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UNIVERSITÀ DEGLI STUDI DI TORINO

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Polyphenolic compounds present in Sardinian wine extracts protect against inflammation induced by oxysterols in CaCo-2 human enterocyte-like cells.

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Abstract

Cholesterol auto-oxidation products, namely oxysterols, are widely present in cholesterol-rich foods. They are thought to potentially interfere with homeostasis of the human digestive tract, playing a role in intestinal mucosal damage. This report concerns the marked up-regulation in differentiated CaCo-2 colonic epithelial cells of two key inflammatory interleukins, IL-6 and IL-8, caused by a mixture of oxysterols representative of a hyper-cholesterolemic diet.

This strong pro-inflammatory effect appeared to be dependent on the net imbalance of red-ox equilibrium with the production of excessive levels of reactive oxygen species through the colonic NADPH-oxidase NOX1 activation. Induced NOX1 hyper-activation was fully prevented by CaCo-2 cell pre-incubation with polyphenols extracted from wines selected from the most typical grape varieties grown in Sardinia. Oxysterol-dependent NOX1 activation, as well as interleukin' synthesis, were completely prevented by Cannonau red wine extract that contains an abundant polyphenolic fraction. Conversely, cell pre-treatment with Vermentino white wine extract, with smaller polyphenolic fraction, showed only a partial NOX1 down-regulation and was ineffective in interleukin' synthesis induced by the dietary oxysterol-mixture.

It is thus likely that the preventive effects of Sardinian wine extracts against intestinal inflammation induced by dietary oxysterols are mainly due to their high polyphenolic content: low doses of polyphenols would be responsible only for direct scavenging oxysterol-dependent ROS production. Besides this direct activity, an excess of polyphenols, such as detectable in red wine, may exert an additional indirect action by blocking oxysterol-related NOX1 induction, thus totally preventing the pro-oxidant and pro-inflammatory events triggered by dietary oxysterols.

Keywords: oxysterols, wine extracts, polyphenols, inflammation, NADPH oxidase NOX1

1. Introduction

Intestinal inflammation is usually associated with a net imbalance of red-ox equilibrium with excessive levels of reactive oxygen species (ROS). Together with pro-inflammatory cytokine production, these most likely contribute to functional impairment of the enteric mucosa [1].

The intestinal tract is often exposed to the action of food-derived lipid oxidation compounds that can induce intestinal damage; increasing interest has been shown in the beneficial effect of a dietary intake of polyphenols, because of their anti-oxidant and anti-inflammatory properties.

Oxysterols are among the lipid oxidation products that have been shown to exert various *in vitro* and *in vivo* biochemical activities, of both physiologic and pathologic relevance; they are the oxidized products of cholesterol metabolism, deriving from either enzymatic or non-enzymatic cholesterol oxidation [2]. Their presence in the plasma, and their accumulation in the human body, may be due either to dietary consumption or to their *in vivo* synthesis. Compared to cholesterol, the presence of additional oxygen groups make these compounds more polar and more easily diffusible through cell membranes; consequently they are more reactive, and can promote and sustain inflammation, fibrosis and programmed cell death. Different forms of oxysterols have been reported to be especially implicated in the pathogenesis of degenerative diseases characterized by chronic inflammation, such as atherosclerosis and Alzheimer's disease [3].

The impact of oxysterols on the intestinal tissue is not yet known in detail; they play a role as intermediates in sterol biosynthesis and cholesterol catabolism, and are necessary for bile acids synthesis [2,3]. Only in the last few years, oxysterols have started to be considered for their possible involvement in the pathogenesis of human inflammatory bowel diseases.

