine degradation and behaved as MAO inhibitors and SSAO inhibitors only in appearance, as schematized in Figure 7. Only resveratrol and quercetin shared a limited capacity to alter partially the MAO activity found in hAT homogenates, at doses between 0.1 and 1 mM, that is, at concentrations that can be qualified as supranutritional.

3.6. Computational Docking in the Active Site of Amine Oxidases. Calculations were performed to simulate the docking of the four tested phenolic compounds into human MAO-A, MAO-B, and SSAO. All these small soluble molecules were able to approach very nearly the catalytic sites of the oxidases (Figure 8).

In the case of MAO-A, all the tested agents could dock in a close vicinity to the FAD. Possible aromatic π-π interactions were observed with Phe208, Phe352, Tyr407, and Tyr444. Some H-bonds were also formed between the hydroxyl groups of the ligands and polar amino acids Asn181, Tyr197, and Tyr444. Docking scores calculated by glide varied between −8.300 and −5.682 with the standard precision method (SP) and between −11.568 and −5.563 with the extra precision level (XP). The predicted activity order was as follows: quercetin > *cis*-resveratrol > *trans*-resveratrol > pterostilbene > caffeic acid, with both SP and XP.

In the case of MAO-B, our computational studies allowed adding resveratrol to the list of chemical compounds that are not amines but which noncovalently bind to MAO within a wide range of affinity: diphenylbutane, farnesol, coumarin. . ., and so on. The formation of π-π interactions was indicated with the aromatic rings of Tyr326, Tyr398, and Tyr435; H-bonds were found with Cys 172, Tyr188, and Tyr435. The backbone oxygen atom of Pro102 seemed to be an H-bond acceptor for several ligands. Scores were slightly higher than those with MAO-A, since SP scores ranged from −9.312 to −6.849 and XP scores ranged from −11.020 to −6.997.

Regarding SSAO, both *cis*-resveratrol and *trans*-resveratrol could be docked into the pocket surrounding the cocrystallized inhibitor (a pyridazinone analogue) though

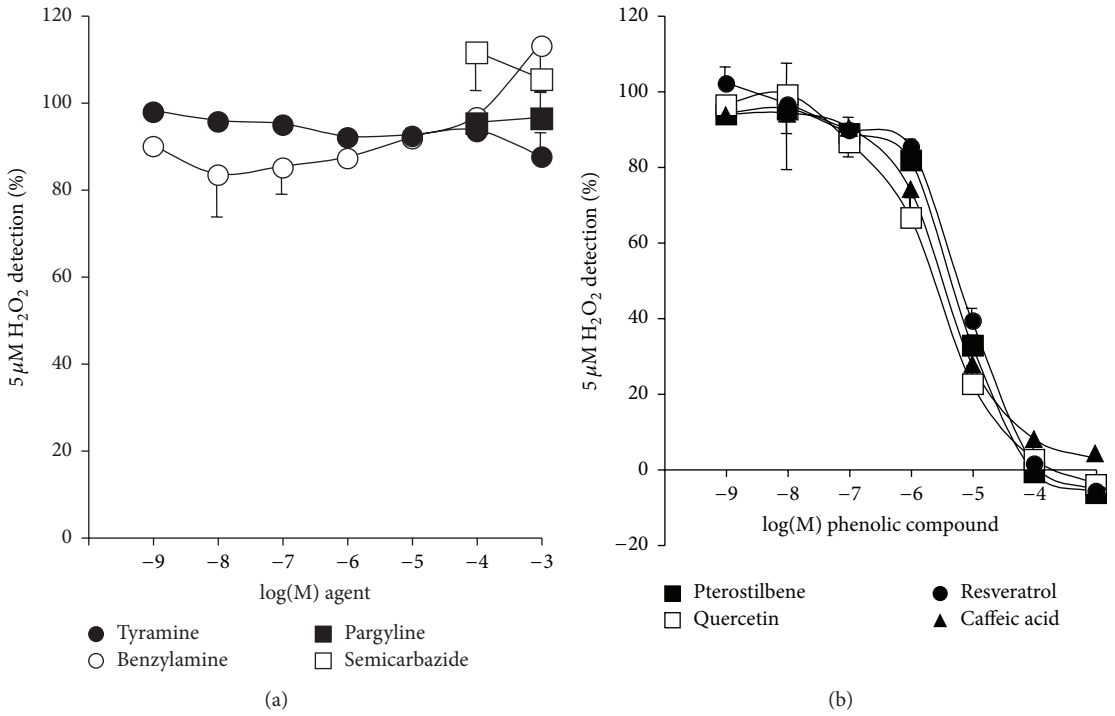

Figure 4: Influence of amine oxidase substrates or inhibitors and phenolic compounds on fluorescent-based detection of hydrogen peroxide. The chromogenic mixture used for Amplex Red-based fluorometric detection of hydrogen peroxide was incubated with 5 μM H_2O_2 alone (control set at 100%) or in the presence of the indicated final concentrations of tested chemicals. (a) Lack of influence of MAO and SSAO substrates or inhibitors. (b) Dose-dependent abolishment by phenolic compounds of the fluorescent signal elicited by 5 μM hydrogen peroxide. Means ± SEM of 7 measurements without any human material.

with very small scores, indicating that there is a possible binding position but not ascertaining that resveratrol can behave as a noncovalent inhibitor. Indeed, the docking of the stilbene, as well as that of the other phenolic compounds tested, was established relatively far away from both topaquinone and the copper ion, two mandatory cofactors for the enzyme catalysis. π-π stacking interactions were formed with the following residues: Phe173, Tyr176, Phe389, and Tyr394; H-bonds were formed with Asp180, Thr210, and Tyr394. Backbone oxygen atom of Thr467 and backbone nitrogen atom of Leu469 could also form additional H-bonds. Docking scores were weaker than those with MAO-A and MAO-B: SP scores varied between −6.857 and −5.156, and XP scores varied between −9.261 and −4.100. These weaker scores of the tested polyphenols were in agreement with their lack of inhibition of [^{14}C]-benzylamine oxidation.

Though being not all genuine AO inhibitors, the tested natural phenolic compounds could nevertheless interact with the MAO and SSAO roles since they were able to modify the fate of one of their common end-products of reaction, namely, H_2O_2. It was therefore investigated whether the phenolic compounds were modulating the response to benzylamine in human adipose cells.

3.7. Phenolic Compounds and Benzylamine-Stimulated Glucose Uptake in Human Adipocytes. It was confirmed that the previously reported insulin-like effect of benzylamine on glucose uptake was readily detectable in human adipocytes from subcutaneous abdominal depots. Figure 9 shows that, in human fat cells, 100 nM bovine insulin stimulated basal glucose transport by 3-fold to 4-fold. At 100 μM, benzylamine partially mimicked the action of the pancreatic hormone, since its activation of hexose uptake averaged 25 to 30% of maximal insulin effect. However, this modest insulin-like effect was highly significant and demonstrated to be dependent on amine oxidation. Indeed, semicarbazide, together with pargyline, did not modify basal or insulin-stimulated glucose uptake, while it clearly inhibited benzylamine action (Figure 9).

On the other hand, phenolic compounds were tested on 1 mM benzylamine stimulation of deoxyglucose uptake and the results were expressed as percentage of insulin maximal stimulation (which at 100 nM elicited a 3-fold to 4-fold activation over baseline in a subset of 7 individuals, Figure 9). At 100 μM, resveratrol, pterostilbene, and quercetin counteracted the effect of 1 mM benzylamine. No clear-cut inhibition was found with caffeic acid. Although phenolic compounds did not inhibit SSAO activity in hAT, they were likely altering the fate of the hydrogen peroxide produced during SSAO activation by benzylamine. We propose that this was preventing the interaction of hydrogen peroxide with intracellular components, such as phosphatases, already

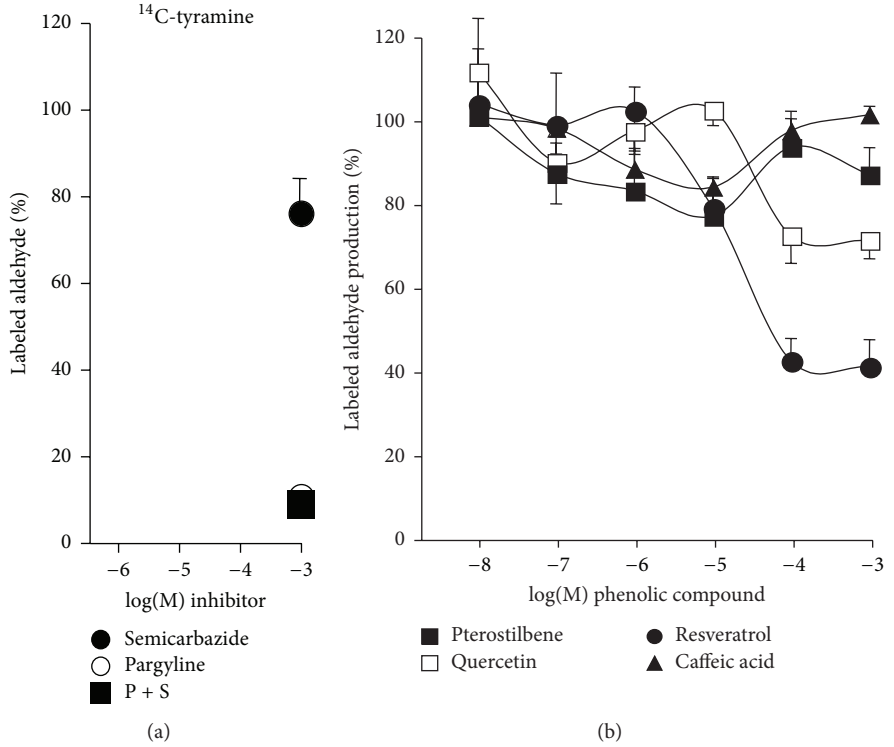

Figure 5: Influence of phenolic compounds and MAO inhibitors or SSAO inhibitors on tyramine oxidation by hAT. The production of radiolabelled aldehydes resulting from the 30 min incubation of fat tissue homogenates with 1 mM isotopic dilution of [^{14}C]-tyramine was set at 100% in the absence of added inhibitor, while the background radioactivity detected at $t0$ was set at 0%. (a) Inhibition by pargyline only of MAO-dependent tyramine oxidation. (b) Influence of phenolic compounds on tyramine oxidation. Each point is the mean ± SEM of 8 (inhibitors) to 12 (phenolic compounds) homogenates.

reported to mediate the actions of SSAO substrates on glucose carrier translocation [38].

Therefore, when directly applied to human adipose cells, phenolic compounds appeared to behave as antioxidants preventing hydrogen peroxide actions. Nevertheless they impaired responses to AO substrates, especially those dependent on hydrogen peroxide, such as SSAO-mediated activation of glucose transport.

4. Discussion

The present study of polyphenol properties was performed with samples of human subcutaneous abdominal adipose depots. In the following lines we justify this tissue selection. Brain contains neuronal MAO, but its SSAO expression is limited to vessels and meninges [39], while liver is rich in MAO and also expresses SSAO/VAP-1 [40]. However, these tissues are not readily available from healthy volunteers. On the opposite side, hAT offered valuable advantages: it is considered as a surgical waste when obtained in healthy patients undergoing plastic surgery and is able to oxidize amines much more efficiently than blood [41]. Yet, hAT has been shown to express the mitochondrial MAO, with an approximate ratio of MAO-A to MAO-B of 80/20 [33], and to be one of the richest tissues in SSAO/VAP-1 [34]. In fact, SSAO is present at the cell surface of adipocytes and is even considered as a marker of adipogenesis [35, 42].

Our first set of observations clearly showed that resveratrol and other phenolic compounds hampered the detection of hydrogen peroxide generated during benzylamine oxidation by hAT preparations, naturally rich in SSAO/VAP-1 [34]. These results were obtained by using a method that allows measuring MAO or SSAO/VAP-1 activity, based on a fluorometric detection in which Amplex Red is oxidized to resorufin (the real fluoroprobe) by a peroxidase, in a manner that depends on the hydrogen peroxide generated during amine oxidation [31]. Though such method has been already employed with human fat tissue preparations [30], our test of the capacity of polyphenols to inhibit human MAO and SSAO/VAP-1 was accompanied with a complete check aiming at verifying whether they interfere or not with Amplex Red-based fluorescence assay. Such verification avoided misinterpretations of the observed fluorescence readouts, since numerous natural functional ingredients have led to erroneous signals, owing to uncorrected quenching and autofluorescent or antioxidant properties [43].

Thus, the "older" radiochemical determination of AO activity [23] solved the inconsistencies found with the fluorometric method. By using radiolabelled substrates and measuring after extraction of the neosynthesized labelled aldehydes, our comparative approach was able to definitely state

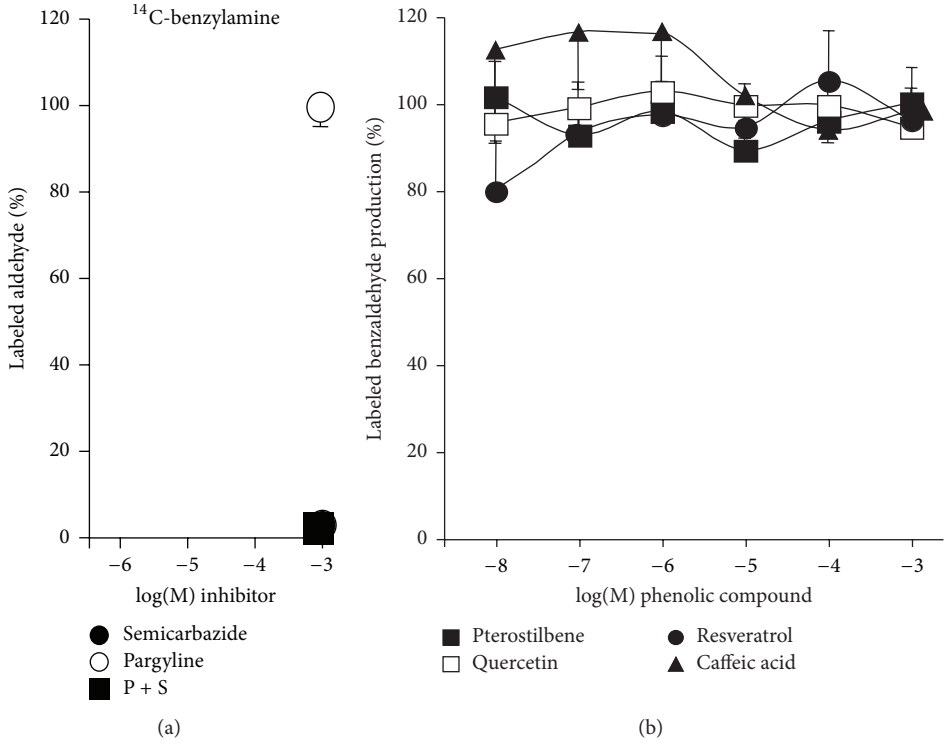

Figure 6: Influence of phenolic compounds and MAO inhibitors or SSAO inhibitors on benzylamine oxidation by hAT. (a) Thirty-minute oxidation of 1 mM [^{14}C]-benzylamine by homogenates generated amounts of radiolabelled benzaldehyde that were separated by extraction as described in Materials and Methods and set at 100% signal (control), while 0% corresponded to the extractible radioactivity at $t0$. The reference MAO inhibitors (pargyline) or SSAO (semicarbazide) inhibitors were present, separately or in combination at 1 mM, 15 min before the addition of the radiolabelled substrate. (b) Indicated doses of resveratrol (dark circles), pterostilbene (dark squares), caffeic acid (black triangles), or quercetin (open squares) and pargyline (dark circles) were tested on the oxidation of 1 mM [^{14}C]-benzylamine in the same conditions as for reference inhibitors. Each point is the mean ± SEM of 8 homogenates.

about the direct interaction between the tested polyphenols and human MAO or SSAO/VAP-1. By using this analytical method, phenolic compounds were unable to inhibit the oxidative deamination of radiolabelled [^{14}C]-benzylamine, suggesting that they were acting as antioxidants rather than genuine SSAO inhibitors (as illustrated in Figure 7). Resveratrol, pterostilbene, quercetin, and caffeic acid could not clearly block AO activities, whereas they could modify the fate of one of the end-products of amine oxidation, hydrogen peroxide. This resulted in an impairment of the interaction between hydrogen peroxide and the chromogenic mixture in fluorometric assays, which could be misinterpreted as an AO inhibition. However, resveratrol and other phenolic compounds altered the consequences of amine oxidase activities that rely on hydrogen peroxide production in hAT, since they were impairing the insulin-like action of benzylamine on glucose uptake in adipocytes. A blockade of these metabolic actions of SSAO substrates has been already reported for SSAO pharmacological inhibitors and for antioxidants (catalase, N-acetylcysteine, and ascorbic acid) [34] but never for natural phenolic compounds.

Thus, our supposed inhibition of SSAO activity by phenolic compounds was not experimentally supported, though a dose-dependent repression of SSAO/VAP-1 was apparent for all phenolic compounds with the misused Amplex Red-based method. However, several pitfalls and complementary experiments rendered irrelevant the shortcut interpretations of fluorescent assays.

Firstly, the fact that resveratrol and caffeic acid totally inhibited benzylamine-induced hydrogen peroxide formation was a little puzzling since the reference inhibitor semicarbazide itself failed to abolish completely the signal. In fact, when hydrogen peroxide formation was measured in the presence of benzylamine, selective pharmacological inhibition of SSAO leaved unaltered approximately 15–20% of the signal. This component was likely independent from SSAO or MAO activity and was resulting from basal ROS production by hAT preparations, as observed in the absence of added amine. A similar spontaneous generation of hydrogen peroxide has been reported in mouse adipose tissue and considered as a baseline ROS production, in which NADPH-oxidase was partly involved [44]. This production represented a minor component compared to that found in response to 1 mM benzylamine treatment. Its detection was impaired by phenolic compounds as was the detection of hydrogen peroxide alone, added at 5 μM in assays without biological material, or even the detection of H_2O_2 produced by AO activation.

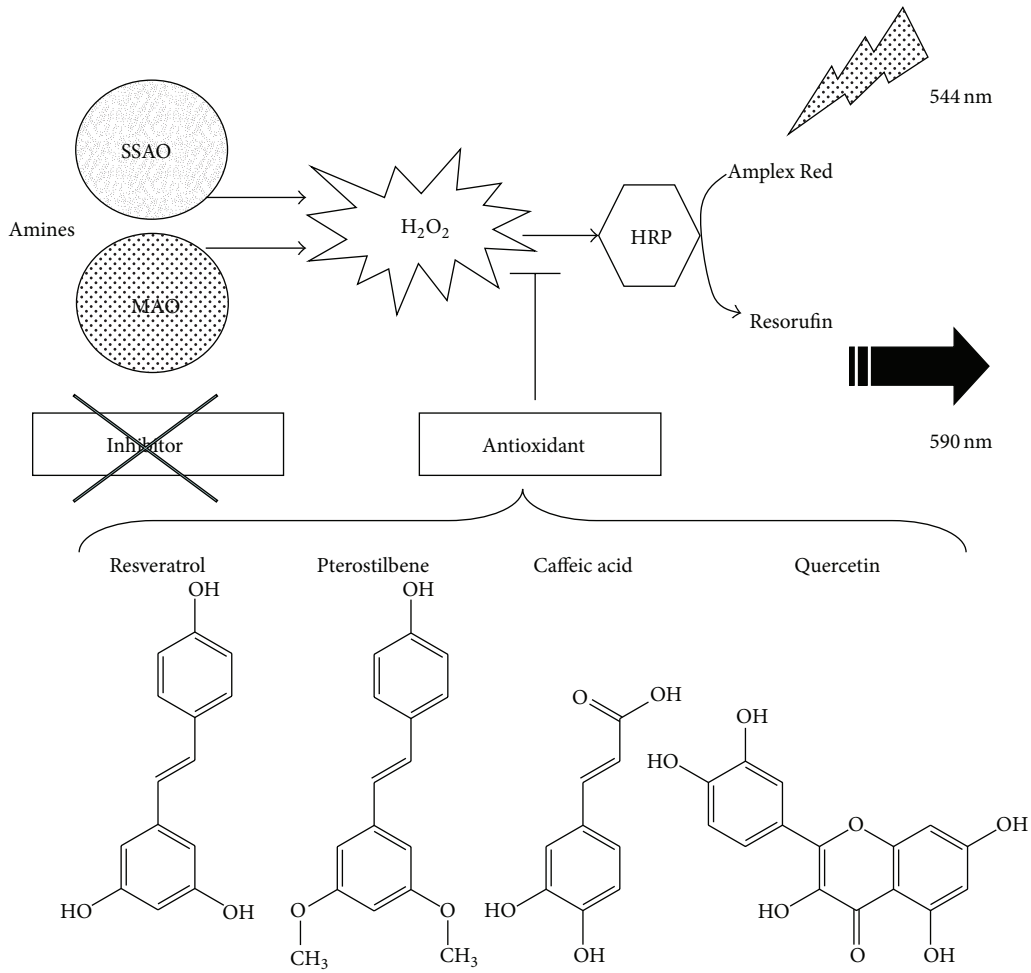

Figure 7: Chemical structures and proposed mechanism of action of phenolic compounds in the Amplex Red-based fluorometric determination of amine oxidase activity in tissue preparations. The tested phenolic compounds with given structures did not behave as full inhibitors of MAO and SSAO present fat tissue, since only resveratrol partially inhibited [^{14}C]-tyramine oxidation by human subcutaneous adipose tissue. However, all of them inhibited hydrogen peroxide detection by preventing the oxygen species from reacting with horseradish peroxidase (HRP) and hampering the generation of the oxidized Amplex Red, that is, the fluorescent probe, resorufin.

Secondly, no inhibition of SSAO/VAP-1 was observed with any dose of the four phenolic compounds tested, even up to 1 mM, when using the radiochemical method. This latter determination was undoubtedly more specific than the H_2O_2-based assays since the SSAO-dependent proportion (i.e., sensitive to semicarbazide by definition) represented 100% of the [^{14}C]-benzylamine-induced response. Obviously, there was no possible spontaneous release of radiolabelled benzaldehyde by hAT preparations not incubated with [^{14}C]-benzylamine.

Importantly, the artefacts detected during the determination of SSAO activity also occurred for MAO activity, since the clear-cut inhibition by phenolic compounds of the hydrogen peroxide signal in response to tyramine was not totally reproduced when MAO activity was assessed via the quantification of the radiolabelled end-product of [^{14}C]-tyramine oxidation. Only a partial MAO inhibition was found with resveratrol and quercetin during hAT short-term incubations, which was in agreement with their capacity to dock in computed models of the catalytic site of MAO-A and MAO-B.

Thus, our observations contrast with previous reports indicating that resveratrol is a valuable inhibitor of human MAO [2]. In fact, in their pioneering observations, Yáñez and coworkers reported that clorgyline and selegiline were 1000-fold to 10000-fold more potent than resveratrol in an artificial model consisting in insect cells overexpressing the recombinant forms of human MAO-A or MAO-B. Only the comparison with a rather "historical" MAO-inhibitor, iproniazid, that exhibited very poor affinity for the enzymes when compared with novel generation of MAO blockers permitted consideration of the MAO inhibition by 10–100 μM resveratrol of relevant interest [2]. Since Amplex Red was employed in such studies, one can expect that there was an overestimation of the capacity of resveratrol to repress MAO activity. At the same doses, quercetin has been reported

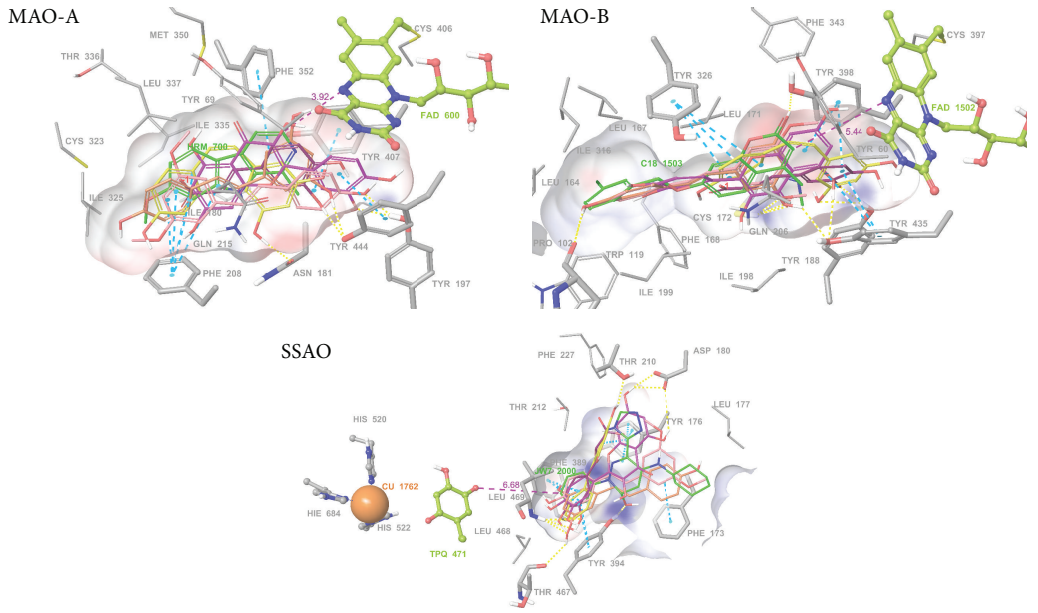

FIGURE 8: Docking poses of resveratrol, pterostilbene, quercetin, and caffeic acid in the active sites of human MAO-A, MAO-B, and SSAO. For all enzymes, amino acid residues surrounding the catalytic site are shown and numbered in grey, while for MAO-A and MAO-B, FAD cofactor is shown in green yellow. For SSAO, topaquinone and copper are shown in green and orange. Green: cocrystallized inhibitor (see Section 2). Yellow: *cis*-resveratrol. Orange: *trans*-resveratrol. Pink: pterostilbene. Purple: quercetin. Maroon: caffeic acid. π-π interactions are marked with light blue dotted lines, while H-bond interactions are marked with yellow dotted lines.

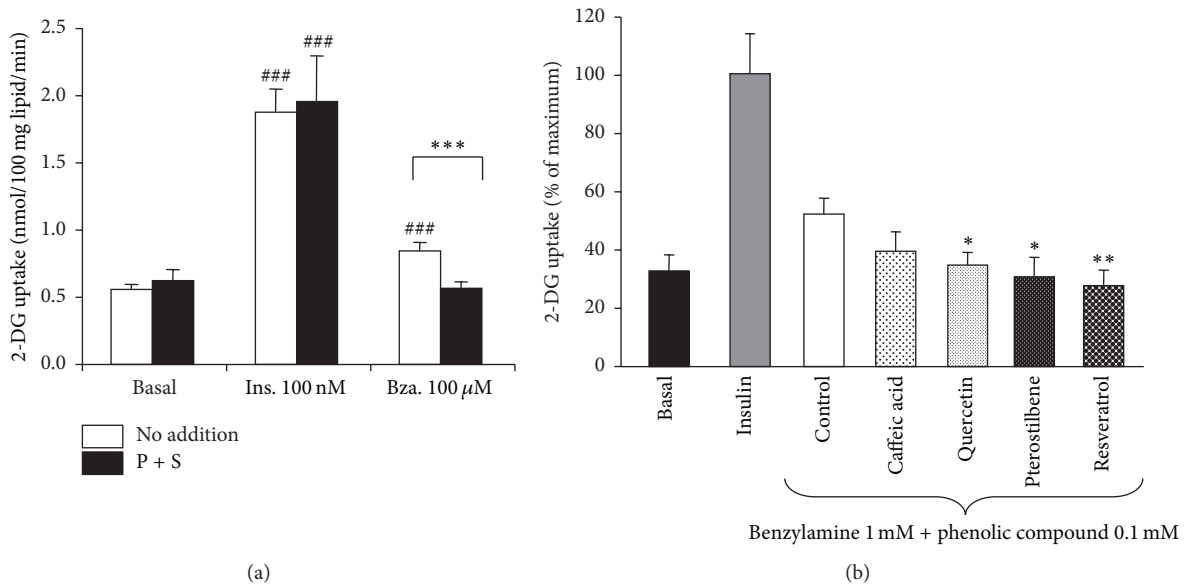

FIGURE 9: Effects of phenolic compounds, benzylamine, and insulin on glucose transport in human adipocytes. Freshly isolated adipocytes were incubated for 45 min without (basal) or with insulin 100 nM (ins.) or with benzylamine and phenolic compounds at the indicated doses. Then 2-DG uptake assays were performed on 10 min as detailed in Section 2. (a) Blockade of benzylamine insulin-like stimulation of glucose uptake in human adipocytes by amine oxidase inhibitors. Basal, insulin-stimulated, or benzylamine-stimulated 2-DG uptake was tested without (no addition, open columns) or with pargyline + semicarbazide at 1 mM each (P + S, dark columns). Mean ± SEM of 39 cases. Different from basal alone at $^{###}P < 0.001$. Different from benzylamine alone at $^{***}P < 0.001$. (b) Insulin was used as the agent of reference for maximal transport stimulation, set at 100%. Benzylamine was tested at 1 mM alone (control, open column) or in the presence of 100 μM of the indicated phenolic compounds. Mean ± SEM of 7 observations. Different from benzylamine alone at $^{*}P < 0.05$ and $^{**}P < 0.01$.

already to inhibit MAO-A in a complete manner, though via a peroxidase-based detection of hydrogen peroxide production, therefore probably with a substantial overestimation of its inhibitory properties [45]. Hence, we propose here that resveratrol and quercetin have to be considered as poorly potent MAO inhibitors since they hamper [^{14}C]-tyramine oxidation (mainly catalyzed by MAO-A, predominant in human adipocytes) at doses that are closer to the millimolar than to the micromolar range, that is, levels that cannot be reached by nutritional intake. Indeed, maximal plasma resveratrol levels are estimated to reach 3-4 μM after ingestion of 1 mg/kg, according to the freely available database http://www.phenol-explorer.eu/ [28]. Nevertheless, it is important to take into account the possibility that a full mixture of natural phenolic compounds may be effective at lower doses, due to potential synergistic effects.

Many, if not all, effects of resveratrol ingestion should be considered therefore as mainly independent from MAO inhibition. This is not incompatible with the neuroprotective activity of the stilbene since it has been proposed that neuroprotection is not linked to MAO inhibition, even for various MAO inhibitors [9, 11]. An attention that must be given to resveratrol in this view, that is, relative to the highly selective, reversible inhibitors of recent generation with high affinity for MAO, is that it is so far a natural product found in foods and beverages and not a prescribed drug.

Our measurements with the radiochemical method using [^{14}C]-benzylamine clearly indicated that resveratrol did not acutely inhibit human SSAO/VAP-1 while semicarbazide did. Of note, semicarbazide used for reference blockade in our assays is no more the best SSAO inhibitor available to date; SSAO has even been renamed primary amine oxidase to signify that it is better defined by its substrates (endogenous or exogenous) rather than by its inhibitor(s) [46]. Although millimolar dose of the reference inhibitor semicarbazide was needed to totally slow down SSAO activity, its blocking action remained selective and was far from being reproduced with the tested phenolic compounds, at least with the radiochemical method.

Most of our computational analyses of resveratrol docking in the catalytic site of human SSAO/VAP-1 were incoherent with a pharmacological binding of high affinity and revealed that SSAO can be hardly considered as a target for this phenolic compound. Regarding MAO, the computationally obtained results were therefore in agreement with the corresponding experimental values and extended the growing list of natural molecules able to interact with functional site of MAOs.

The demonstration that phenolic compounds hampered the detection of hydrogen peroxide when added directly to the fluorometric assay, while not pargyline or semicarbazide, brought further evidence that it was not the generation of hydrogen peroxide occurring during catalyzed oxidative deamination that was prevented by the phenolic compounds but rather the interaction of the MAO-generated or SSAO-generated hydrogen peroxide with chromogenic mixture. On the opposite side, MAO and SSAO genuine pharmacological inhibitors impaired the ROS generation and not its interaction with Amplex Red/peroxidase. Moreover, it was verified that once hydrogen peroxide has completely reacted with the chromogenic mixture the fluorescence readouts were unaltered by subsequent addition of phenolic compounds to the assays. This indicated that phenolic compounds do not quench fluorescence but rather alter the fate of hydrogen peroxide, preventing its interaction with the chromogenic mixture used in our and other fluorometric assays [2] or hampering its messenger role in fat cells (see below).

Though not preventing [^{14}C]-benzylamine oxidation, quercetin, pterostilbene, and resveratrol counteracted the benzylamine activation of glucose transport in human adipocytes, a biological effect previously demonstrated to be dependent on hydrogen peroxide [34]. In other words, the fact that phenolic compounds interfere not only with the fluorometric detection of hydrogen peroxide by the chromogenic mixture but also with other fat cell components might have unexpected consequences for future research. Regarding upcoming determinations of MAO and SSAO activities and their interactions with various "novel" agents supposed to act as antioxidants, the verification of putative interaction of these agents with hydrogen peroxide or chromogenic mixture should carefully accompany future tests. Relative to the effectiveness of ingested nonenzymatic antioxidants, it can be advanced that they probably share with classical AO inhibitors the capacity to alter the effects of elevated doses of dietary amines, as illustrated here by the opposite effects of phenolic compounds and benzylamine on glucose transport in human fat cells.

In this view, it is recognized that resveratrol shares with several SSAO inhibitors a strong antiadipogenic action [47–49]. Nonetheless, the stilbene improves glucose tolerance in rodent model of obesity and diabetes [50, 51], while semicarbazide and related agents reduce body weight gain and adiposity without exhibiting noticeable beneficial effect on glucose homeostasis [52, 53]. Curiously, a few drugs share with resveratrol the properties of being both antidiabetic and MAO inhibitors. This is the case of the PPARγ activators known as glitazones, for which the inhibition of the mitochondrial MAO belongs to the list of their "off-target" actions [54], together with their neuroprotective secondary effects [55]. In this line, glitazones have been recently described as MAO inhibitors devoid of SSAO inhibitory properties [30].

5. Conclusion

To summarize, the capacity of resveratrol to inhibit MAO-A activity has been confirmed here on the human form in mature human adipocytes that naturally express mainly MAO-A. Nevertheless, we propose that this inhibition has been probably overestimated in various previous reports. We also observed that resveratrol and quercetin could dock into the active sites of human MAO-A and MAO-B with acceptable scores, a finding confirmed during the completion of our work by a recent report of drug-design strategy showing that derivatives of resveratrol plus coumarin were successfully designed to develop a target scaffold to inhibit MAO in a reversible manner [56]. Our limited comparison

with four natural phenolic compounds did not indicate which biomolecule could be more beneficial than resveratrol for limiting oxidative stress in experimental models or useful for supplements to human nutrition targeting brain or peripheral MAOs. Nevertheless, our comparative study clearly indicated that none of the tested phenolic compounds directly inhibited primary amine oxidase, also named SSAO/VAP-1. This property cannot be added to their antioxidant action. However, dietary phenolic compounds can hamper the hydrogen peroxide-dependent consequences of SSAO activation, at least in human fat cells. Therefore the coexistence of dietary amines (which will be oxidized once ingested) or other antioxidant molecules present in foods, beverages, or nutritional supplements should be checked carefully. Finally, in a context in which various selective inhibitors of SSAO/VAP-1 have been patented for their anti-inflammatory properties [57, 58], it can be advanced that phenolic compounds exert anti-inflammatory properties independently from direct SSAO/VAP-1 inhibition.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors express gratitude to all the members of the Consortium for Trans-Pyrenean Investigations on Obesity and Diabetes for valuable discussions and to Keith Tipton (Dublin, Ireland) for his knowledge about AO-interacting drugs and elixir of life. The authors would like to thank the staff of Plastic Surgery Dpt. of Rangueil Hospital (Toulouse, France) for facilitating access to postsurgical wastes. This work is partly supported by DIOMED & REF-BIO/POLYFrEsNOL projects, Instituto de Salud Carlos III (CIBERobn), Government of the Basque Country (IT-512-13), and University of the Basque Country (UPV/EHU) (ELDUNANOTEK UFI11/32).

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 2572606, 11 pages
http://dx.doi.org/10.1155/2016/2572606

Research Article

Effects of Polyphenol, Measured by a Biomarker of Total Polyphenols in Urine, on Cardiovascular Risk Factors After a Long-Term Follow-Up in the PREDIMED Study

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Received 24 August 2015; Revised 16 October 2015; Accepted 21 October 2015

Academic Editor: Ilaria Peluso

Several epidemiological studies have shown an inverse association between the consumption of polyphenol-rich foods and risk of cardiovascular diseases. However, accuracy and reliability of these studies may be increased using urinary total polyphenol excretion (TPE) as a biomarker for total polyphenol intake. Our aim was to assess if antioxidant activity, measured by a Folin-Ciocalteu assay in urine, is correlated with an improvement in cardiovascular risk factors (blood pressure and serum glucose, cholesterol, HDL-cholesterol, LDL-cholesterol, and triglyceride concentrations) in an elderly population at high risk. A longitudinal study was performed with 573 participants (aged 67.3 ± 5.9) from the PREDIMED study (ISRCTN35739639). We used Folin-Ciocalteu method to determine TPE in urine samples, assisting with solid phase extraction. Participants were categorized into three groups according to changes in TPE. Multiple linear regression models were used to assess relationships between TPE and clinical cardiovascular risk factors, adjusting for potential confounders. After a 5-year follow-up, significant inverse correlations were observed between changes in TPE and plasma triglyceride concentration ($\beta = -8.563$; $P = 0.007$), glucose concentration ($\beta = -4.164$; $P = 0.036$), and diastolic blood pressure ($\beta = -1.316$; $P = 0.013$). Our results suggest that the consumption of more polyphenols, measured as TPE in urine, could exert a protective effect against some cardiovascular risk factors.

1. Introduction

Cardiovascular diseases (CVDs) are considered to be the leading global cause of death, accounting for 17.3 million deaths per year, which is predicted to rise to more than 23.6 million by 2030 [1]. The main causes of CVDs involve nonmodifiable risk factors, such as age, sex, and family history of coronary heart disease (CHD), and modifiable risk factors, such as an unhealthy diet, lack of physical activity, smoking, and excessive alcohol intake [2, 3]. Therefore, an improvement of dietary habits could help to prevent CVDs.

Several studies have described protective roles of polyphenols in the cardiovascular system. The cardiovascular protection by polyphenol consumption can be explained by various mechanisms, including their anti-inflammatory properties, antioxidant capacity, improvement in endothelial function, inhibition of platelet aggregation and antithrombotic properties, and mechanisms that are not mutually exclusive [4–8]. Hence, further exploration of polyphenol consumption will help to discern its beneficial effects on human health. Prior information on polyphenol intake has often been collected through food frequency questionnaires (FFQs) or dietary recalls, whose bias can result in data not so accurate [9]. Therefore, in order to analyse associations between polyphenol intake and main cardiovascular risk factors, there is a need for biomarkers that can accurately reflect polyphenol intakein human studies.

The Folin-Ciocalteu method, an antioxidant assay based on electron transfer that measures the reductive capacity of an antioxidant, has been widely applied for measuring total polyphenol content in plant-derived food and recently in biological samples for clinical studies [10, 11]. Briefly, polyphenols from urine samples react with the Folin-Ciocalteu reagent to form a blue complex in alkaline medium, measured in spectrophotometry at 765 nm [12]. A solid phase extraction method is used to clean up the sample from possible interferences. This measurement of total urinary polyphenol excretion (TPE) has been considered as reliable biomarker of total polyphenol intake in recent years [8, 13, 14].

Several studies have addressed the relationship between polyphenol intake and cardiovascular risk factors; however, the results have led to mixed and inconsistent conclusions. Two studies conducted in healthy participants observed that improvement in cardiovascular health was due to higher HDL levels after intake of polyphenol-rich foods [15, 16]. Different results were obtained in other two studies in overweight subjects: one showed cardioprotective effects due to a reduction in body weight and an improvement in total cholesterol and LDL concentration after ingestion of a polyphenol extract from *Ecklonia cava*, while the other study observed a reduction in fasting glucose concentration when supplied with polyphenol-rich dark chocolate [17, 18]. Additionally, reduction in systolic blood pressure was observed in hemodialysis patients after the consumption of a polyphenol-rich beverage for one year [19]. Moreover, in the frame of the PREDIMED study, we found that specific categories of polyphenols, calculated through yearly FFQs and the Phenol-Explorer database, were significantly associated with decreased CVD risk [20].

Most of the aforementioned studies were conducted in small populations or over short periods of time. The association between polyphenol intake and cardiovascular risk factors has also been evaluated in large, long-term epidemiological trials, but with the limitations associated with using FFQs [21–24]. Therefore, the aim of the present study was to apply the reliable and validated antioxidant activity test, the Folin-Ciocalteu method, in urine samples as a biomarker of total polyphenol intake, to analyse the association between polyphenol intake and cardiovascular risk factors in an elderly population at high cardiovascular risk after a long-term follow-up (median: 4.8 years).

2. Methods

The present study was conducted within the frame of the PREDIMED study, which aimed to assess effects of the Mediterranean diet on the primary prevention of CVDs in Spain. The protocol and recruitment methods have been reported in detail elsewhere [25]. Eligible participants were men aged 55–80 and women aged 60–80 years without any history of cardiovascular disease but fulfilling at least one of the following two criteria: type-2 diabetes or three or more cardiovascular risk factors (family history of early-onset CVDs, hypertension, current smoking, low HDL-cholesterol, high LDL-cholesterol, and overweight or obesity). Exclusion criteria included any severe chronic illness, previous history of CVDs, alcohol or drug abuse, body mass index (BMI) of more than 40 kg/m^2, and history of allergy or intolerance to olive oil or nuts. The trial was stopped after a median follow-up of 4.8 years due to the benefit of the Mediterranean diet with respect to major cardiovascular events: myocardial infarction, stroke, or death from cardiovascular causes (analysis performed by the Drug and Safety Monitoring Board of the trial), compared to a control low-fat diet [26].

The present longitudinal analysis included 612 volunteers, randomly selected from two recruitment centers in Spain. All participants provided written informed consent, and the protocol was approved by the Institutional Review Boards of the participating centers and registered.

2.1. Nutritional Assessments. Dietary habits of participants were assessed through a validated 137-item FFQ [27]. Nutrient intake was adjusted by calories using the residuals' method. Information about lifestyle, health condition, education, history of illnesses, and medication use was collected by a 47-item general questionnaire. The degree of adherence to the Mediterranean diet was assessed by a 14-point questionnaire [28]. Physical activity was assessed using the validated Spanish version of the Minnesota Leisure-Time Physical Activity Questionnaire [29]. All questionnaires were administered and repeated annually during the follow-up by trained staff in face-to-face interviews.

Information on polyphenol intake was obtained using the FFQ and the Phenol-Explorer database. The relationship between food items in the FFQ and the database has been described previously [30]. The content of total polyphenol intake equals the sum of all the individual polyphenol from each food item.

2.2. TPE Measurements. Urine samples were collected and coded and then immediately shipped to a central laboratory, to be stored at −80°C until analysed. The Folin-Ciocalteu method was applied to determine the content of TPE, using a clean-up procedure with solid phase extraction (SPE) performed in 96-well plate cartridges (Oasis MAX), which helped to remove urinary interferences. Finally, TPE was expressed as mg gallic acid equivalent (GAE)/g of creatinine.

All details have been previously described by Medina-Remón et al. [14].

2.3. Clinical Measurements. Weight and height were measured with light clothing and no shoes with a calibrated balance and a wall-mounted calibrated stadiometer, respectively. BMI was calculated as weight in kilograms divided by the square of height in meters. For the measurement of blood pressure (BP), a validated semiautomatic sphygmomanometer (Omron HEM-705CP) was used by trained nurses. Measurements were taken at 5-minute intervals with participants in a seated position. Data were collected as an average of 2 measurements in each arm, repeated twice [31].

Plasma glucose, total cholesterol, and triglyceride concentrations were measured using standard enzymatic automated methods. Levels of HDL-cholesterol were measured by an enzymatic procedure after precipitation, and LDL-cholesterol was estimated by the Friedewald formula [32].

2.4. Statistical Analysis. Results were expressed as mean ± SD for continuous variables or percentages for categorical variables. Kolmogorov tests were applied to examine the normality distribution and skewness. All participants were divided into three categories according to changes in TPE during the follow-up ($\Delta TP < -11.4$ mg gallic acid/g creatinine, $-11.4 \leq \Delta TP \leq 24.6$ mg gallic acid/g creatinine, and $\Delta TP > 24.6$ mg gallic acid/g creatinine). Changes in nutrient and key food consumption during the follow-up were assessed with ANOVA for repeated measurements analysis. Bonferroni *post hoc* test and paired *t*-test were used to compare each variable within and between groups.

Multivariate linear regression models were used to assess the relationship between serum glucose, total cholesterol, HDL, LDL, triglyceride concentrations, systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate, and tertiles of changes in TPE during the follow-up period, adjusted for potential confounders (sex, age, intervention groups, BMI, smoking status, family history of CHD, physical activity, hypertension, diabetes, dyslipidemia, medication use, and 14-unit Mediterranean diet score at baseline). Sensitivity analyses were used to further assess the relationship between specific cardiovascular risk factors and subcategories.

General Linear Model (GLM) approach to ANCOVA was used to determine differences between tertiles of changes in TPE after 5-year follow-up, adjusted for potential confounders as did in multivariate linear regression models.

All analyses were performed using SPSS software V21.0 (Chicago, USA). All models were tested for the detection of outliers, multicollinearity, homoscedasticity, and normality and independence of errors. All statistical tests were two-tailed, and the significance level was $P < 0.05$.

3. Results

After 5 years of follow-up of 612 participants randomly selected for this substudy of the PREDIMED trial, 39 were excluded because of extreme TPE values, hence a total of 573 participants were included in the present study. Baseline characteristics of participants grouped by tertiles of changes in TPE during the follow-up are shown in Table 1. According to the study design, the average age was 67.3 ± 5.9 years with a BMI of 29.2 ± 3.3 kg/m^2. Most of the participants gathered a high number of cardiovascular risk factors: 41.5% had diabetes; 80.5% had hypertension; 66.8% had dyslipidemia; 16.9% were current smokers, and 37.5% had a family history of CHD. In the second tertile, individuals were less likely to be women and had a higher body weight.

Table 2 shows changes in key food consumption during the follow-up. Most key foods changed considerably after the long-term intervention, with the exception of legumes and chocolate. Table 3 summarizes information on nutrient intake at baseline and 5 years according to changes in TPE during the follow-up. Comparing nutrient intake at 5 years versus baseline, we observed a significant increase in total fat, fibre, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), K, and Mg, while other items such as total carbohydrates, protein, saturated fatty acids (SFA), Na, and cholesterol remained unchanged. This may be due to dietary changes based on recommendations to adhere to a Mediterranean diet, which is characterized by a high consumption of vegetables, fruits, olive oil, wine, and nuts and a low consumption of red meat, high-fat dairy products, and sweets. However, there were no significant changes when comparing tertiles and their interaction. In addition, we found that significant changes in TPE did not significantly affect the intake of nutritional elements among groups.

Several antioxidant substances such as sulfur dioxide, ascorbic acid, sugar, aromatic amines, organic acid, Fe(II), and nonphenolic organic substances might affect total polyphenol when applying Folin-Ciocalteu assay; however, after a solid phase extraction (SPE), the aforementioned interfering substances were eliminated through the cleaning-up process [14].