The oxysterols most widely present in cholesterol-rich foods are reported to be 7 α -hydroxycholesterol, 7 β -hydroxycholesterol, 7-ketocholesterol, 5 α ,6 α -epoxycholesterol, and 5 β ,6 β -epoxycholesterol, in concentrations ranging from 10 to 100 μ M [4,5]. In a previous study we investigated the potential pro-inflammatory effect of a mixture of the main dietary oxysterols, stemming from 30 μ M cholesterol after heating at 180°C, on differentiated human epithelial colonic CaCo-2 cells, which spontaneously undergo differentiation into absorptive enterocyte-like phenotype after 18-21 days' plating. In this experimental model, the pro-inflammatory effect of the oxysterol mixture was mainly detected as an up-regulation of interleukin (IL)-8 expression and synthesis, and appeared to be mediated by ROS generation through hyper-activation of colonic NADPH-oxidase (NOX) isoform 1 [6]. Further, this study also gave evidence of anti-inflammatory properties of epigallocatechin-3-gallate, which is very abundant in green tea, and which prevented the oxysterol mixture-related NOX1 activation. Epigallocatechin-3-gallate is a flavonoid belonging to a broader heterogeneous group of plant secondary metabolites, the polyphenols, which are distributed in a wide variety of plant-derived foods and beverages, including among others grains, legumes, fruits, vegetables, green and black tea, and wine [7].

Polyphenols are present in food as a complex mixture; their concentration is extremely variable due to the seasonal and geographic variations of plant growth, and may also depend on storage and food preparation methods. Interest in food polyphenols has recently been increasing, because of their antioxidant and anti-inflammatory properties and their possible beneficial implications in various human diseases.

The main classes of polyphenols are flavonoids, phenolic acids, lignans and stilbenes; chemically, they are characterized by the presence of a number of phenolic rings, with two or more hydroxyl groups. Flavonoids are further subdivided into subclasses: flavones, isoflavones, flavonols, flavanols, flavanones, anthocyanins and anthocyanidins. Due to the

presence of hydroxyl groups in their chemical structure, polyphenols are effective free radical scavengers and metal chelators [8,9].

The bioavailability of polyphenols is not generally very high in the blood and tissues, because they are poorly absorbed and rapidly catabolized. Conversely, the intestine is closely involved in the absorbance and metabolism of polyphenols, which achieve higher concentrations in that organ than elsewhere. They might thus exert their health benefits by protecting the intestinal mucosa from oxidative damage occurring during digestion, contributing to the enhancement of the antioxidant status as a whole [10]. Polyphenols exert anti-inflammatory action by modulating cell signaling pathways; this action is mainly activated in response to oxidative and inflammatory stimuli, which have the red-ox sensitive transcriptional factors nuclear factor- kappaB (NF-kB) and activator protein-1(AP-1) as principal down-stream effectors [9].

Wine contains many phenolic compounds, which originate from the seeds and skin of the grapes and are mainly responsible for its color. Polyphenolic wine components vary depending on the fruit source as well as processing during the initial phases of fermentation. Maceration allows flavonoids and other substances to be extracted in quantities depending on the temperature and duration of contact with the grape skin. Among the polyphenols, flavonoids, benzoic and hydroxycinnamic acids are the major classes present in wines. Red wine contains more phenolic compounds than white wine, due to the grape variety and variations in the fermentation process.

Wine polyphenols have been shown to have cardioprotective, anticancer, anti-diabetic, antimicrobial, anti-inflammatory, neuroprotective, nephroprotective and anti-ageing properties, as well as improving vascular function and having positive effects on atherosclerosis [11,12,13]. The differences between red and white wines with regard to their possible antioxidant properties are still debated. Most of the beneficial effects of red wine

have been ascribed to its higher polyphenol content than white wine. However, it is also reported that white wine can have the same health benefits as red wine, as far as cardioprotection is concerned [14,15].

This report concerns the marked up-regulation in differentiated CaCo-2 cells of two key inflammatory chemokines, IL-6 and IL-8, caused by a mixture of oxysterols representative of a hyper-cholesterolemic diet. The pro-inflammatory effect of the dietary oxysterol mixture found in this experimental model, which mimics normal intestinal mucosa quite closely, appeared to be dependent on over-activation of colonic NOX1.

Two phenolic extracts, obtained from wines selected from the most typical grape varieties grown in Sardinia (Cannonau red wine and Vermentino white wine) were investigated for their potential anti-inflammatory effects at cell supplementation. The possible variation of the anti-inflammatory effect of wine depending on its polyphenolic content was examined [16]. As red wine has a larger number of polyphenols than white wine, the effect on NOX1 activity of Cannonau red wine polyphenolic extract was compared with that of Vermentino white wine extract.

2. Materials and methods

2.1. Reagents

Unless otherwise specified, all reagents and chemicals were from Sigma–Aldrich (Milan, Italy). Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX (4 500 mg/L glucose and 110 mg/L sodium pyruvate) and fetal bovine serum (FBS) were from Invitrogen (S. Giuliano Milanese, Italy). The protein assay dye reagent and 2-Mercaptoethanol and ECL Western Blotting System were from Bio-Rad (Milan, Italy). Protein A–Sepharose resin was from GE Healthcare (Milan, Italy). Goat anti-Nox1 (anti-Mox1) and mouse anti-mouse HRP-conjugated secondary antibodies were from Santa Cruz (Tebu-Bio s.r.l., Magenta, Milan,

Italy), and mouse anti-NoxA1 antibody was from Abcam (Cambridge, MA, USA). Human IL-6 and IL-8 ELISA Kits were from Gen-probe (France). 25 cm² plastic flasks and 96 multi-well plates were from Falcon, Becton Dickinson Labware Europe (Meylan Cedex, France).

2.2. Cell culture

Human colon adenocarcinoma cells CaCo-2 (passages 20–25; from Cell Bank Interlab Cell Line Collection, Genoa, Italy) were cultured (1×10^6 /ml density) in DMEM-GlutaMAX supplemented with 10% heat-inactivated FBS, 1% antibiotic/antimycotic solution (100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B and gentamicin (0.04 mg/ml) at 37°C in a 5% CO₂ humidified atmosphere. Cells were cultivated for 21 days, replacing the medium thrice weekly, to allow their spontaneous differentiation.

2.3. Preparation of wine extracts

The wine extracts were obtained from Cannonau red wine and Vermentino white wine, both deriving from pure grape varieties typical of Sardinia. The wines were produced following standard procedures by Argiolas S.p.A. (Serdiana (Cagliari), Italy). The wines were less than 6 months of age and neither aged or fermented in oak casks. Liquid-liquid extraction methods were performed on 100 ml of wine and polyphenols were identified and quantified as previously described by Deiana and coworkers [16].

2.4. Cell treatments

Differentiated CaCo-2 cells were placed in DMEM with 5% FBS and pre-treated or not with Cannonau or Vermentino wine extracts (25µg/ml) or with polyphenols present in wine extracts added individually (caffeic acid 10 µM, gallic acid 10 µM, catechin 10 µM, epicatechin 10 µM, quercetin 1 µM) for 60 minutes. Cells were then challenged with the

oxysterol-mixture (Oxy-mixture) (30 and 60 μ M final concentrations) for 4 hours or 24 hours, at 37°C depending on different analyses. The percentage composition of the Oxy-mixture used was 7-ketocholesterol (7 K) 42.96%, 5 α ,6 α -epoxycholesterol (α -epox) 32.3%, 5 β ,6 β -epoxycholesterol (β -epox) 5.76%, 7 α -hydroxycholesterol (7 α -OH) 4.26%, 7 β -hydroxycholesterol (7 β -OH) 14.71%. The concentration of the Oxy-mixture was calculated using an average molecular weight of 403 g/mol. In some experiments cells were also pre-treated with 5 μ M NADPH oxidase inhibitor diphenyl iodonium (DPI) for 30 minutes at 37°C; the solvent used for diluting DPI was dimethyl sulfoxide. For the controls, cells were incubated with the same amount of solvent used to dilute the various substances employed (0.1% ethanol v/v). Cell count and viability were routinely checked by the Trypan blue exclusion method (viability: from 82 to 95%).

2.5. Evaluation of IL-6 and IL-8 protein levels by ELISA

After 24 h treatment, the cell culture medium was collected and centrifuged at 14 000 g for 10 min and the supernatant stored for ELISA detection. The total protein concentration of the cell culture medium was measured using Bio-Rad protein assay dye reagent. Levels of IL-6 and IL-8 were quantified using Human IL-6 and IL-8 ELISA Kits (Gen-probe, France) following the manufacturer's instructions. The plates were then read at 450 nm with wavelength correction of 550 nm in a microplate reader (Model 680 microplate reader Bio-Rad), and data analyzed using SlideWrite Plus software (Advanced Graphics Software). The cytokine profile of each sample was evaluated in duplicate. The values were expressed as pg/mg of proteins.