Linear regression analyses were conducted to assess the relationship between TPE ($Q_1 = -48.70 \pm 30.11$ mg GAE/g creatinine; $Q_2 = 6.95 \pm 10.55$ mg GAE/g creatinine; $Q_3 = 64.48 \pm 31.61$ mg GAE/g creatinine) and clinical possible cardiovascular risk factors (plasma glucose, triglyceride, cholesterol, HDL-c, and LDL-c concentrations, and SBP, DBP, and heart rate). Results are shown in Table 4. Significant inverse associations were found between tertiles of changes in TPE and glucose ($\beta = -4.372$; $P = 0.026$), triglycerides ($\beta = -8.572$; $P = 0.006$), and DBP ($\beta = -1.156$; $P = 0.031$) after adjustment for potential confounders. However, other parameters did not show significant associations. The standardized coefficients (Beta) in the model were used to measure degrees of contribution to different risk factors. Results indicate that, among the CVD risk factors, triglyceride levels showed the highest beneficial effects of dietary polyphenol intake (Beta = −0.126; $P = 0.031$).

We also conducted sensitivity analyses to ascertain whether significant changes were related to specific variables. As shown in Table 5, men were more likely to improve their plasma triglyceride concentration than women, according to tertiles of changes in TPE. In contrast, the lowering effects

Table 1: Baseline characteristics of participants according to tertiles of changes in TPE.

	TPE (mg GAE/g creatinine)						
	Q_1 (ΔTP < −11.4)		Q_2 (−11.4 ≤ ΔTP ≤ 24.6)		Q_3 (ΔTP > 24.6)		P
Number of subjects	191		191		191		
Women, *n* (%)	101	(52.9)	83	(43.5)	112	(58.6)	0.011
Age (y), mean (SD)	66.7	(5.9)	67.3	(5.8)	68.00	(6.0)	0.113
Weight (kg), mean (SD)	73.9	(10.6)	77.1	(11.6)	74.5	(10.7)	0.01
BMI (kg/m^2), mean (SD)	28.9	(3.1)	29.6	(3.5)	29.2	(3.2)	0.103
Systolic BP (mm Hg), mean (SD)	149.8	(17.9)	151.6	(16.9)	152.8	(18.6)	0.238
Diastolic BP (mm Hg), mean (SD)	84.3	(9.8)	85.9	(10.0)	85.5	(10.4)	0.269
Hypertension, *n* (%)	151	(79.1)	152	(79.6)	158	(82.7)	0.621
Diabetes, *n* (%)	78	(40.8)	85	(44.5)	75	(39.3)	0.567
Dyslipidemia, *n* (%)	136	(72.3)	117	(61.3)	128	(67)	0.074
Smoking status							0.641
Current, *n* (%)	35	(18.3)	34	(17.8)	28	(14.7)	0.586
Former, *n* (%)	36	(18.8)	43	(22.5)	47	(24.6)	0.388
Never, *n* (%)	120	(62.8)	114	(59.7)	116	(60.7)	0.814
Family history of CHD, *n* (%)	65	(35.3)	75	(40.3)	75	(41.2)	0.460
Medication							
Aspirin, *n* (%)	33	(32.0)	35	(34.0)	35	(34.0)	0.949
Antihypertensive drugs, *n* (%)	131	(68.6)	142	(74.3)	141	(73.8)	0.381
Hypolipidemic drugs, *n* (%)	91	(47.6)	70	(36.6)	78	(40.8)	0.089
Insulin, *n* (%)	10	(5.2)	9	(4.7)	8	(4.2)	0.890
Oral hypoglycemic drugs, *n* (%)	40	(20.9)	46	(24.1)	45	(23.6)	0.736
Vitamin or minerals, *n* (%)	18	(9.5)	16	(8.5)	13	(6.9)	0.644
Educational level							
Primary school, *n* (%)	140	(74.1)	139	(73.5)	146	(76.8)	
High school, *n* (%)	32	(16.9)	28	(14.8)	28	(14.7)	0.793
University, *n* (%)	17	(9.2)	22	(11.6)	16	(8.4)	
Physical activity at leisure time (MET-min/d)	275	(212)	287	(204)	269	(183)	0.696
Polyphenol intake (mg/d)	853.4	(239.8)	831.2	(248.9)	882.7	(247.8)	0.135

BMI: body mass index; CHD: coronary heart disease; GAE: gallic acid equivalent; TPE: total polyphenol excretion.
Data are given as means (SD) for continuous variables and percentages for categorical variables; $P < 0.05$ indicates statistical significance.
* P values calculated by analysis of variance or χ^2 tests.

of higher polyphenol consumption on DBP were greater in women. In addition, when the P-14 was considered separately, higher scoring groups showed significant differences in plasma triglyceride concentration according to tertiles of changes in TPE.

4. Discussion

In this 5-year study of an elderly population at high cardiovascular risk living in a Mediterranean country, we observed that higher polyphenol intake, measured by TPE, was inversely associated with some cardiovascular risk factors. The observed benefits on CVDs were ascribable to a reduction in plasma glucose and triglyceride concentrations and a diminution of DBP. This may partly explain the decreased CVD risk shown by people following a polyphenol-rich diet such as the Mediterranean diet.

The beneficial effects of polyphenols consumption on major cardiovascular events in the PREDIMED cohort have been published before [20]. The difference between our findings and other reported results lies in the measurement of polyphenols in urine as biomarker of polyphenol intake. Given that more than 8000 phenolic structures exist in nature, beneficial effects from polyphenols depend on a variety of factors, including total intake, food cooking processes, digestion, absorption, metabolic pathways *in vivo*, or even differences between individuals [33]. Therefore, TPE, as a biomarker of total polyphenol intake, may provide a more accurate insight into the effects of polyphenols on CVDs than other dietary assessment methods.

Previous clinical studies on the benefits of polyphenols on the cardiovascular system have provided inconsistent results. A 12-week follow-up clinical trial conducted in Korea reported a strong inverse association between consumption of polyphenol extracts from *Ecklonia cava* and serum glucose, SBP, and HDL concentration [17]. In contrast, a recent randomized control trial performed with 67 elderly men at high cardiovascular risk found an increase in HDL

Table 2: Changes in daily intake of key foods after 5 years with energy adjustment categorized by tertile of changes in TPE[a].

		TPE (mg GAE/g creatinine)								
		Q_1 (ΔTP < −11.4)		Q_2 (−11.4 ≤ ΔTP ≤ 24.6)		Q_3 (ΔTP > 24.6)		P[b]		
		Mean	SD	Mean	SD	Mean	SD	Time[c]	Group[d]	Time ∗ Group[e]
Vegetables (g/d)	Baseline	302.1	117.5	293.9	109.0	289.6	118.4	<0.001	0.195	0.369
	5 years	366.0**	122.9	340.7**	115.8	354.3**	120.9			
Fruits (g/d)	Baseline	346.3	176.7	354.7	169.3	385.4	183.6	<0.001	0.477	0.103
	5 years	459.4**	181.4	456.4**	172.6	454.0**	158.8			
Legumes (g/d)	Baseline	18.7	7.2	19.2	7.2	19.69	8.8	0.445	0.149	0.736
	5 years	18.7	8.3	19.1	7.7	19.9	8.1			
Cereals (g/d)	Baseline	240.0	73.2	242.9	79.2	238.9	70.7	<0.001	0.867	0.712
	5 years	221.1**	63.4	216.4**	68.5	216.1**	63.0			
Milk (g/d)	Baseline	368.8	201.3	345.4	193.9	393.2	233.2	0.005	0.300	0.166
	5 years	402.2*	223.4	386.4**	204.3	395.6	196.6			
Meat (g/d)	Baseline	140.9	49.1	140.1	48.8	138.0	45.3	<0.001	0.992	0.431
	5 years	126.6**	41.9	126.9**	43.3	130.1	44.1			
Fish (g/d)	Baseline	94.7	37.2	90.4	39.1	91.7	39.2	<0.001	0.521	0.882
	5 years	101.4	45.6	98.8**	43.2	97.8*	36.2			
Pastries (g/d)	Baseline	26.1	26.1	25.7	27.1	26.6	25.1	0.001	0.846	0.485
	5 years	20.1	24.2	23.1	28.4	21.5	27.3			
EVOO (g/d)	Baseline	24.1	24.2	21.8	23.8	21.3	22.9	<0.001	0.848	0.346
	5 years	48.2**	22.8	48.1**	25.0	49.7**	23.1			
Nuts (g/d)	Baseline	11.0	12.1	9.8	13.1	10.6	13.1	<0.001	0.794	0.656
	5 years	16.0**	12.5	16.1**	13.1	16.7**	12.2			
Wine (g/d)	Baseline	98.3	140.1	105.2	157.2	96.8	136.1	0.002	0.781	0.979
	5 years	80.1**	130.5	89.0	130.8	80.7	123.4			
Folic acid (μg/d)	Baseline	376.7	83.8	379.4	81.1	381.9	87.6	<0.001	0.939	0.322
	5 years	432.6**	75.9	425.3**	87.3	424.5**	72.4			
Coffee (mL/d)	Baseline	38.4	57.0	33.2	44.2	33.9	47.2	0.004	0.902	0.258
	5 years	27.4*	48.0	28.6	46.4	30.7	48.8			
Chocolate (g/d)	Baseline	2.9	5.7	2.5	4.7	3.1	5.9	0.940	0.422	0.203
	5 years	2.2	4.2	3.1*	6.1	3.4	7.9			

[a]Data are given as means (SD); $P < 0.05$ indicates statistical significance. EVOO: extra virgin olive oil; GAE: gallic acid equivalent; TPE: total polyphenol excretion. Values with asterisks are statistically different from baseline values by the paired-samples t-test ($^*P < 0.05$; $^{**}P < 0.01$).

[b]Data analysed by repeated-measures 2-factor ANOVA.

[c]Comparison between the time before and after intervention.

[d]Comparison between tertiles of TPE changes.

[e]Comparison between measurements obtained before and after intervention and between tertiles of TPE changes.

after consumption of red wine, whereas fasting glucose was kept constant throughout the study, which differs from our observation [34]. Another contrasting result was found in participants with type-2 diabetes, who improved their HDL level and decreased total cholesterol after the consumption of polyphenol-rich chocolate [35]. In addition, a group of overweight participants consuming polyphenol-rich dark chocolate had lower plasma glucose, SBP, and DBP after the intervention, which partly agrees with our findings [18]. However, in the present study, we found no association between polyphenol intake and cholesterol profiles or SBP.

Participants who increased their polyphenol intake showed a reduction in plasma glucose concentrations, adding to the evidence that polyphenol-rich diets protect the cardiovascular system by improvements in glycemic control. A similar clinical trial performed on 78 participants at high cardiovascular risk, administration of polyphenol-rich foods, improved glucose metabolism by increasing early insulin secretion and insulin sensitivity [36]. Another cross-sectional study in an elderly population reported that green tea consumption was inversely associated with fasting blood glucose concentrations, though without adjusting for potential

Table 3: Changes in nutrient intake after 5 years with energy adjustment categorized by tertile of changes in TPE[a].

		TPE (mg GAE/g creatinine)								
		Q_1 (ΔTP < −11.4)		Q_2 (−11.4 ≤ ΔTP ≤ 24.6)		Q_3 (ΔTP > 24.6)		P[b]		
		Mean	SD	Mean	SD	Mean	SD	Time[c]	Group[d]	Time ∗ Group[e]
Total carbohydrates (g/d)	Baseline	235.6	36.5	238.4	43.2	239.9	35.9	0.736	0.964	0.41
	5 years	239.7	63.1	235.0	68.9	235.6	61.0			
Protein (g/d)	Baseline	88.4	36.5	91.2	43.2	92.7	35.9	0.12	0.649	0.498
	5 years	94.5	18.6	92.7	19.5	94.4	17.7			
Total fat (g/d)	Baseline	102.5	12.8	100.8	12.6	102.7	13.6	<0.001	0.528	0.331
	5 years	110.7**	23.1	112.9**	25.6	113.4**	24.4			
MUFA (g/d)	Baseline	53.5	13.6	52.3	17.4	51.6	15.2	<0.001	0.97	0.19
	5 years	58.3**	12.7	59.6**	13.5	59.8**	13.5			
SFA (g/d)	Baseline	25.5	9.0	24.6	10.1	24.0	9.7	0.949	0.887	0.114
	5 years	24.2	6.4	24.8	7.4	25.1	7.3			
PUFA (g/d)	Baseline	15.7	4.7	15.7	6.1	15.6	5.4	<0.001	0.716	0.675
	5 years	19.0**	5.9	19.0**	5.6	19.6**	5.5			
Alcohol (g/d)	Baseline	14.1	5.2	13.3	5.4	13.4	4.8	0.039	0.979	0.75
	5 years	11.9	14.6	12.4	15.2	12.2	14.7			
Fibre (g/d)	Baseline	24.2	6.0	24.6	5.6	25.2	6.4	<0.001	0.564	0.204
	5 years	26.6**	7.5	25.8	7.4	26.4	7.0			
Cholesterol (mg/d)	Baseline	352.4	84.6	353.1	94.6	350.5	94.0	0.2	0.946	0.975
	5 years	359.9	90.9	358.0	98.7	356.8	92.7			
Na (mg/d)	Baseline	2322.4	479.6	2273.1	528.7	2263.7	479.9	0.736	0.963	0.41
	5 years	2229.8	644.5	2230.8	728.0	2253.7	652.0			
K (mg/d)	Baseline	4230.9	723.7	4164.3	682.7	4300.6	796.1	<0.001	0.234	0.542
	5 years	4654.5**	826.8	4546.0**	963.7	4614.7**	805.9			
Mg (mg/d)	Baseline	359.5	62.7	358.4	58.1	367.1	61.8	<0.001	0.432	0.365
	5 years	398.5**	82.1	388.1**	86.4	394.3**	80.8			

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, GAE: gallic acid equivalent; TPE: total polyphenol excretion.
[a]Data are given as means (SD); $P < 0.05$ indicates statistical significance. Values with asterisks are statistically different from baseline by paired-samples t-test ($^*P < 0.05$; $^{**}P < 0.01$).
[b]Data analysed by repeated measures 2-factor ANOVA.
[c]Comparison between the time before and after intervention.
[d]Comparison between tertile changes in TPE.
[e]Comparison between measures obtained before and after intervention and between tertiles of TPE changes.

confounders [37]. Despite the abundance of results from different clinical trials, animal models, and *in vitro* tests, the mechanisms for hypoglycemic effects of polyphenols still warrant discussion. Potential explanations for these putative protective effects include reduced absorption of total carbohydrate in the intestine, modulation of enzymes related to glucose metabolism, stimulation of insulin secretion, improvement of β-cell function and insulin action, reduction in oxidative stress, inhibition of glucose transport, and enhanced vascular function [36, 38–40].

Triglycerides are considered the highest source of energy, and inhibition of triglyceride absorption also plays a role in the prevention of CVDs [41]. In the present study, increasing polyphenol intake was inversely associated with triglyceride levels, in agreement with some previous studies. Sugiyama et al. investigated the inhibitory effect of oligomeric procyanidins from apples on triglyceride absorption, explained by the inhibition of pancreatic lipase activity *in vivo* and in animal models [42]. Data from animal models indicated that such lowering effects could be attributed to the very low-density lipoprotein (VLDL) secretion rates and a decrease in apolipoprotein B secretion [43]. In addition, a study of haemodialysis patients fed with polyphenol-rich pomegranate juice also reported improvements in triglyceride levels, but this was explained by an inhibition of intestinal absorption and clearance of plasma triglycerides *in vivo*[19]. The variety of plausible mechanisms put forward to explain these effects, such as absorption, metabolism, and elimination during metabolic processes, reflect the highly varied chemical structure of polyphenols. Unlike the current study, most clinical trials have focused on a single

Table 4: Multivariate linear regression analyses with changes in cardiovascular risk factors as dependent variables and tertiles of changes in TPE in spot urine samples (mg GAE/g creatinine) as exposure variables, adjusted for potential confounders.

		β	SE	Beta	Sig.	95% CI	
Change in GLU (mg/dL)	Model 1	−4.164	1.979	−0.095	0.036	−8.053	−0.275
	Model 2	−4.316	1.981	−0.098	0.030	−8.208	−0.424
	Model 3	−4.355	1.949	−0.099	0.026	−8.186	−0.525
	Model 4	−4.372	1.953	−0.099	0.026	−8.209	−0.534
Change in COL (mg/dL)	Model 1	−2.51	2.001	−0.057	0.210	−6.442	1.421
	Model 2	−2.236	2.011	−0.050	0.267	−6.187	1.715
	Model 3	−1.845	2.013	−0.042	0.360	−5.800	2.109
	Model 4	−1.802	2.015	−0.041	0.372	−5.762	2.157
Change in HDL (mg/dL)	Model 1	0.102	0.448	0.010	0.820	−0.778	0.982
	Model 2	0.135	0.448	0.014	0.763	−0.744	1.015
	Model 3	0.133	0.456	0.014	0.771	−0.764	1.030
	Model 4	0.174	0.454	0.018	0.701	−0.718	1.067
Change in LDL (mg/dL)	Model 1	−0.205	1.775	−0.005	0.908	−3.693	3.283
	Model 2	−0.039	1.784	−0.001	0.983	−3.545	3.467
	Model 3	0.448	1.783	0.012	0.802	−3.056	3.952
	Model 4	0.469	1.786	0.012	0.793	−3.041	3.979
Change in TG (mg/dL)	Model 1	−8.356	3.06	−0.123	0.007	−14.369	−2.344
	Model 2	−8.563	3.058	−0.126	0.005	−14.572	−2.554
	Model 3	−8.627	3.094	−0.127	0.006	−14.708	−2.546
	Model 4	−8.572	3.099	−0.126	0.006	−14.662	−2.483
Change in SBP (mm Hg)	Model 1	−1.367	0.994	−0.058	0.169	−3.319	0.585
	Model 2	−1.222	1.001	−0.052	0.222	−3.188	0.744
	Model 3	−1.127	1.003	−0.048	0.262	−3.098	0.843
	Model 4	−1.098	1.005	−0.046	0.275	−3.071	0.876
Change in DBP (mm Hg)	Model 1	−1.316	0.531	−0.104	0.013	−2.359	−0.273
	Model 2	−1.254	0.532	−0.099	0.019	−2.298	−0.209
	Model 3	−1.153	0.532	−0.091	0.031	−2.198	−0.108
	Model 4	−1.156	0.533	−0.091	0.031	−2.203	−0.109
Change in HR	Model 1	−0.002	0.555	0.000	0.997	−1.091	1.087
	Model 2	0.043	0.559	0.003	0.938	−1.055	1.142
	Model 3	−0.011	0.567	−0.001	0.985	−1.125	1.103
	Model 4	−0.074	0.565	−0.006	0.895	−1.184	1.035

GLU: glucose, COL: total cholesterol, HDL: high-density lipoprotein, LDL: Low-density lipoprotein, TG: triglycerides, SBP: systolic blood pressure, DBP: diastolic blood pressure, and HR: heart rate.

β: nonstandardized coefficient (regression line coefficient); SE: standard error; Beta: standardized coefficient; CI: confidence interval; P: two-sided test of significance.

Model 1: unadjusted; Model 2 adjusted for sex, age, and intervention groups; Model 3 adjusted as in Model 2 plus BMI, smoking status, family history of CHD, physical activity, hypertension, diabetes, dyslipidemia, and medication use: antihypertensive drugs, vitamins, insulin, oral hypoglycemic drugs, aspirin, or other antiplatelet drug; Model 4 was adjusted as in Model 3 plus 14-unit Mediterranean diet score.

polyphenol-rich food such as dark chocolate, wine, or green tea. Therefore, considering that the Mediterranean diet is a constellation of several polyphenol-rich foods, it is difficult to draw a single mechanism to explain the lowering effects found on triglycerides.

Hypertension is a well-established risk factor for CVDs [44]. There is evidence from our study and others that increasing polyphenol intake is associated with lower BP. Both DASH (Dietary Approaches to Stop Hypertension) and SUN (Seguimiento Universidad de Navarra) studies emphasize that the consumption of plant-derived foods, particularly fruits, vegetables, nuts, and olive oil, is inversely associated with BP [45–47]. We previously reported that greater TPE was inversely associated with BP [13]. However, we found significant associations only for DBP, and not SBP. Another PREDIMED clinical substudy based on a 4-year intervention also supports our findings [48]. Mechanisms of the BP lowering effect could involve endothelial nitric oxide (NO) production. NO plays a fundamental role in the regulation of the vascular system, and vascular homeostasis is achieved only when NO levels are adequate [6]. Briefly, polyphenols induce NO production by promoting endothelial nitric oxide synthase (eNOS) expression, generating vascular relaxing factors such as prostacyclin (PGI2) and

Table 5: Sensitivity analysis of clinical cardiovascular risk factors.

			Change in TG (mg/dL)							Change in GLU (mg/dL)							Change in DBP (mm Hg)						
		N	Q_1		Q_2		Q_3		P^a	Q_1		Q_2		Q_3		P^a	Q_1		Q_2		Q_3		P^a
			Mean	SD	Mean	SD	Mean	SD		Mean	SD	Mean	SD	Mean	SD		Mean	SD	Mean	SD	Mean	SD	
Gender	Male	236	6.41	43.58	−17.86	62.54	−14.80	44.68	0.007	2.35	38.03	−0.32	35.56	−6.12	35.99	0.36	−1.24	10.04	−2.43	9.73	−2.14	11.67	0.714
	Female	249	8.22	46.57	9.25**	53.14	−5.01	68.69	0.194	10.31	38.68	9.23	30.44	1.51	35.04	0.193	−1.46	10.24	−3.67	10.75*	−5.29	9.84	0.026
Age, years	≤67	242	8.71	47.80	−9.75	70.39	−8.77	62.27	0.089	7.08	41.13	1.56	30.86	−2.23	36.48	0.262	−0.64	9.70	−1.41	10.78	−2.77	11.73	0.396
	≥68	243	5.96	42.21	−2.06	46.74	−9.68	57.35	0.129	6.06	35.60	6.37	36.39	−1.36	34.91	0.285	−2.18	10.58	−4.50*	9.36	−4.99	9.75	0.123
P-14	<9	274	−0.89	57.27	−5.27	58.57	−10.63	67.61	0.676	7.29	28.72	5.65	34.89	−3.68	42.90	0.178	−1.36	11.63	−2.39	9.87	−4.98	10.05	0.094
	≥9	211	12.07	35.90	−6.49	61.19	−8.33	53.71	0.007	6.18	43.25	2.69	32.89	−0.43	29.55	0.428	−1.35	9.20	−3.36	10.40	−3.27	11.17	0.24

P-14: 14-point Mediterranean diet score test; GLU: glucose; TG: triglycerides; DBP: diastolic blood pressure.

[a]P value tested by ANOVA.

Values with asterisks are statistically different from the baseline by the paired-samples t-test ($^*P < 0.05$; $^{**}P < 0.01$).

inhibiting synthesis of the vasoconstrictor endothelin-1 (ET-1) in vascular endothelial cells [49]. Strong and positive association between polyphenol intake and plasma NO levels has been previously demonstrated by our group [8].

Some study limitations deserve to be noted. First, given that this substudy was conducted only among elderly subjects at high cardiovascular risk, it is difficult to extrapolate the results to the general population. Second, even though we adjusted potential confounders relative to CVD risk, residual confounding could still exist. Nonetheless, our study adds new evidence in support of a preventative effect of a long-term polyphenol intake on CVDs.

Compared with previous studies, the present study also has several strengths. Firstly, even though biomarkers are necessary to assess the compliance of the intervention, it is difficult to find a reliable and available biomarker. TPE in urine could be useful as a marker of compliance in intervention studies with foods with high-polyphenol content such as fruits, vegetables, wine, chocolate, tea, and coffee, while other markers are not suitable; moreover, in comparison with measuring the total polyphenol intake through self-reported information based on FFQ, the use of TPE, a biomarker of polyphenol intake, could provide more precise evidence [20, 50]. Secondly, the long duration of the intervention should also be considered as strength, since only few studies have tested associations between polyphenols and cardiovascular risk factors in such long-term intervention [8, 51, 52]. Thirdly, the selection of participants is a group of free-living individuals reproducing real-life conditions with home-prepared, energy-unrestricted foods. Fourthly, the Folin-Ciocalteu assay is a rapid, cheaper, and environmentally friendly measurement without requirement of dedicated instrumentation, which could be suggested to be applied in large intervention studies in the future.