2.6. Evaluation of IL-6 and IL-8 mRNA levels by real-time polymerase chain reaction (RT-PCR)

After 4 h cell treatment, total RNA was extracted from differentiated CaCo-2 cells using TRIzol reagent and following the manufacturer's instructions. The concentration of extracted RNA was determined by measuring the absorbance at 260 nm; the ratio of the absorbances 260/280 was used to assess the purity of RNA ($A_{260}/A_{280} > 1.8$). Total RNA was stored at -80°C until used. cDNA was synthesized by reverse transcription from 4 μg RNA with a commercial kit and random primers (High-Capacity cDNA reverse transcription kit) following the manufacturer's instructions.

Singleplex RT-PCR was performed on 50 ng of cDNA using TaqMan gene expression assay kits prepared for human IL-6, IL-8 and actin, TaqMan Fast Universal PCRmaster mix, and the 7500 Fast Real-Time PCR system. Cycling parameters were 40 cycles of 3 seconds at 95°C (melting) and 30 seconds at 60°C (annealing/extension). The RT-PCR fluorescence signal for each gene was evaluated with reference to a threshold curve. The results were then normalized against the expression of actin, as housekeeping gene. Relative quantification of target gene expression was expressed as the fold increase induction versus control values.

2.7. Evaluation of activated NADPH oxidase complex by immunoblotting

NOX1 activity was evaluated through Western blotting. Membrane extracts were immunoprecipitated for the membrane enzyme component Nox1, and then immunoblotted for cytosolic subunit NoxA1. Briefly: after 30 min treatment, cells were trypsinized and harvested by centrifugation at 80 g for 5 min, washed with cold 0.1 M PBS. Cell membrane proteins were extracted using Mem-PER Eukaryotic Protein Extraction Reagent Kit (Thermo Scientific, Rockford, IL USA). After protein evaluation with Bio-Rad protein assay dye reagent, 100 μg of membrane proteins were immunoprecipitated overnight at 4°C with goat anti-Nox1 polyclonal antibody and then purified on protein A-Sepharose resin. Specimens

were denatured in boiling Laemmli buffer [200 mM Tris-HCl, pH 7.4, glycerol 36% (v/v), SDS 7% (w/v), 1M 2-mercaptoethanol, bromophenol blue 0.1% (w/v)] and separated by SDS-PAGE (10% gel acrylamide), followed by transfer to Hybond ECL nitrocellulose membrane. After saturation of nonspecific binding sites with 5% (w/v) nonfat milk in 0.1M PBS-Tween 0.05% (w/v), the membrane was immunoblotted overnight at 4°C with mouse anti-NoxA1 polyclonal primary antibody (1:200 dilution) and probed with goat anti-mouse HRP-conjugated secondary antibody (1:1000 dilution). The ECL Western Blotting System (Bio-Rad) was used to detect chemiluminescence. The immunoreactive protein bands were densitometrically analyzed, using Image Tool software. Values were expressed as percentage compared to control (taken as 100%).

2.8. Statistical analyses

Results were expressed as Mean \pm Standard Deviation (SD). Statistical differences between groups were evaluated using the one-way ANOVA test associated with Bonferroni's multiple comparison post test. Data were analyzed with GraphPad InStat software (San Diego, USA).

3. Results

3.1. A representative mixture of oxysterols of dietary origin induces IL-6 and IL-8 synthesis and expression in differentiated CaCo-2 cells

Because of the significant role exerted by cholesterol auto-oxidation products from a hypercholesterolemic diet, in inducing inflammation of intestinal mucosa, we evaluated the pro-inflammatory effect on differentiated CaCo-2 cells of a 60 μ M representative dietary oxysterol mixture (Oxy-mixture) and compared its effect to that of the same Oxy-mixture at a

lower concentration (30 μ M). The main oxysterols formed exogenously were mixed in the proportions resulting from the complete auto-oxidation of 30 or 60 μ M cholesterol (see section 2.) [17].