In conclusion, in this 5-year study within the frame of the PREDIMED trial conducted in subjects at high cardiovascular risk, we found that polyphenol intake measured by TPE was inversely associated with some clinical cardiovascular risk factors, namely, plasma glucose and triglycerides concentrations and SBP, suggesting that intake of polyphenols provides protection against CVDs throughout these mechanisms. Further research is needed to confirm the current findings in the general population.

Disclosure

None of the funding sources played a role in the design, collection, analysis, or interpretation of the data or in the decision to submit the paper for publication.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This study was supported by CICYT (AGL2013-49083-C3-1-R) from the Spanish Ministry of Economy and Competitiveness (MEC), the Generalitat de Catalunya (GC) 2014 SGR 773 and Instituto de Salud Carlos III, ISCIII (CIBEROBN). CIBEROBN is an initiative of ISCIII, Spain. Xiaohui Guo received support from China Scholarship Council (CSC). Alexander Medina-Remón thanks the "Juan de la Cierva" postdoctoral program (JCI-2012-13463) from MEC. The Fundación Patrimonio Comunal Olivarero (Madrid, Spain), California Walnut Commission (Sacramento, CA), Borges SA (Reus, Spain), and Morella Nuts SA (Reus, Spain) donated the olive oil, walnuts, almonds, and hazelnuts, respectively, used in the study.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 1594616, 9 pages
http://dx.doi.org/10.1155/2016/1594616

Research Article

The Potential Health Benefits of Polyphenol-Rich Extracts from *Cichorium intybus* L. Studied on Caco-2 Cells Model

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Received 7 September 2015; Revised 28 October 2015; Accepted 28 October 2015

Academic Editor: Gregor Drummen

Phytochemicals can exert their bioactivity without reaching the systemic circulation; scarcely absorbed antioxidants might reach the large bowel contributing to protection from oxidative damage-induced gastrointestinal diseases. In the present work, we aimed to study the relationship between potential activity of polyphenol-rich extracts from *Cichorium intybus* L. and changes in morphological characteristics on Caco-2 cells. Phytochemicals content (carotenoids and flavonoids) and total antioxidant activity of Red Chicory of Treviso and Variegated Chicory of Castelfranco were evaluated. The bioactivity of polyphenol-rich extracts from chicories was studied in *in vitro* Caco-2 cell monolayers model. Morphological characteristics changes to test the antioxidant and/or prooxidant effect were verified by histological analysis and observed by Electronic Scansion Microscopy (SEM). On Caco-2 cell model, the polyphenols fractions from chicories have indicated a moderate antioxidant behavior until 17 μM concentration, while 70 μM and 34 μM exert cytotoxic effects for Treviso's and Castelfranco's Chicory, respectively, highlighted by TEER decreasing, increased permeability, and alteration of epithelium. Our findings support the beneficial effects of these products in counteracting the oxidative stress and cellular damage, induced *in vitro* on Caco-2 cell model, through interaction with the mucopolysaccharide complexes in the glycocalyx, maintaining *in vivo* a healthy and effective intestinal barrier.

1. Introduction

Chicory, a plant genus typical of Mediterranean area, is native to Europe, Western Asia, and North America and its colour varies from white to red [1]. "Red Chicory of Treviso" and "Variegated Chicory of Castelfranco," with PGI according to EU rules, are strongly linked to their territory and grown according to traditional cultivation techniques. These products have acquired great interest for their organoleptic and nutritional characteristics. As well known, the consumption of phytochemicals from fruits and vegetables can improve the prevention of several chronic degenerative pathologies [2, 3]. Phytochemicals content may be affected by several factors: genetic characteristic, environmental aspects, agronomic practices, and postharvest conditions [4, 5]. Genetic factors exert great influence on nutritional and phytochemicals content, between and within vegetables species. Climate condition, light exposure, temperature, relative humidity, and luminous intensity are specific parameters that affect food quality. In particular, the choice of an appropriate agronomic practice could improve the levels and the profile of bioactive compounds. Among the vegetal crops, red chicories are attractive because they may be consumed either raw or cooked. In particular, they are commonly eaten raw in salad during winter months when most vegetables are not in season. In fact, red chicories are particularly resistant to low temperatures [6] and their availability throughout the year is an important source of micronutrients during the coldest season. The red color is caused in large part by the presence of water-soluble pigments, anthocyanins, but several works show that the red-leaved varieties of *Cichorium intybus* L. have the highest content of polyphenols among the leafy vegetables that are consumed raw [7, 8]. Changes in phytochemicals content in agricultural production take on

a particular importance in our diet. The bioactive compounds in foods, such as vitamins, carotenoids, and polyphenols, seem to be able to modulate one or more metabolic processes, which result in the promotion of better health [9]. The most accepted explanation for the protective effect of food probably derives from the observation that different plant phytochemicals may act as "scavengers" of free radicals, "quenchers" singlet oxygen, or metal chelators [10, 11] and therefore induce protection against oxidative damage through antibacterial, anti-inflammatory, hepatoprotective, anticarcinogenic, and vasodilator actions.

Recently, D'Evoli et al. [12] have shown that the high levels of antioxidant anthocyanins present in Red Chicory exert a direct scavenging effect against ROS formation in terms of antioxidant and cytoprotective activities as well as antiproliferative activity in Caco-2 cell. In the present work, we aimed to study the relationship between potential activity of polyphenol-rich extracts from chicories and morphological and chemical/physical changes in Caco-2 cellular line. To this purpose, bioactive compounds content (carotenoids and flavonoids) and total antioxidant activity were evaluated in Early and Late Red Chicory of Treviso and Variegated Chicory of Castelfranco. In addition, the bioactivity of polyphenol-rich extracts from chicories in *in vitro* Caco-2 cell monolayer model was studied. Morphological characteristics changes to test the antioxidant and/or prooxidant effect were verified by histological analysis and observed by Electronic Scansion Microscopy (SEM).

2. Materials and Methods

2.1. Reagents. All solvents were purchased from Carlo Erba (Milan, Italy), BDH (Poole, England), and Merck (Darmstadt, Germany). 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) was from Fluka (Switzerland). Phosphate-buffered saline (PBS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and ascorbic acid were provided by Sigma-Aldrich Srl. Commercial standards were also from Sigma-Aldrich Srl (Milan, Italy). AAPH (2,2′,-azobis (2-amidinopropane) dihydrochloride) (WACO Chem., Richmond, VA, USA) was used as a source of hydrophilic radicals. Double distilled water (Millipore, Milan, Italy) was used throughout the study.

2.2. Cichorium intybus L. Red Chicories of Treviso (two varieties: Early and Late) samples were grown in the region including Quinto (Treviso), Zero Branco (Treviso), and Scorzè (Venezia), while PGI samples of Variegated Chicory of Castelfranco come from Due Carrare (Padova), Mira (Venezia), and Monselice (Padova). A total weight of 5 kg of each variety for each locality was collected at harvesting time and delivered to the laboratory. Only the edible portion of the samples was utilized for analysis, according to the following methods.

2.3. Analytical Methods. The extraction of some flavonoids (quercetin, kaempferol, and apigenin) was performed as described by Hertog et al. [13] and the quantitative analysis through a system-ESA HPLC with electrochemical detector as reported by Azzini et al. [14]. Carotenoids were extracted from Treviso's and Castelfranco's chicory according to the method described by Sharpless et al. [15]. For the quantification, the samples were analyzed by high pressure liquid chromatography (HPLC) as reported by Maiani et al. [16]. Total Antioxidant Capacity (TAC) was evaluated using two different assays: Ferric Reducing Antioxidant Power (FRAP) [17] and Trolox Equivalent Antioxidant Capacity (TEAC) method [18, 19]. The results of each analysis have been performed in triplicate.

2.4. Transepithelial Electrical Resistance (TEER) Evaluation. For TEER evaluation, the polyphenol fractions from Red Chicory of Treviso and Variegated Chicory of Castelfranco hydrolysed extracts have been studied on Caco-2 cell monolayers to test the changes in tight-junction (TJ) permeability by TEER, the phenol red passage, and histological analysis.

The cellular line was treated with increasing concentration of two polyphenols extracts (0.2, 1.3, 10, 17, 34, and 70 μM), for 180 minutes, to simulate *in vivo* physiological processes. The variations of transepithelial potentials and phenol red permeability were recorded at time intervals (30′). In the experiment, the cells were seeded onto polycarbonate filter cell culture chamber inserts (diameter 6.5 mm, area 0.33 cm^2, and pore diameter 0.4 μm), at density of $1.5 \cdot 10^5$ cells per filter and placed in a multiwell Falcon. The filter divided the chamber into two parts, apical and basal, that represent the lumen and the basal area of the gastroenteric system. Into two chambers, TEER measurement for assessment of tight-junction permeability was performed using the Millicell ERS apparatus (Millipore, Bedford, MA, USA) according to the manufacturer's instruction. After calibrating the resistance system, the electrical resistance of the monolayer was measured by placing one electrode on either side of the polycarbonate filter [20, 21]. The results of each analysis have been performed in triplicate and expressed as OHM $\times$ cm^2.

Cells reached confluence and differentiation within 15–20 days. During this time, cellular morphology was monitored and checked with a Leitz Diavert inverted light microscope.

2.5. Histological Analysis. The cellular monolayer was isolated together with the filter and fixed in Bouin (an aqueous solution composed of picric acid, acetic acid, and formaldehyde) for about 12 hours, then dehydrated using the alcohol ascending scale (70%, 80%, 90%, 95%, and 100%), and finally enclosed into resin blocks of polymerized epoxy (GMA, J134K polyscience Inc., Warrington, PA, USA). Then, the samples were glued on embedded stubs and cut into 3 μm sections with a Micron rotary microtome (Zeiss Germany) appropriately assembled so as to use a crystal blade according to Ralph's modification [22, 23]. The sections glued to the microscope slides were stained with Harris' hematoxylin and eosin [24, 25]. The preparations were made permanent with slide covers and sealed with resin (Eukitt, mounting medium, BDA Laboratories Supplies Pool, England). All sections (3 μm) were examined for histological changes by Diaplan 22 light microscopy (Leitz Germany) as shown in Figure 1. All

Table 1: Flavonoid identification from Red Chicory of Treviso and Variegated Chicory of Castelfranco extracts.

Peak analyte	Red Chicory of Treviso			Variegated Chicory of Castelfranco		
	RT (min)	Dominant channel height	Ratio accuracy	RT (min)	Dominant channel height	Ratio accuracy
Quercetin	51.26	637 nA	0.925	51.19	22.7 nA	0.947
Kaempferol	53.28	174 nA	0.800	53.13	123 nA	0.807
Apigenin	55.61	37.3 nA		55.52	36.4 nA	

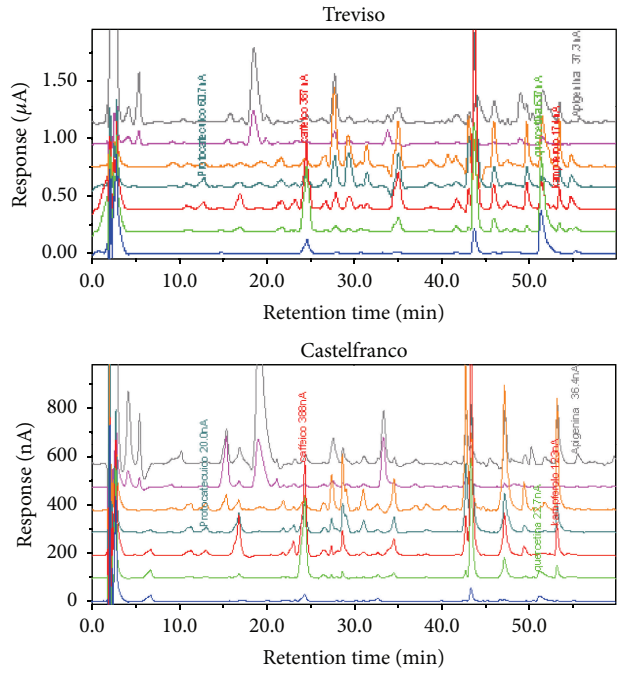


Figure 1: Representative HPLC-ECD gradient array chromatograms of polyphenol-rich extracts analyzed under specified conditions from Red Chicory of Treviso and Variegated of Castelfranco extracts.

pictures (microphotographies) have a magnification of 356x and were performed with Leica microscope Dialux 22.

2.6. SEM Analysis. At the end of each experiment (180 minutes), the cellular monolayer was fixed for 12 hours in formaldehyde (10%) and glutaraldehyde (2.5%) and then dried with alcohol solution. Then, the sample was dehydrated by Critical Point Drying (Emitech K850, Quorum Technologies, West Sussex, UK). The final dehydration was achieved in CO_2. Finally, the sample was sputtered with gold for 120 s at 30 mA in a modified atmosphere with 2% Argon and analyzed using SEM (EVO LS10, Carl Zeiss Microscopy GmbH, Jena, Germany).

2.7. Statistics. The results are expressed as mean ± SD. Statistical data analysis was performed using the ANOVA test followed by Bonferroni post hoc test (significance at $P < 0.05$).

3. Results

3.1. Phytochemical Characterization. According to several authors, flavonoid components could be used as potential markers for the analysis of herbs and plants for human consumption [26–28].

With regard to the quantitative analysis of flavonoids (free plus conjugated forms), Figure 1 displays representative HPLC-DAD gradient array chromatograms, while their identification is reported in Table 1. The varied flavonoids content amongst different varieties and growing locations is summarized in Table 2. Comparing the growing locations, there were no significant differences in the flavonoids content, while significant differences were recorded between varieties. Quercetin was found as the most abundant flavonoid in Red Radish of Treviso varieties (90.04 ± 25.16 mg/kg and 97.88 ± 33.94 mg/kg, resp., in Late and Early Red Chicory) with respect to the Variegated Chicory of Castelfranco (14.20 ± 4.51 mg/kg).

The kaempferol was significantly different among cultivars and varieties. Its content in the Late Red Chicory of Treviso (22.80 ± 5.84 mg/kg) variety was significantly higher ($P < 0.05$) comparing to Early Red Chicory of Treviso (12.35 ± 7.65 mg/kg) and Variegated Chicory of Castelfranco (11.80 ± 3.64 mg/kg).

There were no significant differences in the apigenin content between cultivar and production area on average range from 2.60 ± 1.40 to 3.58 ± 0.29 mg/ kg, respectively, for Early Red Chicory of Treviso and Variegated Chicory of Castelfranco.

Our data (Table 3) indicate that lutein and β-carotene were the main carotenoids (ranging from 1.19 ± 0.24 to 2.40 ± 0.61 mg/kg and from 0.19 ± 0.02 to 0.48 ± 0.15 mg/kg, resp.). A significant difference ($P < 0.05$) was present between β-carotene content of Late Red Chicory of Treviso (0.38 ± 0.08 mg/kg) and Early Red Chicory of Treviso (0.22 ± 0.04 mg/kg). Variegated Chicory of Castelfranco showed a mean of β-carotene content equal to 0.35 ± 0.14 mg/kg. In the Early Red Chicory of Treviso, the mean lutein content was 2.16 ± 0.28 mg/kg with significantly higher levels ($P < 0.05$) than Late Red Chicory of Treviso (1.27 ± 0.20 mg/100 g) and Variegated Chicory of Castelfranco (1.20 ± 0.27 mg/100 g).

After evaluating the content of individual antioxidants, the cooperative action of bioactive molecules in the different specimens of *Cichorium intybus* L. was evaluated by total antioxidant capacity (TAC). Figure 2 shows the synergistic effects of various antioxidants measured by FRAP (mmol/kg) and TEAC (mmol trolox/kg) methods.

Table 2: Flavonoids content amongst different varieties by growing locations (mg/kg).

	Quercetin (mg/kg)	Kaempferol (mg/kg)	Apigenin (mg/kg)
Late Red Chicory of Treviso			
Zero Branco	83.92 ± 15.08	21.43 ± 2.19	2.10 ± 0.61
Scorzè	95.89 ± 26.51	26.23 ± 10.48	3.24 ± 0.82
Quinto	90.31 ± 33.9	20.94 ± 4.86	3.05 ± 1.12
	90.04 ± 25.16[a]	22.80 ± 5.84[a]	2.80 ± 0.85
Early Red Chicory of Treviso			
Zero Branco	101.50 ± 40.20	14.42 ± 8.40	3.98 ± 1.81
Scorzè	100.23 ± 42.4	14.51 ± 9.80	1.51 ± 0.80
Quinto	91.89 ± 29.22	8.11 ± 5.38	2.30 ± 1.60
	97.88 ± 33.94[a]	12.35 ± 7.65[b]	2.60 ± 1.40
Variegated Chicory of Castelfranco			
Monselice	15.32 ± 3.54	8.68 ± 2.16	3.33 ± 0.44
Mira	14.47 ± 4.92	13.5 ± 3.81	3.78 ± 0.38
Due Carrare	12.8 ± 5.08	13.21 ± 4.97	3.62 ± 0.05
	14.20 ± 4.51[b]	11.80 ± 3.64[b]	3.58 ± 0.29

ANOVA: $P < 0.05$ a versus b by column.

Table 3: Carotenoids (lutein and β-carotene) means values (mg/kg) by different cultivars of *Chicorium intybus* L. and by production area.

	Lutein (mg/kg)	*beta*-carotene (mg/kg)
Late Red Chicory of Treviso		
Zero Branco	1.27 ± 0.17	0.27 ± 0.06
Scorzè	1.19 ± 0.24	0.20 ± 0.01
Quinto	1.41 ± 0.07	0.19 ± 0.02
	1.27 ± 0.20[b]	0.22 ± 0.04[b]
Early Red Chicory of Treviso		
Zero Branco	2.12 ± 0.89	0.45 ± 0.02
Scorzè	1.97 ± 0.11	0.35 ± 0.01
Quinto	2.40 ± 0.61	0.34 ± 0.09
	2.16 ± 0.28[a]	0.38 ± 0.08[a]
Variegated Chicory of Castelfranco		
Monselice	1.28 ± 0.13	0.48 ± 0.15
Mira	1.26 ± 0.36	0.35 ± 0.11
Due Carrare	1.07 ± 0.4	0.23 ± 0.01
	1.20 ± 0.27[b]	0.35 ± 0.14[ab]

ANOVA: $P < 0.05$ a versus b by column.

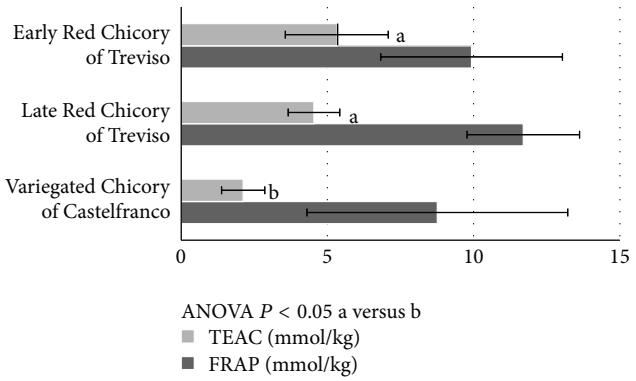


Figure 2: Total antioxidant capacity: FRAP (mmol/kg) and TEAC (mmol trolox/kg) values by different cultivars of *Cichorium intybus* L.

Our results highlighted the highest FRAP values of Late Red and Early Red Chicories of Treviso with respect to Variegated Chicory of Castelfranco (11.70 ± 1.92 mmol/kg and 9.93 ± 3.11 mmol/kg versus 8.76 ± 4.46 mmol/kg, resp.). No differences by varieties and production area were present in FRAP (mmol trolox/kg) results.

Significant differences were present in TEAC (mmol trolox/kg) levels. TEAC values of Late Red Chicory of Treviso (4.54 ± 0.88 mmol trolox/kg) and Early Red Chicory of Treviso (5.32 ± 1.76 mmol trolox/kg) were higher ($P < 0.05$) compared with Variegated Chicory of Castelfranco (2.12 ± 0.74 mmol trolox/kg).

3.2. Cell-Based Assays. Differently from other food components, phytochemicals can exert their bioactivity without reaching the systemic circulation. Scarcely absorbed antioxidants might reach the large bowel contributing to protection from oxidative damage-induced gastrointestinal diseases [29]. There are several reports about pharmacological actions and anti-inflammatory effects of chicory [30, 31].

TEER measurements are routinely used to characterize monolayer integrity in the context of cell monolayer permeability experiments, or to quantify permeability changes, for example, as a consequence of paracellular permeability enhancers [32, 33].

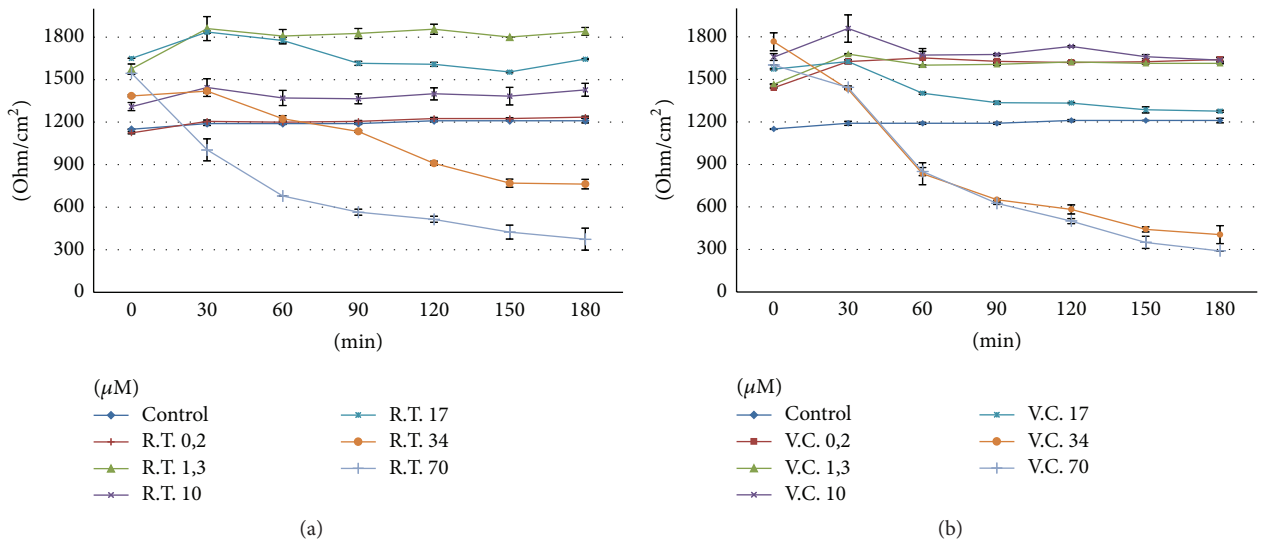
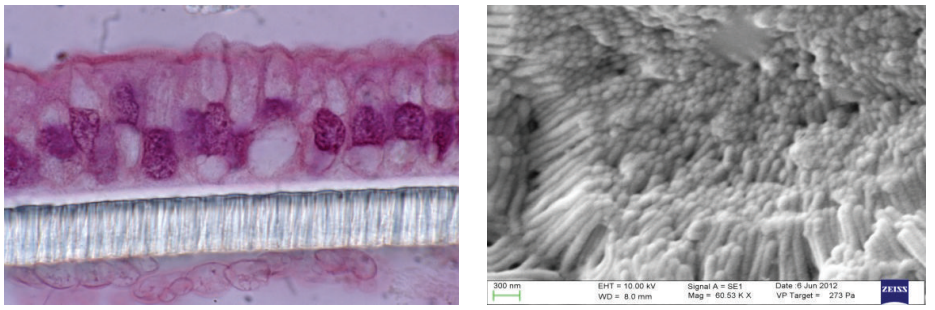


Figure 3: TEER changes at different concentration of polyphenol extract: (a) Red Chicory of Treviso (RT) and (b) Variegated Chicory of Castelfranco (VC) polyphenol extract.



(a) (b)

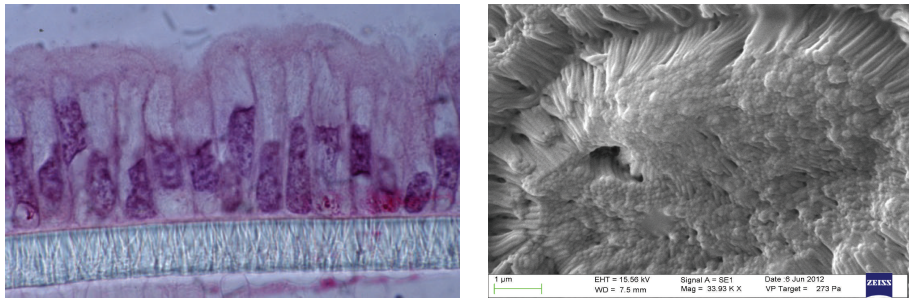
Figure 4: Histological analysis (a) and scanning electron micrographs (b) of normal Caco-2 *pattern* treated with polyphenolics extract of Treviso Red (17 μM).

Utilizing polyphenol fractions from Red Treviso's and Castelfranco's Chicory, the changes in TJs permeability were tested on Caco-2 cell line in monolayers culture. TEER measurements at increasing phenolic concentrations are shown in Figures 3(a) and 3(b), respectively, for Treviso Red Chicory and Variegated Chicory of Castelfranco.

Upon treatment with Red Chicory of Treviso's and Variegated Chicory of Castelfranco's polyphenolics extract, as indicated by TEER values, the results showed a monolayer equilibrium model (healthy monolayer cells) at 0.2-1.3-10-17 μM extract concentration. Until 17 μM polyphenolic extracts concentration, the TEER measurements hold out a plateau, indicating the tightness of TJs and the absence of direct interaction between epithelial Caco-2 cells and chicory extracts as confirmed by histological analyses. Figures 4(a) and 4(b) display the 17 μM effect upon both treatments. The ultrastructural cytological analysis by SEM highlighted and confirmed the absence of morphological change or extracts activity on the cellular monolayer (Figures 5(a) and 5(b)).

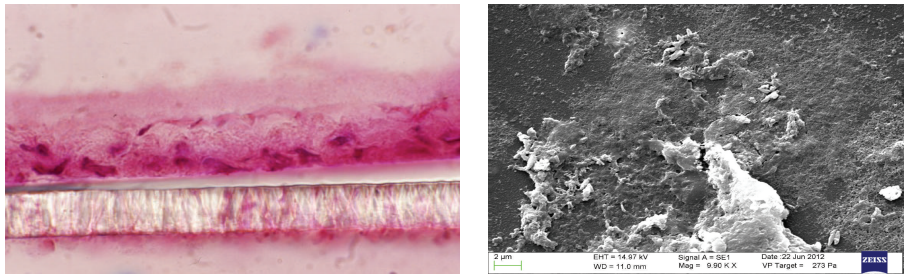
The concentrations of 70 μM and 34 μM showed a high toxicity, respectively, for Treviso's and Castelfranco's Chicory extracts tested. These treatments produce lowering of TEER values (Figures 3(a) and 3(b)), highlighted by increased permeability of TJs (phenol red) and by alteration of epithelium. These concentrations promote cellular necrosis in Caco-2 cells monolayer as shown in histological analysis and confirmed by SEM observations (Figures 6(a), 6(b) and 7(a), 7(b) for Treviso's and Castelfranco's Chicory, resp.).

To better understand their bioactivity and attempting to demonstrate the probable prebiotic role of these extracts, Caco-2 oxidative stress was induced by adding 2,2′azobis (2-amidinopropane) dihydrochloride (AAPH) to the cell culture medium. The interactions between polyphenol-rich Red Chicory of Treviso extracts against AAPH-induced oxidative stress are reported in Figure 8(a). The progressive TEER increases indicated that, upon 0.2, 1.3, and 10 μM, chicory extracts treatment displayed a strong antioxidant activity that appeared to be able to counteract the peroxidative trigger



(a) (b)

Figure 5: Histological analysis (a) and scanning electron micrographs (b) of normal Caco-2 *pattern* treated with polyphenolics extract of Variegated Chicory of Castelfranco (17 μM).



(a) (b)

Figure 6: Histological analysis (a) and scanning electron micrographs (b) showing Caco-2 necrotic *pattern* upon treatment with polyphenolics extract: Treviso Red extract (70 μM).

(a) (b)

Figure 7: Histological analysis (a) and scanning electron micrographs (b) showing Caco-2 necrotic *pattern* upon treatment with polyphenolics extract: Variegated Chicory of Castelfranco extract (34 μM).

induced by AAPH, suggesting a cellular membrane integrity and confluency restoring. Only 0.2 μM Variegated Chicory of Castelfranco extract exhibited a low antioxidant effect (Figure 8(b)) that promotes a cell damage recovery resulting by slight TEER increase.

4. Discussion

The antioxidant properties of several varieties of *Cichorium* genus vegetables have been attributed, in part, to the presence of phytochemicals including hydroxycinnamic acid derivatives, mono- and diglycosides of flavonoids, and anthocyanins. As reported by Rossetto et al. [6], the presence of these phenolics confers to red chicories an exceptionally high peroxyl radical scavenging activity in terms of both capacity and efficiency, particularly in their early stage of growth. The lower carotenoid values observed in the present study could be due to the limited exposure to sunlight and the lower temperature, during winter, because carotenoids biosynthesis is not stimulated as in vegetables in open fields. As reported by Niizu and Rodriguez-Amaya [34], the green chicory showed a higher average content of 53.7 ± 8.3 (mg/kg) and 35.3 ± 5.0 (mg/kg) for lutein and β-carotene, respectively. As discussed elsewhere [35], green chicory from Lazio exhibited higher carotenoids content. In particular, lutein and β-carotene values were 14.10 ± 3.30 mg/kg and

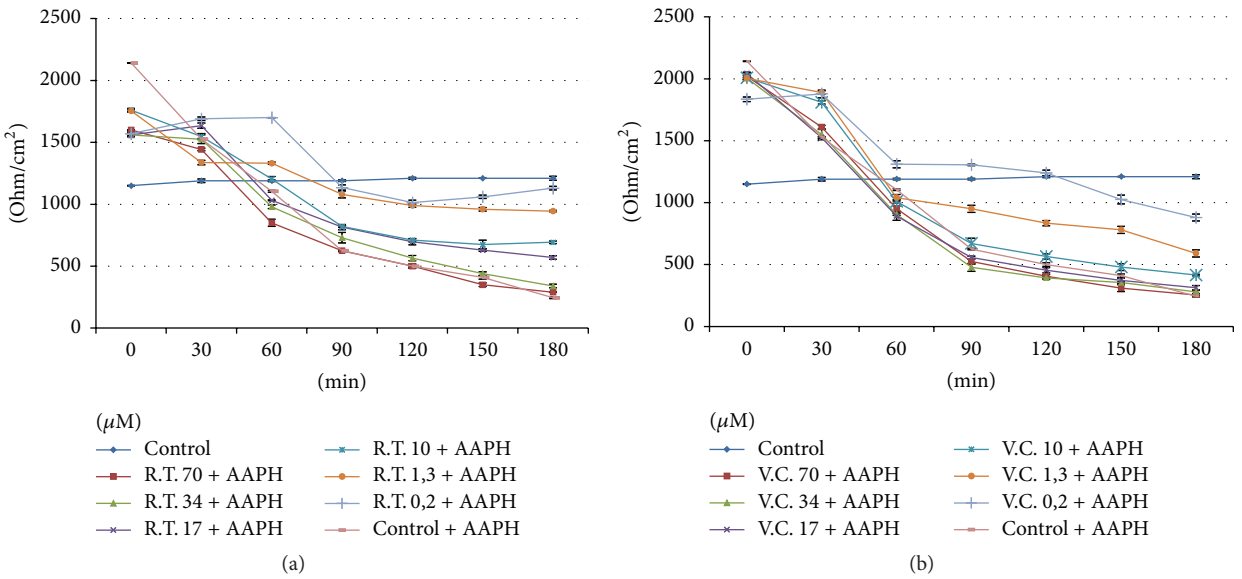


FIGURE 8: TEER changes at different concentration of polyphenol-rich extract after AAPH-induced oxidative stress. (a) Red Chicory of Treviso (RT) extract and (b) Variegated Chicory of Castelfranco (VC) polyphenol extract.

42.12 ± 5.99 mg/kg, respectively, in wild type, while the average content was 15.51 ± 3.28 mg/kg for lutein and 40.62 ± 2.15 mg/kg for β-carotene in cultivated chicory.

The total antioxidant capacity is a parameter strongly influenced by type of food (i.e., species and varieties for the same species), but also by growing conditions, environmental factors, and preservation techniques. Variegated Chicory of Castelfranco exhibited the lowest TAC values for the FRAP and TEAC methods, respectively. Red Chicory of Treviso (Early and Late) results, analyzed by the FRAP and TEAC assays, were according to Pellegrini et al. [36]. As reported elsewhere [35], changes in antioxidant composition of several vegetables were linked to agricultural practices and environmental factors. Regarding cell-based assays, TEER measurement represents a physical measure to evaluate the physiological state of cell cultures detecting the early intestinal barrier function *in vivo* damage. As known, defect in epithelial permeability caused by alteration of TJs is seen in several inflammatory bowel diseases. Different elements, including robust innate immune responses, epithelial paracellular permeability, and epithelial cell integrity, as well as the production of mucus, contribute to the integrity of the intestinal barrier [37]. Our experimental system was the confluent monolayer of Caco-2 cells at equilibrium. In this condition, the epithelial culture is more susceptible to variations of the chicory extracts concentration. So, small relative changes in concentrations of extracts correspond to chemical and physical altered cellular patterns and dramatic cell morphological changes. The tightness of TJs, as confirmed by histological analyses (Figures 4(a) and 5(a)) and the absence of morphological change or extracts activity on the cellular monolayer by SEM (Figures 4(b) and 5(b)), does not specify if these extracts maintain the protective effect of the antioxidant mixture but indicates the absence of direct interaction between epithelial Caco-2 cells and chicory extracts. These extracts could be compartmentalized in the mucopolysaccharides of the glycocalyx like prebiotic mixture. As known, the glycocalyx is a meshwork of glycoprotein molecules that binds to mucus largely composed of mucopolysaccharides produced by goblet cells. The glycocalyx and mucus form a flexible coat which provides cells protection from mechanical and chemical damage. Nutrients can diffuse into the mucosa, be acted upon by the enzymes in the glycocalyx, and create an area at high concentration of more easily adsorbed molecules by concentration gradient [38]. At the moment, our research is focused on clustering of the food extracts by glycocalyx interaction.

The greatest protective effect on cell cytotoxicity, deriving from Treviso Red radish extract (70 μM) with respect to Variegated Chicory of Castelfranco extract (34 μM), could be due to specific composition in polyphenol-rich fractions. The main flavonoids in above-mentioned extracts are reported in Table 1 and the TJs respond to various naturally plant-derived and food extracts. TEER measurements present dose-response curve patterns, indicating the absence of alteration in Caco-2 cell monolayer, as underlined by our results from 0.2 to 17 μM chicory extracts treatments (Figure 3). Moreover, *Cichorium intybus* L. seems to counteract and improve the oxidative stress and cellular damage induced by AAPH *in vitro* Caco-2 cell model (Figure 8). As previously reported [39], when the prooxidant (AAPH) is added, the TEER measurements dramatically decrease until cellular necrosis; in addition, as reported by Finotti et al. [40], the presence of oxidant (AAPH) induces an increase in the mucopolysaccharides secretion located at microvilli glycocalyx. Overall, a better healthy action by Red Chicory of Treviso polyphenol-rich extract on Caco-2 (at 0.2-1.3-10 μM concentrations) could be assumed which does not interact with monolayer cells while as exogenous substance it helps to maintain optimal cellular functions by its strong antioxidant activity.

On the other hand, Red Chicory is characterized by a high content of anthocyanin pigments [41] that could exert several beneficial health or nutraceutical effects [12, 42, 43].

5. Conclusion

It could be concluded that the TJs response depends on the dose exposure and particular chemical composition of food extracts by synergic and interdependent antioxidant effects (enzymatic and nonenzymatic) with the epithelial glycocalyx. Even if the redox balance does not originate from a single cause, our study suggests that the interaction between antioxidant extracts and the mucopolysaccharide complexes in the glycocalyx could protect the *in vivo* lining of gut from damage maintaining a healthy and effective intestinal barrier.

Abbreviations

Caco-2: Human colon carcinoma cell line
FRAP: Ferric Reducing Antioxidant Power
TEAC: Trolox Equivalent Antioxidant Capacity
TEER: Transepithelial Electrical Resistance
TJ: Tight-junction
SEM: Scanning Electron Microscopy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The present study was performed within the Projects BIOVITA and TERRAVITA funded by the Italian Ministry of Agriculture, Food, and Forestry Policies.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 5630985, 10 pages
http://dx.doi.org/10.1155/2016/5630985

Clinical Study

Relationship between the Peroxidation of Leukocytes Index Ratio and the Improvement of Postprandial Metabolic Stress by a Functional Food

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Received 18 August 2015; Revised 5 October 2015; Accepted 7 October 2015

Academic Editor: Steven McAnulty

For the first time, we investigated the relationship between postprandial dysmetabolism and the Peroxidation of Leukocytes Index Ratio (PLIR), a test that measures the resistance of leukocytes to exogenous oxidative stress and their functional capacity of oxidative burst upon activation. Following a blind, placebo controlled, randomized, crossover design, ten healthy subjects ingested, in two different occasions, a high fat and high carbohydrates meal with Snello cookie (HFHCM-S) or with control cookies (HFHCM-C). Snello cookie, a functional food covered by dark chocolate and containing glucomannan, inulin, fructooligosaccharides, and *Bacillus coagulans* strain GanedenBC30, significantly improved postprandial metabolic stress (insulin, glucose, and triglycerides) and reduced the postprandial increase of uric acid. HFHCM-S improved PLIR of lymphocytes, but not of monocytes and granulocytes. Both meals increased granulocytes' count and reduced the lipoperoxidation induced by both exogenous free radicals and reactive oxygen species (ROS) produced by oxidative burst. Our results suggest that the healthy status of the subjects could be a limitation of this pilot study for PLIR evaluation on cells that produce ROS by oxidative burst. In conclusion, the relationship between PLIR and postprandial dysmetabolism requires further investigations.

1. Introduction

Postprandial dysmetabolism has been linked to atherosclerosis and inflammation [1]. Therefore, fatty meal consumption represents a model of acute inflammatory response and has been applied to study the effect of antioxidant-rich foods, beverages, or nutritional supplements, but results are scarce and controversial [2, 3].

Despite the fact that the consumption of high fat and high carbohydrates meals (HFHCM) has been associated with oxidative stress and with a decline in antioxidant defences in plasma, increases in plasma nonenzymatic antioxidant capacity have been reported following HFHCM [4, 5]. Furthermore, in healthy subjects, both increased [6] and reduced [7] reactive oxygen species (ROS) generation were observed in peripheral blood mononuclear cells (PBMC) during the postprandial period.

In this context, although oxidative stress is involved in metabolic syndrome, decreases in oxidative burst of neutrophils occurred in some conditions, such as hypercholesterolaemia [8] and non-insulin-dependent diabetes mellitus (NIDDM) [9].

Based on the potential protective effects against the onset of metabolic syndrome [10], functional foods containing probiotics, prebiotics, and/or polyphenols were placed on the market. Improvement of metabolic profile, oxidative stress, and inflammation has been reported also for glucomannan [11, 12].

de Luis et al. [13] pointed out that one of the problems of dietetic therapy is the lack of patient adherence and

Table 1: Macronutrient composition of the two meals.

	Kcal	Lipids (saturated)	Proteins	Carbohydrates (sugars)
HFHCM-C (total)	**831.4**	**40.9 (29.6)**	**6.9**	**105.7 (63.3)**
Panna cotta with caramel (240 g)	647.4	34.3 (27.4)	4.3	77.5 (54.5)
Control cookies (40 g)	184	6.6 (2.2)	2.6	28.2 (8.8)
HFHCM-S (total)	**846.4**	**40.6 (31.2)**	**6.8**	**108.8 (65.0)**
Panna cotta with caramel (240 g)	647.4	34.3 (27.4)	4.3	77.5 (54.5)
Snello cookie (48 g)	199	6.3 (3.8)	2.5	31.3 (10.5)

C: control cookies; HFHCM: high fat and high carbohydrates meals; S: Snello cookie.

suggested that one possibility to overcome this problem is to include functional cookies in the diet. In particular, significant decreases of cholesterol and C reactive protein were observed in obese patients after the consumption of an alpha linolenic acid, fructooligosaccharides (FOS), and inulin-enriched cookie [13]. Improvements of glycemic control and lipid profile have been reported after consumption of glucomannan-enriched biscuits in subjects with impaired glucose tolerance, reduced high density lipoprotein (HDL) cholesterol, elevated serum triglycerides, and moderate hypertension [14].

In this pilot study, we aimed to investigate the relationship between the improvement of postprandial dysmetabolism by a functional cookie, covered by dark chocolate and containing glucomannan, inulin, FOS, and *Bacillus coagulans* strain GanedenBC30 [15], and the Peroxidation of Leukocytes Index Ratio (PLIR), a test that measures the resistance of leukocytes to exogenous oxidative stress and their functional capacity of oxidative burst upon activation [16].

2. Methods

2.1. Subjects' Selection. We recruited 10 healthy subjects who volunteered in response to advertisements.

Selection of subjects was made according to the following criteria: being healthy, being aged between 25 and 50 years, and taking no drugs, supplements, probiotics, or functional foods. Exclusion criteria include smoking habits and adherence to special diets (vegetarian, vegan).

Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP), and heart rate (HR) were measured. MEDScore [17, 18] was calculated by the MedDietScore Software [19], and physical activity was calculated according to the "Guidelines for Data Processing and Analysis of the International Physical Activity Questionnaire" (IPAQ) [20, 21].

2.2. Study Design and Meals' Composition. For 2 days prior to each feeding study, the subjects followed a low antioxidant and low purine diet (washout), by avoiding fresh fruit and vegetables with high antioxidants' content and their products (juices or soups), tea, cocoa, nuts, coffee and wine, meat, and fish. Subjects were asked to refrain from exercise 2 days before the study. Compliance with dietary instructions was evaluated through dietary records and all subjects ingested pasta (160 ± 40 g/day), white bread (160 ± 50 g/day), croissant or cookies (50 ± 10 g/day), eggs (1/2 days), cheese (70 ± 20 g/day), fresh cheese (150 ± 50 g/day), milk (200 ± 50 mL/d), only a fruit/day (apple or pear), and less than 60 g/d of vegetables (zucchini, endive, or fennel).

Following a blind, placebo controlled, randomized, crossover design, subjects were allocated into Group A (n = 5) (HFHCM + control cookies) (HFHCM-C) or Group B (n = 5) (HFHCM + Snello cookie) (HFHCM-S). Panna cotta with caramel and control cookies were purchased from the supermarket. Snello cookie, a functional food commercially available in Italy, covered by dark chocolate and containing glucomannan (2.4 g/48 g), inulin (2.3 g/48 g), FOS (0.2 g/48 g), and *Bacillus coagulans* strain GanedenBC30 (3×10^6 UFC/g), was provided by Nutripharma S.r.l.

After 12 days, subjects followed again 2 days of washout and 14 days after the first test the groups were crossed over to the alternative cookies. The macronutrient composition of the two meals, given as breakfast with 500 mL of water, is depicted in Table 1.

In patients with coronary artery disease (CAD) [22] or type 2 diabetes (T2D) [23], 2-hour breakfast tests [22, 23] revealed the improving effects of lipid lowering (fibrate) [22] or oral hypoglycaemic (mitiglinide) [23] drugs on the phorbol ester-activated leukocyte ROS production [22], plasma malondialdehyde (MDA), oxidized low density lipoproteins (oxLDL), plasma total radical-trapping antioxidant parameter (TRAP), and inflammatory cytokines [23]. Notwithstanding the above, the Ethics Committee approved a 3-hour test meal considering that ROS generation by polymorphonuclear (PMN) cells reached a peak 3 hours after meal in healthy subjects [6].

On the day of the study, after an overnight fast, venous blood samples were collected before ($T0$) and 30 minutes ($T0.5$), 2 hours ($T2$), and 3 hours ($T3$) after meal.

2.3. Clinical Markers. Blood was collected in Silicone-Coated tubes. The serum was stored at −80°C.

Serum levels of triglycerides (TG), glucose (GLU), and uric acid (UA) were quantified enzymatically using colorimetric kits (Sentinel CH. SpA, Italy). Plasma insulin was measured with an enzyme-linked immunosorbent assay (ELISA) kit (Li StarFish S.r.l., Italy).

2.4. PLIR Method. Blood was collected in EDTA tubes. After red blood cells' lysis and 4,4-difluoro-5-(4-phenyl-1, 3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY, Invitrogen, final concentration 1 μM)

staining, leukocytes were treated as previously described [24] with phorbol 12-myristate 13-acetate (PMA, Sigma, final concentration 1 μg/mL), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, Sigma, final concentration 10 mM), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma, final concentration 10 μM), PMA 1 μg/mL + Trolox 10 μM, or AAPH 10 mM + Trolox 10 μM. After 30 min at 37°C, cells were stored in ice, to stop reactions, and rapidly analyzed on an Accuri C6 BD cytometer.

C11-BODIPY, used in the PLIR method, modifies its fluorescence from red (FL2) to green (FL1) as a result of oxidation [25]. Treatment with AAPH or PMA changed the C11-BODIPY fluorescence in a different manner compared to unstimulated cells (Figure 1(a)), showing that oxidative burst induced ROS production only in activated cells (Figure 1(b)), while all cells were sensitive to exogenous (AAPH) ROS injury (Figure 1(c)). Trolox did not affect neither baseline levels of fluorescence (Figure 1(d)) nor the PMA-induced change in fluorescence of monocytes and granulocytes (Figure 1(e)) but decreased the AAPH-induced change in fluorescence of lymphocytes, monocytes, and granulocytes (Figure 1(f)).

Both FL1 and FL2 are higher in monocytes and granulocytes compared to lymphocytes in unstimulated samples (Figure 1(a)). Therefore, in order to normalize for cell incorporation of the probe into membrane, data acquired on the cytometer were exported in FCS format and analyzed by FCS Express software (De Novo Software) to calculate the ratio of oxidation of the probe C11-BODIPY (FL1/FL2). This ratio, being independent of the concentration of the probe, has been used to calculate PLIR, applying the previously described [16, 24] formula:

$$\text{PLIR} = \frac{(\text{RATIO AAPH} \times \text{RATIO PMA Trolox})}{(\text{RATIO AAPH Trolox} \times \text{RATIO PMA})}. \quad (1)$$

PLIR is a functional index that measures the ratio between the resistance to exogenous (Trolox μM equivalents AAPH) and resistance to endogenous (Trolox μM equivalents PMA) ROS injury [16]. Although PLIR is independent of the baseline levels of oxidation, this functional index is sensitive to the difference between leukocytes isolated from fresh and stored blood [24].

Also Side Scatter (SS) was recorded and leukocytes' count was measured as previously described [26].

2.5. Statistics. Two-Way Repeated Measures Analysis of Variance (Two-Factor Repetition ANOVA), with cookies and time as within-subject factors, was performed. Student-Newman-Keuls post hoc analysis (All Pairwise Multiple Comparison Procedure) was used to isolate differences between groups.

All statistical evaluations were performed using the SigmaStat and Sigmaplot software (Jandel Scientific, Inc.).

3. Results

3.1. Characteristics of Subjects. Based on the exclusion criteria, ten subjects (6 men and 4 women), with a mean age of 36.0 ± 2.9 years and a mean body mass index of 23.3 ± 1.4, were recruited. Volunteers had a mean homeostasis model assessment of insulin resistance (HOMA-IR) of 1.6 ± 0.3 and were normotensive (SBP: 122.9 ± 3.1 mmHg, DBP: 75.9 ± 2.1 mmHg, and HR: 73 ± 2.5 beats/min).

Subjects had a MEDScore of 35.0/55 ± 1.9 (63.6% adherence's level to Mediterranean diet) [19] and a moderate physical activity (1089 ± 180 MET-minutes/week) [21].