As reported in Fig.1 A and B, 60 μ M Oxy-mixture was 1.6 fold as efficient as 30 μ M Oxy-mixture at inducing both IL-6 and IL-8 synthesis, which was quantified by the ELISA method as protein release by the interleukins into the cell incubation medium after 24 hours' treatment. The higher Oxy-mixture concentration was also able to induce high IL-6 and IL-8 mRNA levels (4 and 9.5 fold increase vs. controls, respectively) after 4 hours' cell incubation (Fig. 1 C, D).

Based on these data, all subsequent experiments used 60 μ M oxysterol concentration added as a mixture.

3.2. Colonic NADPH oxidase is involved in IL-6 and IL-8 synthesis induced by the dietary oxysterol mixture in differentiated CaCo-2 cells.

Addition to the cell incubation medium of 5 μ M DPI, a widely-used NADPH inhibitor, 30 min prior to challenging the cells with the 60 μ M oxysterol mixture, inhibited the oxysterol-related stimulation of both IL-6 and IL-8 synthesis (Fig. 2 A, B). These data are in agreement with previous results evidencing the major role of the colonic NADPH oxidase isoform NOX1 in inducing IL-8 production by CaCo-2 cells treated with 30 μ M oxysterol mixture [6], and suggest that up-regulation of NOX1 activity by oxysterols may also be responsible for the observed increase of IL-6 synthesis, as well as of the synthesis of IL-8.

3.3. CaCo-2 cell pre-treatment with polyphenol Sardinian wine extracts prevents the dietary oxysterol mixture from activating intestinal NADPH oxidase

The efficacy of the antioxidant polyphenol compounds present in extracts of two Sardinian wines, red Cannonau and white Vermentino, on modulating NOX1 activity was assessed. As shown in Fig. 2C, NOX1 activity in differentiated CaCo-2 cells was up-regulated by cell treatment with 60 μ M Oxy-mixture (215% increase) in comparison with control (solvent alone).

Differentiated CaCo-2 cells were pre-treated for 1 hour with 25 μ g wine extracts / ml cell incubation medium, and treated with 60 μ M Oxy-mixture. Cell pre-treatment with Sardinian wine extracts prevented NOX1 hyperactivation by the Oxy-mixture (Fig.2C). In particular, the Cannonau extract exerted a protective effect on oxysterol-related NOX1 activation (80% decrease) more efficiently than the Vermentino extract. Pre-treatment with the white wine extract only lowered oxysterol-dependent NOX1 activation by 25%, the difference being significant also versus the experimental group treated with Vermentino extract alone.

3.4. Different anti-inflammatory effects of Sardinian wine polyphenol extracts on differentiated CaCo-2 cells treated with the dietary oxysterol mixture

The anti-inflammatory efficacy of extracts of the two Sardinian wines was evaluated, in terms of both IL-6 and IL-8 expression and synthesis. IL-6 and IL-8 mRNA cell levels were determined after 60 minutes' cell pre-treatment with wine extract, and after 4 hours' treatment with the 60 μ M Oxy-mixture. The pro-inflammatory effect exerted by oxysterols mixture was abolished by the Cannonau extract, but not by the Vermentino extract (Fig. 3 A, B).

As in the case of gene expression, the enhancement of IL-6 and IL-8 synthesis induced by the oxysterol mixture was significantly prevented by cell pre-treatment with red wine extract, but not by white wine extract (Fig. 3 C, D).

3.5. Effect on IL-6 and IL-8 production of the principal individual polyphenolic components of Sardinian wine extracts.

Based on the evidence that Cannonau exerts a stronger anti-inflammatory effect than Vermentino, we hypothesized that a different polyphenol concentration of the two Sardinian wines might be responsible for their differing properties.

We then, investigated the anti-inflammatory roles of the polyphenols that were detected by our laboratory in both wine extracts but were present in Cannonau in larger amounts [16]. Among the polyphenol components of wine extracts, gallic acid (belonging to the group of hydroxybenzoic acids), caffeic acid (a hydroxycinnamic acid), the two flavanols catechin and epicatechin, and the flavonol quercetin, were considered, because of their widely-recognized anti-oxidant and anti-inflammatory effects.

As shown in Fig. 4A and B, most of these polyphenols were able to reduce both IL-6 and IL-8 synthesis; in particular gallic and caffeic acids ($p < 0.001$) exerted more significant reduction of the interleukin synthesis induced by 60 μ M dietary oxysterol mixture than did the other phenolic compounds.

4. Discussion

It is generally accepted that extensive changes to lifestyles in Western and industrialized countries have brought with them significant changes in dietary habits, and these are associated with an increase in chronic inflammatory, autoimmune and vascular diseases, as well as cancer. As far as intestinal injury is concerned, a marked dietary intake of cholesterol in the form of animal fats makes its oxidation products available to the intestinal mucosa, and exposes it to a state of persistent inflammation. In turn, this leads to excessive production of pro-inflammatory cytokines and ROS by activated leukocytes. These molecules

most likely contribute to the functional impairment of the enteric mucosa, with consistent intestinal mucosal injury, causing erosion, ulceration, and strictures. These features characterize human inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis.

The association of cholesterol oxidation products with cardiovascular diseases, such as atherosclerosis and neurodegenerative diseases, is now established. However, the role of oxysterols in inflammatory events characterizing the onset and/or progression of intestinal mucosal injury is not yet fully clear.

In this study, differentiated CaCo-2 cells were used as enterocyte-like cells; they possess absorptive properties that mimic the function of the small intestine, since they express microvilli, basolateral membranes with tight junctions, and intestine-specific enzymes.

The study tested the pro-inflammatory effect of a mixture of oxysterols, found most widely in cholesterol-rich foodstuffs, produced by cholesterol auto-oxidation (7K, α -epox, β -epox, 7 α -OH 7 β -OH) [17] when added to differentiated CaCo-2 cells at low or high concentrations (30 μ M or 60 μ M). Dose-dependent interleukin' production by cells treated with the two concentrations of the dietary oxysterol mixture was found, in terms of IL-6 and IL-8 synthesis; the 60 μ M oxysterol mixture had a significant stronger effect than the lower dose, and also significantly enhanced both IL-6 and IL-8 mRNA expression.

A previous study on differentiated CaCo-2 cells detected an enhancement of IL-8 expression and synthesis after treatment with the 30 μ M dietary oxysterol mixture; the increase was mainly mediated by colonic NADPH oxidase NOX1 activity and ROS production [6]. However, no other evidence pointed to IL-6 production by high doses of a dietary oxysterol mixture, using the *in vitro* model of enterocyte-like cells used here. This cytokine, as well as IL-8, is crucial in chronic intestinal inflammation in sustaining intestinal barrier disruption, by furthering release of pro-inflammatory mediators. IL-6 generation is

involved in the function of T helper (Th) 1 lymphocytes, which act against intracellular intestinal pathogens by means of intense phagocyte activity. This cytokine also appears to be involved in the synthesis and activation of Th17 cells, in close synergism with IL-23. The two cytokines have been identified as potent inducers of tissue inflammation, both in experimental models of colitis and in humans [18,19]. A direct correlation between IL-6 serum levels and clinical activity, together with an increase of monocytes, has been found in patients with ulcerative colitis or Crohn's disease [20]. The pro-inflammatory effect of IL-8, as a potent chemoattractant molecule in promoting neutrophil migration into the epithelial layer is widely known. It appears to be the chemokine most abundantly secreted by infected epithelial cells; it can direct the recruitment of phagocytes to the infected site, and their infiltration into the epithelial layer [21].

It has been suggested that intracellular oxysterols act as secondary messengers in cell signal transduction, and some of their biological effects are conducted by the activation of mitogen-activated protein kinase (MAPK) signaling pathways [22,23].

The production of several pro-inflammatory chemokines, including IL-6 and IL-8, has been reported in vascular U937 and THP-1 cells, mainly in the presence of individual 7 β -OH and 25-hydroxycholesterol. In particular, IL-8 secretion in these vascular cells is associated with activation of the mitogen-activated protein/extracellular regulated kinase kinase (MEK)-extracellular regulated kinase (ERK)1/2 signaling pathway [24,25].