3.2. Clinical Markers. Statistical analysis revealed a normal distribution for all markers (Normality Test Shapiro-Wilk passed: GLU: $p > 0.8$; INS: $p > 0.5$; TG: $p > 0.8$; UA: $p > 0.9$).

The glucose and insulin time courses reflected the postprandial load for healthy people. Both glucose and insulin levels peaked 30 min after meal ingestion (Figures 2(a) and 2(b)).

Although glucose and insulin levels began to decrease within one hour with both HFHCM-C and HFHCM-S, they returned to baseline values only with the latter (Figures 2(a) and 2(b)). In particular, glucose and insulin values were significantly higher after HFHCM-C compared to HFHCM-S at 3 hours (HFHCM-S versus HFHCM-C: $p < 0.01$; Figure 1(a)) and 2 hours (HFHCM-S versus HFHCM-C: $p < 0.05$; Figure 2(b)), respectively. Furthermore, insulin remained significantly above preingestion values for 3 hours only after HFHCM-C ($p < 0.05$ versus baseline; Figure 2(b)).

With respect to lipid metabolism, both HFHCM-C and HFHCM-S induced lipaemia at 2 and 3 hours; however, TG increase was significantly lower after HFHCM-S compared to HFHCM-C (HFHCM-S versus HFHCM-C: $p < 0.01$ within 2 hours, $p < 0.001$ within 3 hours; Figure 2(c)). Besides, HFHCM ingestion caused a significant increase in the endogenous antioxidant UA at 2 hours, but the latter was lower with HFHCM-S compared to HFHCM-C (within 2 hours: $p < 0.01$, within 3 hours: $p < 0.05$; Figure 2(d)). Furthermore, only HFHCM-C increased UA at 3 hours ($p < 0.001$ versus baseline; Figure 2(d)).

3.3. PLIR, Count, and Scatter of Leukocytes. Statistical analysis revealed a normal distribution for L, M, and G populations (Normality Test Shapiro-Wilk passed: L: $p > 0.3$; M: $p > 0.2$; G: $p > 0.3$).

HFHCM-S, but not HFHCM-C, significantly decreased PLIR of lymphocytes at 3 hours ($p < 0.01$ versus baseline; HFHCM-S versus HFHCM-C: $p < 0.01$ within 3 hours; Figure 3(a)), whereas a nonsignificant decrease was observed for PLIR of monocytes and granulocytes after HFHCM-S (Figure 3(a)). In particular, considering the major components of PLIR affected by treatment, compared to baseline, the AAPH-induced (exogenous) oxidation of lymphocytes appeared significantly lower after HFHCM-S (difference of means RATIO AAPH versus RATIO UNST: at baseline 0.68, $p < 0.001$, at 3 hours 0.29, $p < 0.01$).

We analyzed also leukocytes' count and scatter after meal. The mean count and scatter from lymphocytes and monocytes remained unchanged after both meals. On the other hand, there was a postprandial increase in granulocytes' count with both meals (HFHCM-C versus baseline: $p < 0.01$; HFHCM-S versus baseline: $p < 0.05$; Figure 3(b)). However,

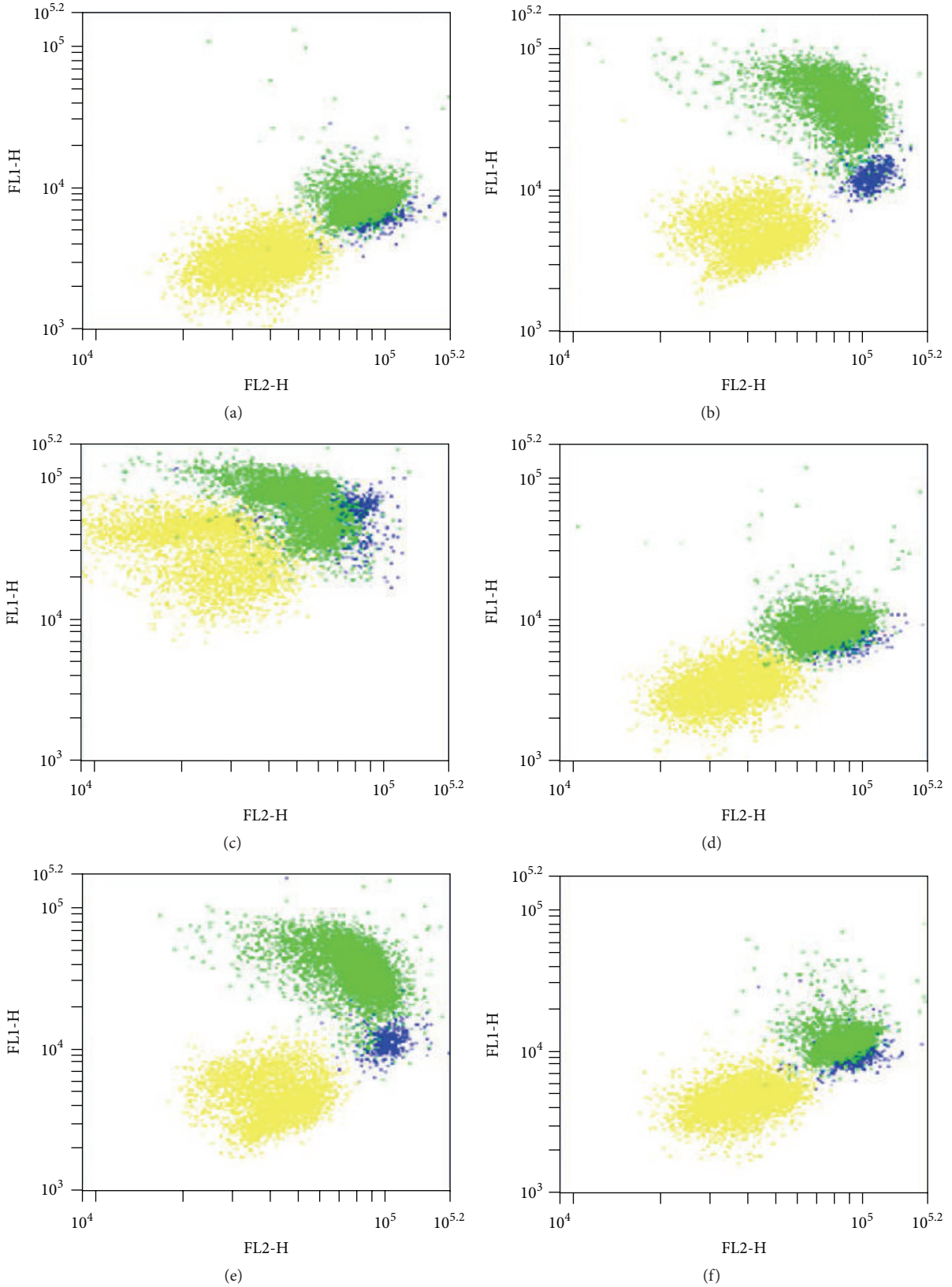

Figure 1: Typical dot plots of C11-BODIPY red (FL2) and green (FL1, oxidized) fluorescence of lymphocytes (yellow), monocytes (blue), and granulocytes (green) in unstimulated (UNST) samples (a) and after treatment with phorbol 12-myristate 13-acetate (PMA, (b)), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, (c)), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, (d)), PMA + Trolox (e), or AAPH + Trolox (f).

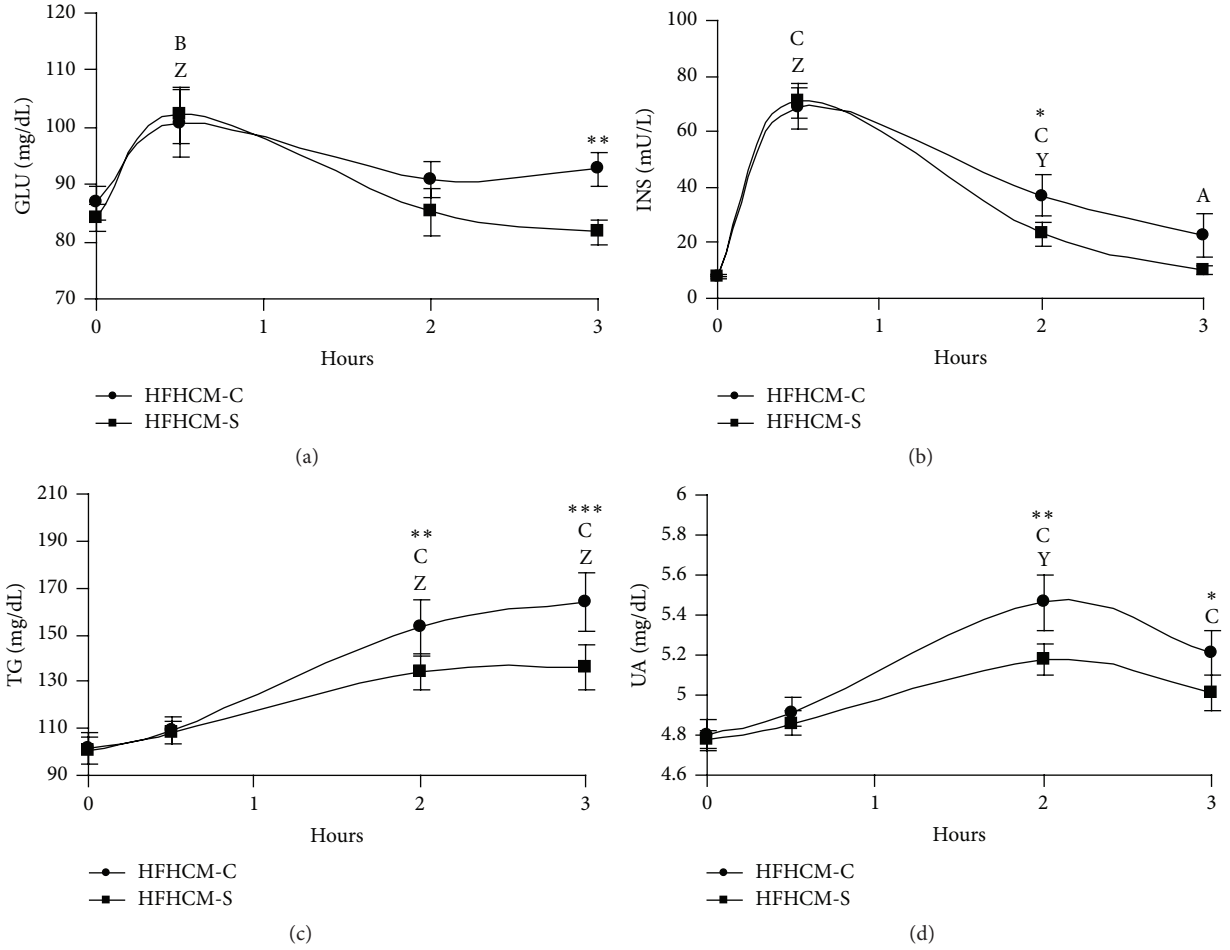

Figure 2: Line plots showing the serum levels as means ± standard errors ($n = 10$) in plasma glucose (GLU, (a)), insulin (INS, (b)), triglycerides (TG, (c)), and uric acid (UA, (d)), following high fat and carbohydrates meal ingestion with control (HFHCM-C) or Snello (HFHCM-S) cookies. Two-Way Repeated Measures ANOVA followed by Student-Newman-Keuls post hoc analysis. A: $p < 0.05$; B: $p < 0.01$; C: $p < 0.001$, single time point versus before meal intake within HFHCM-C; Y: $p < 0.01$; Z: $p < 0.001$, single time point versus before meal intake within HFHCM-S; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, HFHCM-S versus HFHCM-C within time.

this increase, after both meals, was accompanied neither by a reduction of SS in unstimulated samples nor by a different decrease in SS after PMA-activation (Figure 3(c)).

On the contrary, the increase in the RATIO of fluorescence of granulocytes after both PMA and AAPH treatment versus unstimulated samples appeared lower 3 hours after both meals (PMA or AAPH versus UNST: HFHCM-C at baseline: $p < 0.001$; HFHCM-C at 3 hours: $p < 0.01$; HFHCM-S at baseline: $p < 0.001$; HFHCM-S at 3 hours: $p < 0.01$; Figure 3(d)).

Similarly, nonsignificant effects were observed on monocytes with both meals.

4. Discussion

4.1. Postprandial Dysmetabolism. The functional food Snello cookie significantly improved postprandial metabolic stress. In particular, Snello cookie reduced the postprandial TG rise. Furthermore, glucose and insulin levels returned to baseline values at 3 hours after HFHCM-S, but not after HFHCM-C.

The effect on postprandial insulin could be due to the content of glucomannan in Snello cookie. In fact, McCarty [27] suggested that glucomannan reduces the postprandial insulin surge. Besides, results of a meta-analysis of randomized controlled trials pointed out that glucomannan significantly lowered TG and GLU [11].

On the contrary, a systematic review reported that studies evaluating the effects of inulin and FOS on glucose concentration in humans gave contrasting results [28]. On the other hand, inulin-enriched pasta [29] and breakfast cereal containing inulin [30] decreased TG. Inulin markedly increased bifidobacteria count and faecal concentration of lactate [30]. Lactic acid is involved in the immunomodulating effect of lactobacilli [31]. Although the prebiotic effects of inulin-type prebiotics, including FOS and inulin, occur after long term consumption [32], it has been reported that acute inulin ingestion increased postprandial serum short-chain fatty acids and reduced free fatty acids [33, 34].

On the other hand, also the chocolate contained in Snello cookie could improve postprandial dysmetabolism. Although

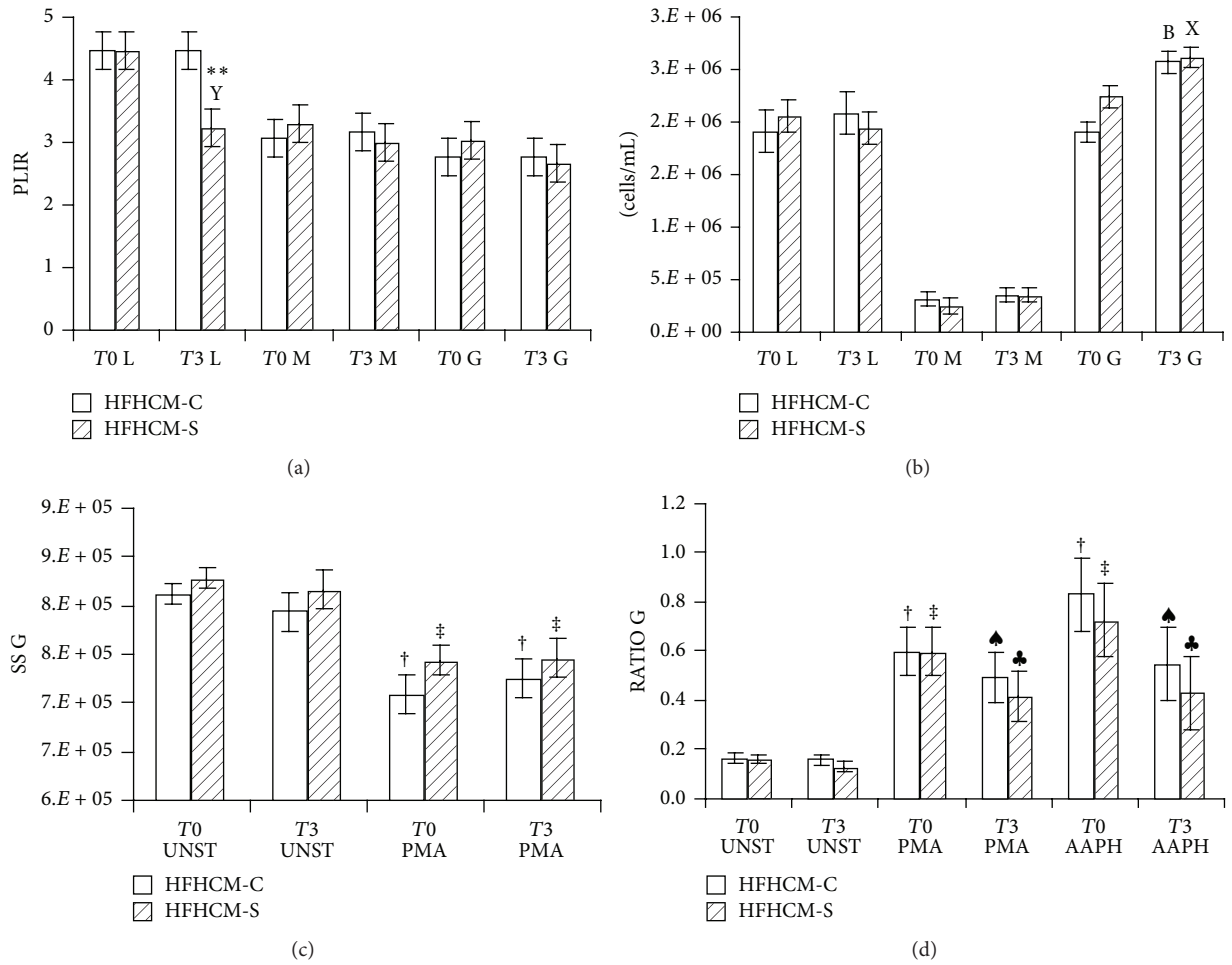

Figure 3: Vertical bars showing the values as means ± standard errors ($n = 10$) of PLIR (a), leukocytes count (b), Side Scatter of granulocytes (c), and RATIO of fluorescence (FL1/FL2) of granulocytes (d), following high fat and carbohydrates meal ingestion with control (HFHCM-C) or Snello (HFHCM-S) cookies. L: lymphocytes; M: monocytes; G: granulocytes. Two-way Repeated Measures ANOVA followed by Student-Newman-Keuls post hoc analysis. B: $p < 0.01$, 3 hours ($T3$) versus before ($T0$) meal intake within HFHCM-C; X: $p < 0.05$; Y: $p < 0.01$, 3 hours ($T3$) versus before ($T0$) meal intake within HFHCM-S; $^{**}p < 0.01$, HFHCM-S versus HFHCM-C within time; $^{\spadesuit}p < 0.01$; $^{\dagger}p < 0.001$, treatment (PMA: phorbol 12-myristate 13-acetate; AAPH: 2,2′-azobis(2-methylpropionamidine) dihydrochloride) versus unstimulated (UNST) samples within HFHCM-C; $^{\clubsuit}p < 0.01$; $^{\ddagger}p < 0.001$, treatment versus unstimulated (UNST) samples within HFHCM-S.

acute cocoa supplementation showed no clear overall benefit on postprandial GLU, INS, and TG [35, 36], it has been reported that an oral supplement of (−)-epicatechin (the major flavanol contained in chocolate) significantly lowered GLU and TG 2 hours after meal [37].

Therefore, the overall improvement of postprandial dysmetabolism induced by Snello cookie could be due to the synergistic effect of its constituents.

4.2. Postprandial Leukocytes' Recruitment and Activation. We observed the previously described [38–41] postprandial increase of granulocytes' count at 3 hours. In this context, the leukocytes' excursion, after meal, was significantly reduced with acarbose in patients with T2D and subclinical inflammation (leucocytes > or = 6.2 gigaparticles/L) [42]. Rosiglitazone reduced the incremental area under the curves for leukocytes, due to a specific reduction of neutrophils (−39%, $p < 0.05$), in patients with T2D [43]. On the contrary, rosuvastatin did not affect baseline leukocytes' count or the postprandial neutrophils' increase in CAD patients [44]. Furthermore, results of statin withdrawal demonstrated that the expression of leukocytes' markers of activation is not affected by the use of statins [45]. However, the studies that investigated the effect of glucose on leukocytes' markers of activation have shown conflicting results in T2D [45–48]. On the other hand, postprandial studies reported that leukocytes are activated by lipids [38, 49, 50]. The expression of leukocytes' markers of activation (i.e., CD11b, CD11c) increased on monocytes or neutrophils after a high fat meal [50, 51]. The extent of upregulation of the expression of leukocytes' markers of activation correlated with TG and was accompanied by an altered scatter profile [50, 51]. In particular, CD11b expression on neutrophils was negatively correlated with the mean SS of neutrophils, reflecting granularity [50].

In our study, the increase in granulocytes' count was accompanied neither by a reduction of SS in unstimulated

samples nor by a different decrease in SS after PMA-activation. In this context, the fact that, even if white blood cells' count increased and intracellular myeloperoxidase decreased within 2–4 hours after meal, waist-to-hip ratio influenced the degranulation of PMN must be taken into account [52]. Besides, recent results showed that only obese subjects had higher postprandial endotoxemia, the mechanism of postprandial leukocytes' activation, despite the lipaemia increased in both normal-weight and obese men after meal [53]. Furthermore, postprandial leukocytes' activation was highest in patients with T2D and hyperlipidaemia [45]. Therefore, our pilot study has the major limitation that subjects were healthy, of normal weight, with a moderate physical activity and none of them presented risk factors for CVD.

4.3. PLIR and UA in the Postprandial Phase. Snello cookie improved PLIR of lymphocytes, but not of monocytes and granulocytes. To understand this result further considerations should be made.

Although PLIR is a functional index that is independent of baseline levels of oxidation, measuring the ratio between the resistance to exogenous and resistance to endogenous ROS injury [16], this ratio calculation could mask the effect of foods that inhibit both the exogenous ROS injury and the oxidative burst. In particular, the calculation of PLIR includes the PMA-induced oxidation. Lower increases in the RATIO of fluorescence of granulocytes after both PMA and AAPH treatment versus unstimulated samples were recorded 3 hours after both HFHCM-C and HFHCM-S compared to baseline. However, the unchanged mean SS (reflecting granularity) after meal suggests that the decrease in the RATIO of fluorescence is more likely due to the antioxidant effect of UA and cocoa flavanols after HFHCM-C and HFHCM-S, respectively, rather than an effect on oxidative burst. In agreement with this hypothesis, Sodré et al. [7] reported that the intracellular ROS in PBMC, assessed by flow cytometry as the ethidium (ETH) fluorescence, decreased 2 and 4 hours after meal not only in monocytes but also in lymphocytes, which do not produce ROS by oxidative burst. On the contrary, others [6] reported that the release of superoxide radical by PMN, as measured by chemiluminescence, was significantly lower when orange juice was added to the meal than when water or glucose was added to the meal. However, extracellular free radicals' measurements, such as the chemiluminescence assay, are deeply affected by cell count and viability. Therefore, the postprandial increase of granulocytes' count [38–41] could bias these methods.

On the other hand, we observed an increase in UA after HFHCM-C. This increase could be due to the healthy status of the subjects. In agreement with this, increases in TRAP and UA have been reported following HFHCM in healthy subjects [4], despite the lower TRAP values after meal in T2D patients [23].

The increase in UA was lower after HFHCM-S. The inhibition of UA increase could be due to the cocoa flavanols contained in Snello cookie. In agreement with this hypothesis, tea flavanols could have UA lowering effect [54, 55] and fruit-based juice drinks, providing exogenous antioxidants, prevented the endogenous antioxidant response to HFHCM, by inhibiting the production of UA [56].

In this context, UA levels could affect PLIR in two different ways: acting as antioxidant [57] on all leukocytes and inducing oxidative burst in ROS-producing cells [58]. The effect of UA depends on its concentration. AAPH-induced lipid peroxidation, in vitro, was strongly inhibited by UA at concentration ranging between 50 and 400 μM (0.84–6.72 mg/dL) [57]. The increase in UA after HFHCM-C ingestion could justify why HFHCM-C did not change PLIR. In this context, it has been suggested that in healthy people the body responds to postprandial stress by inducing endogenous defenses [4]. In the presence of dietary antioxidants (i.e., the chocolate contained in Snello cookie), the resistance to AAPH-induced oxidation is increased in lymphocytes despite the reduced UA increase.

On the other hand, although the level at which UA concentration becomes abnormal is still disputed, ranging between 3.5 and 7.2 mg/dL in adult males and postmenopausal women and between 2.6 and 6.0 mg/dL in premenopausal women [59], a threshold value below the saturation concentrations (<6 mg/dL or <360 μmol/L), in order to prevent monosodium urate (MSU) crystals formation, has been suggested [59, 60]. In fact, in response to MSU, the neutrophils recruited to sites of inflammation undergo oxidative burst [58]. In our study, after HFHCM-C, UA reached the concentration of 5.46 ± 0.13 mg/dL, a value below the threshold value of 6 mg/dL [59, 60]. From that, the healthy status of the subjects could be a limitation of this study for PLIR evaluation on cells that produce ROS (i.e., monocytes and granulocytes).

5. Conclusion

In conclusion, the functional food Snello cookie significantly improved postprandial metabolic stress and reduced the postprandial increase of UA.

After HFHCM-S, PLIR was improved on lymphocytes, but not on monocytes and granulocytes.

The healthy status of the subjects could be a limitation of this pilot study for PLIR evaluation on cells that produce ROS (i.e., monocytes and granulocytes). From that, further studies on subjects who are at risk of cardiovascular diseases are needed in order to investigate the relationship between postprandial dysmetabolism and PLIR.

Ethical Approval

Approval for the study was obtained from the Ethics Committee for Human Nonclinical Research of the Department of Physiology and Pharmacology "V. Erspamer", "Sapienza" University of Rome, and all procedures involving human subjects complied with the Declaration of Helsinki as revised in 2000.

Consent

Written informed consent was obtained from all the participants in accordance with the Italian law (law number

196/2003, Ministry of Health Circular Letter GU number 76/2008).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Ilaria Peluso designed the research, analyzed the data, and drafted the paper. Husseen Manafikhi and Raffaella Reggi performed the analyses. Yaroslava Longhitano assessed the healthy status of the subjects, the MEDScore, and the physical activity. Christian Zanza performed the blood sampling. Maura Palmery critically reviewed the paper and supervised the whole project.

Acknowledgments

This study was supported by Nutripharma S.r.l., Rome, Italy (http://www.nutripharma.it/dettprodotti.php?idx=8&pd=2#.VboVB8sw_IU). The authors thank Fondazione "Enrico ed Enrica Sovena" for the scholarship contribution to Husseen Manafikhi. The authors also thank Claudio Andrew Gobbi for English review of the paper.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 2827567, 11 pages
http://dx.doi.org/10.1155/2016/2827567

Research Article

Administration of a Polyphenol-Enriched Feed to Farmed Sea Bass (*Dicentrarchus labrax* L.) Modulates Intestinal and Spleen Immune Responses

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Received 1 July 2015; Revised 27 August 2015; Accepted 2 September 2015

Academic Editor: Jara Perez-Jimenez

Farmed fish are exposed to a continuous antigenic pressure by microbial and environmental agents, which may lead to a condition of chronic inflammation. In view of the notion that polyphenols, largely contained in fruits and vegetables, are endowed with antioxidant and anti-inflammatory activities, farmed sea bass (*Dicentrarchus labrax* L.) have been administered with red grape polyphenol-enriched feed. Polyphenols were extracted from the seeds of *Canosina* Nero di Troia *Vitis vinifera* and mixed with conventional feed at two different concentrations (100 and 200 mg/kg, resp.). Fish samples collected at days 223 and 273, respectively, were evaluated for intestinal and spleen cytokine release as well as for spleen macrophage (MØ) and melanomacrophage center (MMC) areas and distribution. Data will show that in treated fish decrease of intestinal interleukin- (IL-) 1β and IL-6 and increase of splenic interferon- (IFN-) γ occur. On the other hand, in the spleen reduction of MØ number seems to parallel increase in MMCs. Collectively, these data suggest that polyphenol-administered sea bass generate lower levels of intestinal proinflammatory cytokines, while producing larger amounts of spleen IFN-γ, as an expression of a robust and protective adaptive immune response. Increase of MMCs corroborates the evidence for a protective spleen response induced by feed enriched with polyphenols.

1. Introduction

It is well known that fish immune cells mainly include lymphocytes, dendritic cells, monocytes, macrophages, granulocytes, and thrombocytes [1]. In teleost fish, major lymph reticular tissues are head kidney, spleen, thymus, liver, and mucosa-associated lymphoid tissues as in the gut [2]. Accordingly, these tissues may represent the primitive analogues of fish lymph node germinal centers, even if their morphology and immune function are still under investigation [3–6].

In fish, melanomacrophage centers (MMCs) are defined as a group of pigmented macrophages (MØ) under form of a nodular cluster of MØ characterized by heterogeneous inclusions such as degradation products of cells [7]. MMCs are devoted to the destruction and recycling of exogenous and endogenous antigens [8], even including storage of iron as a consequence of erythrophagocytosis [9]. All these processes generate cell debris, melanin pigments, hemosiderin granules, and lipofuscin residues [10], as well as lipid droplets, basic protein aggregates, and neutral mucopolysaccharides [11]. Moreover, some types of intracellular granules contain trace metals [12–15]. The pigment deposits contained in vacuoles suggest a mechanism of phagocytosis by MMCs as an expression of early antimicrobial defenses [16–18].

Number, size, and pigment distribution of MMCs depend on fish species [19, 20], organs [11, 21, 22], age [23–25], sexual activity [26, 27], nutritional status, and fish health [10, 28–31].

The growth of aquaculture, associated with the intensification of production systems, has increased the demand for high-quality feedstuff in order to improve fish health without any side effects for consumers [32, 33]. Fish meal has traditionally been used as the main feed ingredient in

preparation of fish feed, due to its high protein content and balanced amino acid profile. Because of its recent shortage in global production, coupled with increased demand and competition for its use in livestock and poultry feeds, prices of fish meal have become unaffordable [34]. Therefore, sustainable aquaculture depends on a perfect balance between growth and healthy conditions of fish. The use of antibiotics and chemotherapeutics to combat fish infections may generate resistant pathogens, bioaccumulation, and environmental pollution. Furthermore, commercial vaccines are expensive for fish farming practices and must be specific against particular pathogens [35].

Nowadays, use of plant proteins to replace fish meal without reducing the performance has started. The administration of probiotics and prebiotics to fish seems to favor the growth of a protective microbiota [36, 37], which, for its immunomodulating activities, may represent a very promising biological control for aquaculture. Therefore, investigations of spleen MMCs have provided useful knowledge on the fish health status also in relation to the type of nutrition [38–43].

Among natural products, polyphenols, largely present in fruits and vegetables, have been shown to scavenge oxygen and nitrogen derived free radicals, modulating antioxidant enzymes and cellular redox transcription factors [44]. In particular, the protective effects of polyphenols consist in the continuous removal of various reactive oxygen species from cells, such as singlet oxygen, peroxynitrite, and hydrogen peroxide in order to maintain healthy metabolic functions [45]. They may also affect cell-to-cell signaling, receptor sensitivity, inflammatory enzyme activity, or gene regulation [46, 47]. According to our own studies in both animal models and humans polyphenols from red grapes are endowed with antioxidant and anti-inflammatory activities, also keeping in equilibrium the immune machinery [48, 49].

A few studies have been conducted on the use of polyphenols in farmed fish in order to evaluate their antioxidant and anti-inflammatory effects. One study investigated the *in vitro* effects of resveratrol, mangiferin, and (–)-epigallocatechin-3-gallate on the histiophagous ciliate *Philasterides dicentrarchi*, which causes fatal scuticociliatosis in farmed turbot *Scophthalmus maximus* L. Of the 3 polyphenols, resveratrol showed strongest antiprotozoal activity, reducing ciliate density after 1-week culture. In view of these findings, the potential utility of chemotherapy with polyphenols as a strategy for the control of scuticociliatosis in farmed turbot has been emphasized [50].

In another research, the beneficial effects of polyphenols derived from waste water from an olive mill, obtained by nonplastic molecular imprinting device, were evaluated in a hypercholesterolemic diet on *Carassius auratus*, commonly known as goldfish, that was selected as experimental model. Results show the beneficial activity of polyphenols with a reduction of the damage in the steatotic group, confirming that they may be used for the treatment of diseases by lipid accumulation in feed of farmed fish. This dietary approach may improve the organoleptic and nutritional quality. Finally, the beneficial effects of waste oil extract should be suggested in the context of research programs focused on the products to the health system [51].

In the present study, in farmed sea bass (*Dicentrarchus labrax* L.) the effects of a diet rich in polyphenols on the cytokine release from gut and spleen tissues as well as deposition of MMCs in spleen were evaluated.

2. Materials and Methods

2.1. Polyphenol Extracts. *Canosina* red grape from Nero di Troia is an autochthonous *Vitis vinifera* grape cultivar which grows in Apulia (South Italy). It is characterized by thick skinned and small sized berries. Frozen seeds from berries were extracted by percolation using ethanol/water (70 : 30). Then, the extract was first analyzed by means of liquid chromatography with diode array detection to define the polyphenol composition. Thereafter, the extract was purified on a synthetic adsorbent brominated resin and percentage of polyphenol content was determined.

The extracts were evaluated for their potential antioxidant effects by using the 2,2-diphenyl-1-picrylhydrazyl assay which measures the ability of test agents to scavenge radicals [52].

2.2. Preparation of Feed and Dietary Regimen. Fish diet consisted in conventional feed mixed with two different concentrations of grape extracts, administered 3-4 times a day:

(1) Fish ($n = 30$) fed mix composed of 50 g of grape extracts in 5 kg of cornstarch, namely, 10 g of mix (100 mg of extract) for 1 kg of conventional pellet (100 mg/kg).

(2) Fish ($n = 30$) fed mix composed of 100 g of grape extracts in 5 kg of cornstarch, namely, 10 g of mix (200 mg of extract) for 1 kg of conventional pellet (200 mg/kg). For preliminary experiments, lower concentration of grape extracts, for example, 1, 10, and 50 mg/kg, was not effective in our test system.

Controls ($n = 30$) were represented by fish fed conventional feed.

2.3. Sample Collection. Spleen samples were collected from a total of 90 immature (juvenile) samples of sea bass reared in captivity in a farm near Lesina lake (Foggia, Italy). Sample collection took place during winter at 223 days (T1) and 273 days (T2) from the beginning of the experiment.

Fish, reared in experimental conditions, were treated according to the "Council Directive 86/609 EEC for the protection of animals used for experimental and other scientific purposes" and the "Ethical Justification for the Use and Treatment of Fishes in Research" [53].

2.4. Immunological Investigations. Specimens were taken from spleen and both pyloric (P) and terminal (T) gut (G) segments of treated and untreated fish, respectively.

Gut portions were placed in Petri dishes containing RPMI 1640 (Miltenyi Biotec, Bergisch Gladbach, GE) plus

streptomycin (100 mg/mL) (Biowhittaker, Walkersville, USA) and 1% penicillin (Biowhittaker) and sliced with scissors to obtain GP and GT samples, respectively. Both segments were then incubated in Petri dishes containing RPMI 1640 for 2 and 24 h at 14°C, respectively. Afterwards, supernatants of GP and GT cultures were obtained by centrifugation at 10,000 ×g for 10 min at 4°C and stored at −30°C, until use.

Spleen specimens were placed in Petri dishes containing RPMI 1640 plus 0.2% heparin and passed through a cell strainer with a 70 μm nylon membrane (Becton Dickinson, Bedford, MA), gently forced with a 1 mL syringe plunger, and filtered in medium, to yield a single cellular suspension for each case. After incubation for 2 and 24 h at 14°C, respectively, 1 mL of cell cultures was put into Eppendorf cups and centrifuged at 10,000 ×g for 10′ at 4°C. Finally, culture supernatants were collected and stored at −30°C, until use [54].

Concentrations (pg/mL) of fish interleukin- (IL-) 1β, IL-6, and interferon- (IFN-) γ in supernatants were determined by specific ELISA kits (Cusabio Biotech Co., Wuhan, Hubei, China) according to manufacturer's instructions. Cytokine concentrations were read at 550 nm by means of an ELISA reader (iMark Microplate Absorbance Reader, BioRad, Hercules, California, USA). Concentrations obtained for each cytokine were multiplied by the dilution factor to obtain sample values.

2.5. Basic Histology and Histochemistry. All fishes were anaesthetized with Tricaine 1 : 5000 (Fluka BioChemika, Buchs, Switzerland) according to the guidelines for Euthanasia of Nondomestic Animals American Association of Zoo Veterinarians (2006). GP, GT, and spleen of each fish were removed, fixed in 10% buffered formalin, later washed in running water, dehydrated in increasing ethanol concentrations, and embedded in paraffin wax.

Sections of tissue (5 μm thick) were processed for the following: (a) Hematoxylin-Eosin (H&E) staining (Merck, Darmstadt, Germany); (b) Perls-Van Gieson staining (Bio-Optica, Milan, Italy) to identify ferric iron; (c) Mallory staining (Merck, Darmstadt, Germany) to detect lipofuscin pigments and ceroids. The identification of MØ was performed using the α-Naphthyl Acetate Esterase (Anae) (Sigma Diagnostics, St. Louis, MO, USA) and Peroxidase (Perox) (Sigma Diagnostics) methods.

2.6. Quantification of Melanomacrophage Centers. The surface occupied by MØ and MMC (μm^2 spleen parenchyma) was counted and measured randomly for a number of 100 elements, selected at digital fields. Each digital field was photographed with a 40x objective with a digital camera (XC-003P, Sony, Tokyo, Japan) connected to a light microscope (Laborlux 12, Leitz, Wetzlar, Germany). Measurements were performed using an image analysis software (QWin, Leica, Cambridge, England).

2.7. Statistical Studies. Statistical differences for concentrations of cytokines from gut and spleen tissues after 2 and 24 h incubation, respectively, between untreated and treated samples, both at T1 and T2, and number and surface occupied by MØ and MMCs were compared. Statistical analysis was performed using the GraphPad Prism statistical software release 5.0 for Windows Vista. Bonferroni's test was used for comparison between controls and treated samples. Statistical significance was set at $P < 0.05$.

3. Results

As far as the polyphenol content of the red grape extracts is concerned percentages were the following: proanthocyanidins (101.8%) and catechins plus epicatechin (10.37%).

(a) Cytokine Release from Gut and Spleen following 2 h Incubation. With regard to GP supernatants, concentrations of IL-1β are represented in Figure 1(a). At T2, in treated samples amounts of IL-1β were significantly lower than those observed in the respective controls. No differences were noted in relation to polyphenol concentrations used.

Results related to determination of IL-1β in GT supernatants are expressed in Figure 1(b). A significant increase in IL-1β was observed at T2 with 100 mg/kg polyphenol dose in comparison to other treated samples.

With regard to spleen IL-1β production, no significant differences between the various samples were detected (data not shown).

Determination of IL-6 in the GP supernatants is represented in Figure 2(a). In untreated samples, an increase in IL-6 levels was noted at T1 in comparison to T2. In treated samples with both concentrations of polyphenols levels of IL-6 decreased in a statistically significant manner at T2 versus T1. However, values of these treated samples were not significantly different from those of controls at the same time points.

With special reference to GT supernatants, IL-6 determination is depicted in Figure 2(b). In untreated samples, an increase in IL-6 levels was noted at T1 in comparison to T2. In the case of treated samples, T2 values of IL-6 were lower than T1 levels using 100 mg/kg of polyphenols. However, T1 values with 200 mg/kg were lower than the respective untreated counterpart.

With regard to spleen IL-6 production, no significant differences between the various samples were observed (data not shown).

In the GP supernatants, IFN-γ levels decreased at T1 in treated samples with 200 mg/kg dose in comparison to T2 controls (data not shown). In Figure 3(a), at T2, levels of IFN-γ released from GT were higher with 200 mg/kg dose in comparison to the same concentration at T1.

At T2, spleen IFN-γ release significantly increased in all samples considered (Figure 3(b)). However, at T2, levels of IFN-γ in fish treated with 200 mg/kg were significantly higher than those observed at T1 in both treated and untreated samples.

(b) Cytokine Release from Gut and Spleen following 24 h Incubation. In GP supernatants, at T2, IL-1β concentrations

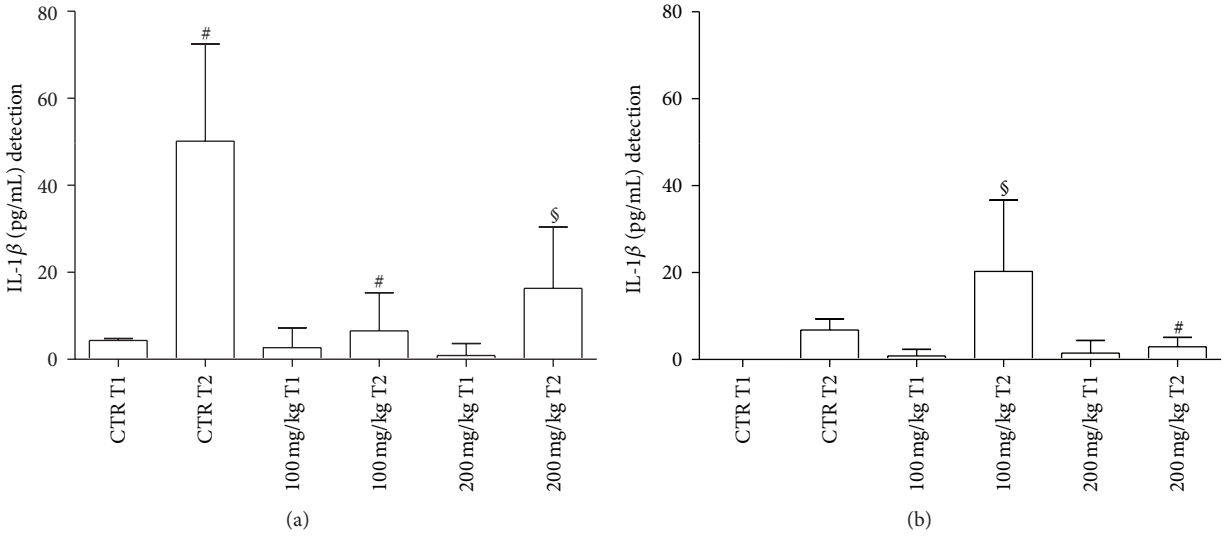


Figure 1: Fish IL-1β levels from GP (a) and GT (b) supernatants after 2 h incubation. CTR = untreated samples; T1 = before treatment; T2 = at the end of treatment. Statistical analysis was performed using the GraphPad Prism statistical software release 5.0 for Windows Vista. Bonferroni's test was used for comparison between the controls and treated samples at both concentrations. Statistical significance was set at $P < 0.05$. (a) $^{\#}P < 0.0001$ CTR T1 versus CTR T2; $^{\#}P < 0.0001$ CTR T2 versus 100 mg/kg T2; $^{\S}P < 0.01$ CTR T2 versus 200 mg/kg T2. (b) $^{\S}P < 0.01$ 100 mg/kg T1 versus 100 mg/kg T2; $^{*}P < 0.05$ 100 mg/kg T2 versus 200 mg/kg T2.

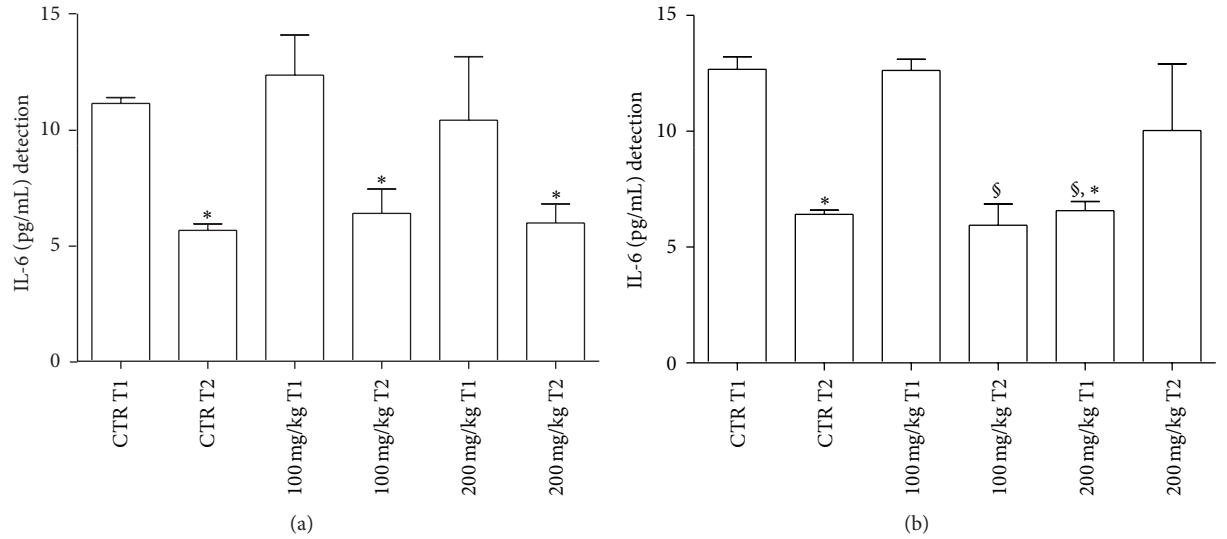


Figure 2: IL-6 levels released from GP (a) and GT (b) supernatants after 2 h incubation. For abbreviations and statistical analysis see Figure 1. (a) $^{*}P < 0.05$ CTR T1 versus CTR T2; $^{*}P < 0.05$ 100 mg/kg T1 versus 100 mg/kg T2; $^{*}P < 0.05$ 200 mg/kg T1 versus 200 mg/kg T2. (b) $^{*}P < 0.05$ CTR T1 versus CTR T2; $^{*}P < 0.05$ CTR T1 versus 200 mg/kg T1; $^{\S}P < 0.001$ 100 mg/kg T1 versus 100 mg/kg T2; $^{\S}P < 0.001$ 100 mg/kg T1 versus 200 mg/kg T1.

of untreated samples were higher in comparison to 200 mg/kg treated samples at T1 (data not shown).

In GT supernatants, no differences in terms of IL-1β concentrations were noted in all samples (data not shown).

Values of spleen IL-1β production were not statistically significant in all samples (data not shown).

In GP supernatants, IL-6 amounts were significantly lower at T2 versus T1 in all samples considered (Figure 4(a)). However, IL-6 values of treated samples were not significantly different when compared to controls.

In GT supernatants, the same pattern of response was observed in terms of a significant reduction of T2 versus T1 values in all samples (Figure 4(b)).

Spleen IL-6 levels in untreated and treated samples were significantly higher at T1 versus T2. In the spleen, T1 values of IL-6 with 200 mg/Kg were lower than the respective untreated counterpart (Figure 4(c)).

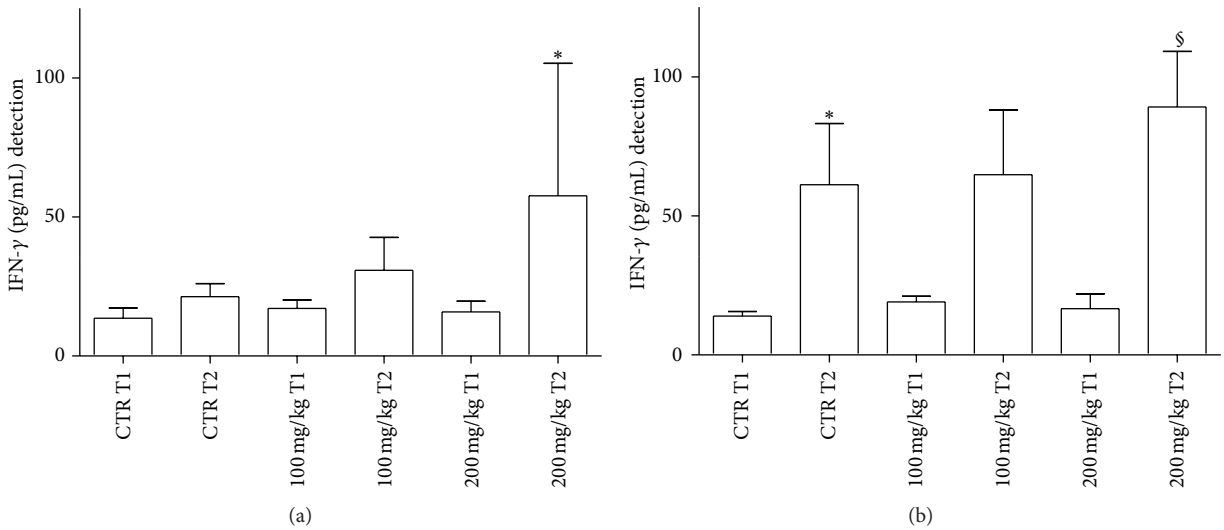


Figure 3: IFN-γ levels released from GT (a) and spleen (b) supernatants after 2 h incubation. For abbreviations and statistical analysis see Figure 1. (a) $^{*}P < 0.05$ 200 mg/kg T1 versus 200 mg/kg T2. (b) $^{*}P < 0.05$ CTR T1 versus CTR T2; $^{\S}P < 0.01$ 200 mg/kg T1 versus 200 mg/kg T2.

In GP supernatants, IFN-γ production was basically the same in all samples (data not shown).

In GT treated samples with 200 mg/kg dose a significant increase in IFN-γ secretion was detected in comparison to all samples, except for T2 controls (Figure 5(a)).

At T2, spleen IFN-γ release significantly increased in treated samples in comparison to the remaining samples. In addition, at T2, values were higher with 200 mg/kg dose in comparison to 100 mg/kg dose. (Figure 5(b)).

3.1. Basic Histology and Histochemistry. The histological appearance of spleen, stained with H&E, is shown in Figure 6(a).

These images show an outer capsule, pink stained, consisting of connective tissue and small trabeculae extended into the parenchyma, which can be divided into a red and white pulp, respectively. However, this arrangement is not in an orderly manner, as can be observed in spleen of higher vertebrates since the two types of tissue are always intermixed. Anyway, the red pulp consists of a reticular cell network supporting blood-filled sinusoids that hold diverse cell population, including MØ and lymphocytes, while the white pulp is composed of small spherical corpuscles also called "ellipsoids" [4], MØ free, and MMCs. Splenic ellipsoids are divided into arterioles forming dense-walled capillaries that are capable of collecting enormous quantities of small particulate antigens. MØ and MMCs exhibit irregular boundaries with densely filled cytoplasmic granules and other unidentified substances. Of note, it becomes difficult to distinguish the nucleus from the rest of the cytoplasm. For this reason, they were cytochemically identified with Perox and Anae staining (Figure 6(b)). The presence of ferric iron and lipofuscin-ceroids in MMCs was detected as dark brown granules with Mallory and Perls-Van Gieson staining, respectively (Figures 6(c) and 6(d)).

3.2. Quantitative Analyses of MMC. The size of MMCs varied greatly within the same section; some formed large clusters measuring up to 77 μm in area, while others were smaller, less than 20 μm, likely monocellular MØ. The single size of MØ and MMCs did not significantly differ between groups. Instead, number and sum of surfaces occupied by MØ and MMC were different in spleen samples from all farmed fish at T1 and at T2, respectively.

MØ percentage was higher in controls than that observed in treated fish at T1 with both polyphenol doses.

MMC percentage was lower in controls than that detected in treated fish with 200 mg/kg dose.

At T2, MMC percentage significantly increased with both concentrations of polyphenols in comparison to untreated fish (Figure 7).

At T1 and T2, the area occupied by control MØ decreased compared to treated fish. On the contrary, the area occupied by MMCs increased in polyphenol-administered fish. In particular, MØ areas showed no statistical difference in controls versus treated fish at T1 and at T2. Instead, MMC areas were higher in treated fish when compared to controls at T2 with both polyphenol concentrations (Figure 8).

4. Discussion

Polyphenols are endowed with antioxidant and anti-inflamma-tory activities as documented by a series of data obtained in animals and humans [55–59]. Stemming from the concept that farmed fish are exposed to a continuous antigenic pressure owing to intensive rearing conditions in aquaculture, a robust stimulation of their immune system likely takes place. In principle, microbial challenges along with environmental insults (e.g., ultraviolet radiation) may trigger an early protective immune response in fish which may be converted into a chronic inflammation in the presence

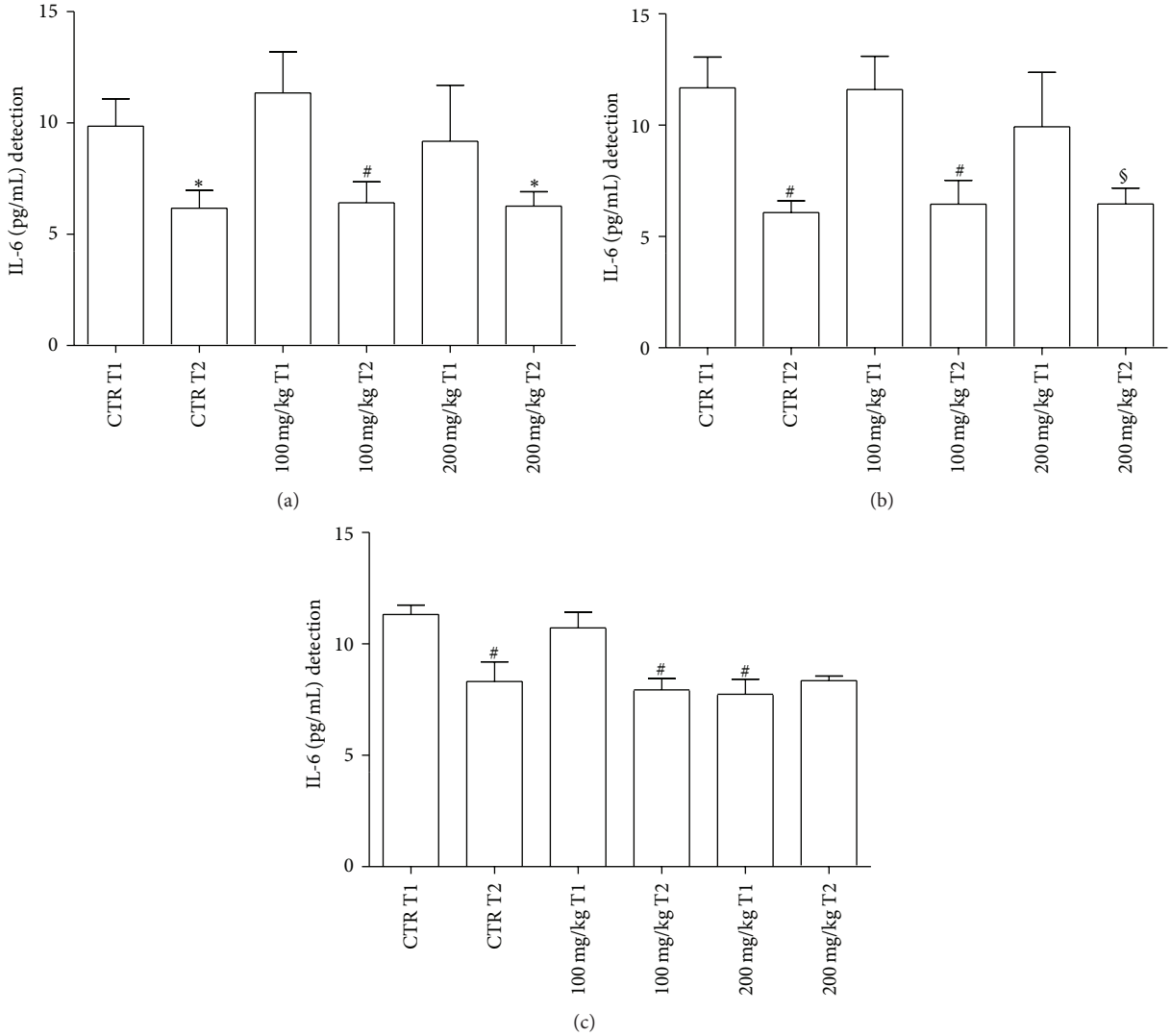


Figure 4: IL-6 levels released from GP (a), GT (b), and spleen (c) supernatants after 24 h incubation. For abbreviations and statistical analysis see Figure 1. (a) $^{*}P < 0.05$ CTR T1 versus CTR T2; $^{\S}P < 0.01$ 200 mg/kg T1 versus 200 mg/kg T2. (b) $^{\#}P < 0.0001$ CTR T1 versus CTR T2; $^{\#}P < 0.0001$ 100 mg/kg T1 versus 100 mg/kg T2; $^{\S}P < 0.01$ 200 mg/kg T1 versus 200 mg/kg T2. (c) $^{\#}P < 0.0001$ CTR T1 versus CTR T2; $^{\#}P < 0.0001$ CTR T1 versus 200 mg/kg T1; $^{\#}P < 0.0001$ 100 mg/kg T1 versus 100 mg/kg T2; $^{\#}P < 0.0001$ 100 mg/kg T1 versus 200 mg/kg T1.

of a persistent immune stimulation [60, 61]. Ultimately, this pathological condition may increase fish mortality in aquaculture and/or lower quality of meat in terms of dietary consumption.

In the light of these considerations, we have treated farmed sea bass with a polyphenol-enriched feed in order to evaluate putative modifications of intestinal and splenic immune responses.

Release of cytokines from gut and spleen of treated and untreated fish has been evaluated under conditions of short term (2 h) or long term culture (24 h). In fact, previously, it has been demonstrated that production of fish cytokines is different according to length of incubation time of immune cells [62].

With regard to IL-1β concentrations, this cytokine decreases in treated samples in GP in comparison to controls after 2 h of incubation. In the spleen, no significant differences are observed between conventionally fed and polyphenol fed animals. It is well known that IL-1β is a proinflammatory cytokine characterized by the functional conservation of its signaling between mammalian and teleost lineages [63]. In principle, IL-1β protects the host against potential pathogens, while generating a detrimental inflammatory milieu in the case of its exaggerated production in response to persistent stimuli. As demonstrated by *in vitro* studies with sea bass, in head kidney leukocytes stimulated with *Vagococcus fluvialis* L-21 upregulation of IL-1β and Tumor Necrosis Factor (TNF)-α suggests an early inflammatory response [64]. This has also been confirmed by experiments in zebrafish following tissue injury [65]. In our experimental model, IL-1β reduction in treated samples seems to correlate with less fish mortality and reduced frequency of infections

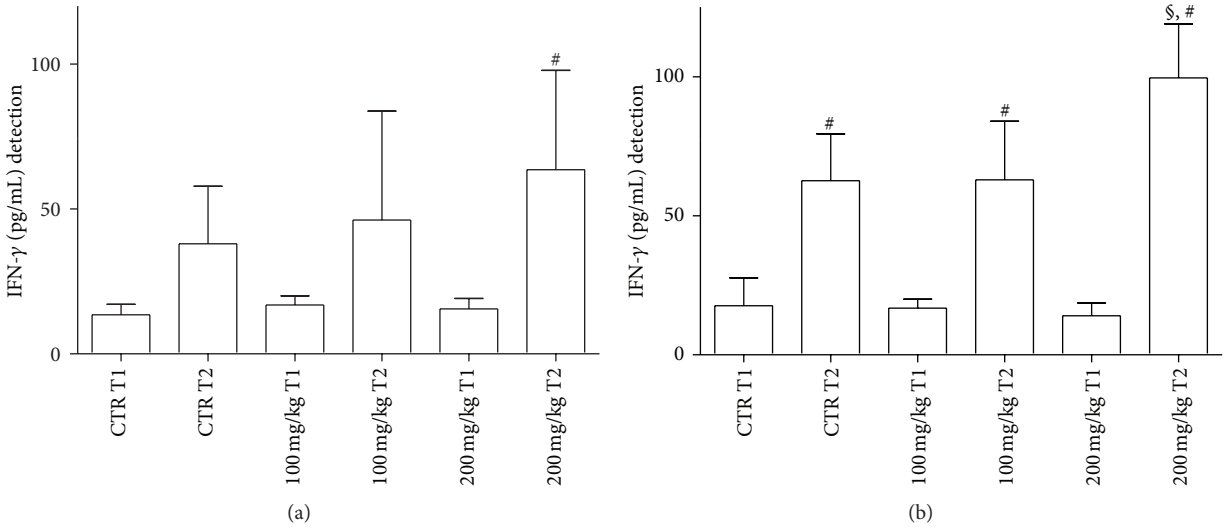


Figure 5: IFN-γ levels released from GT (a) and spleen (b) supernatants after 24 h incubation. For abbreviations and statistical analysis see Figure 1. (a) $^{\#}P < 0.0001$ 200 mg/kg T1 versus 200 mg/kg T2; (b) $^{\#}P < 0.0001$ CTR T1 versus CTR T2; $^{\S}P < 0.01$ CTR T2 versus 200 mg/kg T2; $^{\#}P < 0.0001$ 100 mg/kg T1 versus 100 mg/kg T2; $^{\S}P < 0.01$ 100 mg/kg T2 versus 200 mg/kg T2; $^{\#}P < 0.0001$ 200 mg/kg T1 versus 200 mg/kg T2.

(manuscript in preparation), thus indicating a potential beneficial effect exerted by polyphenols. Quite interestingly, in our fish samples production of splenic IL-1β is much less than that observed in the intestines, thus suggesting a more effective capacity of spleen phagocytes to effectively destroy pathogens, thus leading to their immediate clearance. Instead, in the intestines the reduced contingent of MØ as well as lymphocytes, as documented by Figure 9(b), may lead to pathogen persistence with a continuous triggering of IL-1β release.

In mammals, IL-6 is an acute phase reactant which is also involved in hematopoiesis, inflammation, and immunomodulation, even including antibody production [66]. In fish, scarce information is available on the function of IL-6 despite the recent identification of its gene. In addition, IL-6 induced expression by IL-1β remains to be clarified in fish [67]. In our test system, decreases of IL-6 values occur in the GT (at T1 with 200 mg/kg dose/2 h) and in the spleen (at T1 with 200 mg/kg/24 h).

With regard to IFN-γ production, it is well known that this cytokine, mainly released by T helper (h)-1 cells and Th1-like cells [68, 69], is highly protective for the host against intracellular bacterial and viral infections. In our experiments, IFN-γ mostly increases in the spleen under the influence of polyphenols, thus indicating a more predominant immune adaptive function exerted by this lymphoid organ in comparison to the gut. Quite interestingly, in Atlantic halibut, experimentally infected with nodavirus, increased levels of T cell marker and IFN-γ transcripts have been observed as an example of a robust adaptive immune response. In the same context, *in vitro* experiments have also shown the intervention of IL-6 and IFN-γ with involvement of CD8β+ cells against nodavirus [70]. Furthermore, Jung and associates [71] have reported that recombinant IFN-γ protects the olive flounder (*Paralichthys olivaceus*) against *Edwardsiella* (*E. tarda*, thus increasing survival in comparison to *E. tarda* treated group only.

In relevance to polyphenols used in our experiments, green tea administration [72] to rainbow trout (*Oncorhynchus mykiss*) was able to modulate immune-related gene expression of several cytokines, such as IL-1β, IL-6, IL-8, and IL-10 and TNF-α in spleen, liver, and kidney. In general terms, upregulation of all cytokines was observed except for the reduction of IL-10. Furthermore, an enhancement of the antioxidant system was demonstrated in green tea treated animals.

Finally, as far as polyphenol doses used in our feeds are concerned, no differences have been evidenced between 100 and 200 mg/kg concentrations used.

Fish MMCs are considered as an expression of primitive cellular aggregates where MØ engulf various pigments and cell debris but also exert an early protection against pathogens [16–18]. The analysis of MØ and MMCs demonstrates that treatment with polyphenols induces an increase of MMC numbers and related areas. This means that MØ may form clusters which generate MMCs which, in turn, may afford more protection to fish against pathogens, even including viruses. In fact, we can hypothesize that all trapped material is first engulfed by MØ and then transported to MMCs for their processing. Of note, it is possible to observe MMCs mainly located in close proximity of blood vessels. Reduction in MØ number by effect of polyphenols may explain the decrease of IL-1β while it is conceivable that MMCs may act as antigen presenting cells toward T lymphocytes, which in turn release IFN-γ. Therefore, increase in MMCs seems to contribute to less mortality and reduction of infectious events in farmed sea bass.

In conclusion, our results confirm and extend previous data related to the anti-inflammatory and immune-modulating activities exerted by polyphenols [45, 48, 59].

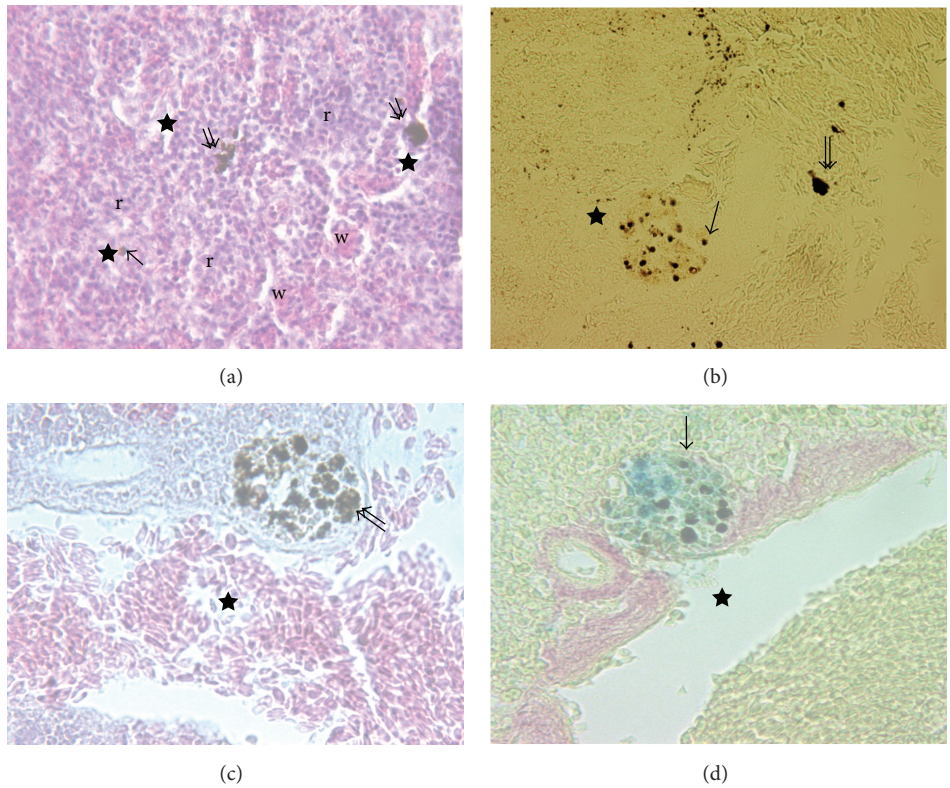


Figure 6: (a) Splenic tissue of *Dicentrarchus labrax* L. organized in areas of red pulp (r) and white pulp (w); the last consisting of ellipsoids, MØ (arrow), and MMC (double arrow) (H&E, 100x). ((b), (c), (d)) At higher magnification (400x) MØ and MMC appear distributed above all near vessels (star) (Anae, Mallory, and Perls-Van Gieson, resp.).

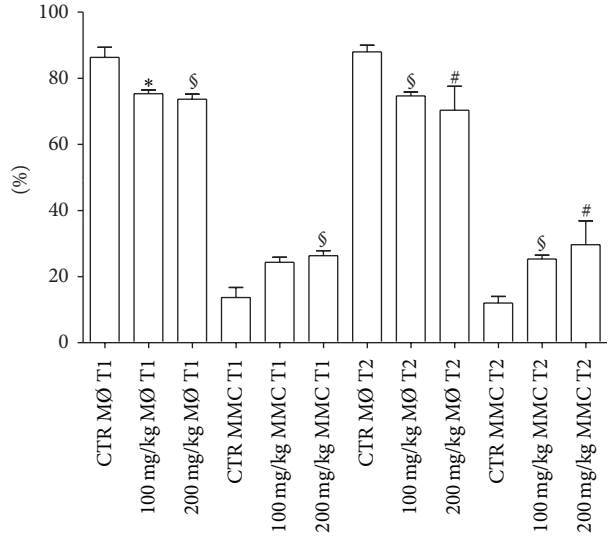


Figure 7: Spleen number percentages of MØ and MMCs from treated and untreated fish. For abbreviations and statistical analysis see Figure 1. $^{*}P < 0.05$ CTR MØ T1 versus 100 mg/kg MØ T1; $^{\S}P < 0.01$ CTR MØ T1 versus 200 mg/kg MØ T1; $^{\S}P < 0.01$ CTR MMC T1 versus 200 mg/kg MMC T1; $^{\S}P < 0.01$ CTR MØ T2 versus 100 mg/kg MØ T2; $^{\#}P < 0.0001$ CTR MØ T2 versus 200 mg/kg MØ T2; $^{\S}P < 0.01$ CTR MMC T2 versus 100 mg/kg MMC T2; $^{\#}P < 0.0001$ CTR MMC T2 versus 200 mg/kg MMC T2.

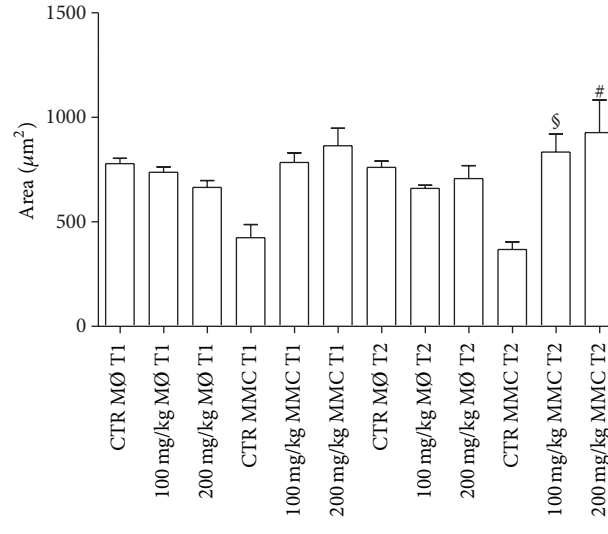
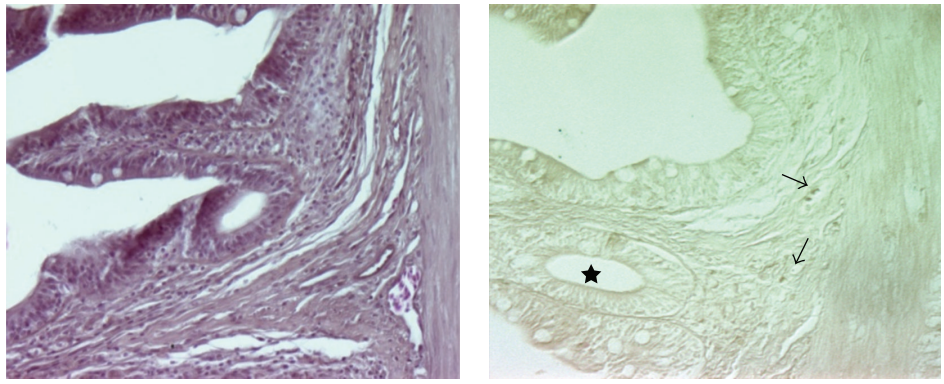


Figure 8: Spleen areas occupied by MØ and MMCs of treated and untreated fish. For abbreviations and statistical analysis see Figure 1. $^{\S}P < 0.01$ CTR MMC T2 versus 100 mg/kg T2; $^{\#}P < 0.0001$ CTR MMC T2 versus 200 mg/kg T2.

This contention is supported by the reduction of IL-1β and IL-6 concentrations and enhancement of spleen IFN-γ release. This dietary approach may be exploitable by farmed fish



(a) (b)

Figure 9: Intestinal sections from farmed sea bass. (a) (H&E staining, 250x) provides a general view of the gut. In (b) (Anae staining, 250x) a few lymphocytes (upper arrows) and one MØ (lower arrow) are evident in the lamina propria. Some blood vessels are present in the section (see star).

companies in terms of either longer fish survival or more beneficial effect for consumers owing to higher quality of meat.

Conflict of Interests

The authors declare that they have no competing interests.

Acknowledgment

This paper was supported by Regione Puglia-Project Vis Maris (Bando FESR 2007-13-Azione 1.2.4 Bando "Aiuti a Sostegno dei Partenariati Regionali per l'Innovazione").

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