The role of oxysterols in activating cell signals could be dependent on their capability to activate NADPH oxidase and generate ROS, which may be the true second messengers of signal transduction pathways, modulating red-ox sensitive MAPK and affecting the activation of inflammation-related genes, i.e. NF- κ B and AP-1 transcription factors and interleukins.

It is known that certain oxysterols exert their cytotoxicity through NADPH oxidase activation in macrophages and vascular cells [26,27]. THP-1 macrophages treated with 7K

and 25-hydroxycholesterol showed an increase of IL-1 β , IL-6, IL-8 and TNF α secretion, and enhanced expression, in a dose-dependent manner, associated with an increased expression of NADPH oxidase and ROS production [28]. 7K is also reported to be effective in enhancing ROS production by inducing the macrophage NADPH oxidase isoform NOX4, and in activating the phosphorylation of ERK1/2, p38MAPK and Jun N-terminal kinase (JNK) [29].

In line with the hypothesis that pro-inflammatory cytokine release may be induced by an over-production of ROS, in this study we observed that 60 μ M dietary mixture of oxysterols, a dose corresponding to a high intake of dietary cholesterol oxidation products, exerted NOX1 up-regulation in differentiated CaCo-2 cells. The observed induction of IL-6 and IL-8 synthesis was prevented by pre-treating cells with the NOX1 chemical inhibitor DPI.

A major and novel finding of the present study is that pre-treatment of differentiated intestinal CaCo-2 cells with polyphenolic extracts of Sardinian wines may counteract NOX1 activation, as well as countering IL-6 and IL-8 production. This agrees with our previous results, which pointed to a preventive effect of the main polyphenolic component of green tea, epigallocatechin-3-gallate, in suppressing oxysterol-related NOX1 hyper-activation and interleukin' expression in differentiated intestinal epithelial cells [6].

The benefits of a dietary intake of polyphenols have widely been suggested to include ameliorating intestinal inflammation [30,9]. A new concept concerning the biological activity of these compounds has received much attention: alongside their simple, direct antioxidant effect as free-radical scavengers, polyphenols are now thought also to exert beneficial effects on human health by indirectly interfering with specific signaling proteins, which mediate gene regulation in response to oxidative stress and inflammation [31,32].

A moderate consumption of wine, in particular red wine, has generally been considered both to protect against coronary heart disease and to be associated with a lower incidence of degenerative diseases [33,34]. The preventive role of polyphenolic compounds in

wine against endothelial dysfunction and the age-related decline in NADPH oxidase down-regulation (due to the decline in physical exercise) has been proved in Wistar rats in a model of chronic administration of red wine polyphenols [35]. An *in vitro* study on human platelets demonstrated that wine polyphenols inhibited Low-density lipoprotein oxidation and protein kinase C (PKC)-mediated NADPH oxidase activity. this inhibitory effects were more marked when the mixture of polyphenols was used at concentrations corresponding to that detected in human circulation after wine intake [36].

It is difficult to distinguish whether these positive properties are due exclusively to wine polyphenols, or also to alcohol intake, since various beneficial effects of ethanol have been reported in many studies, particularly on cardiovascular health [37]. Conversely, other studies have reported an unfavorable role of ethanol, inducing inflammation in patients with coronary artery disease [38]. To our knowledge, ethanol alone, which was the solvent used to prepare the oxysterol solution in our experiments, and was considered as control, did not exert any effect.

The study compared the effect on oxysterol-dependent NOX1 activation of polyphenolic extracts of two Sardinian wines: Cannonau red wine, with an abundant polyphenolic fraction, and Vermentino white wine, with one order of magnitude smaller polyphenolic fraction [16].

The results indicate complete prevention of NOX1 by Cannonau red wine extract, whereas in cells pre-treated with Vermentino white wine extract the inhibition of this enzyme was moderate. Further, both expression and synthesis of IL-6 and IL-8 were significantly lowered by the red wine extract. On the contrary, white wine had no effect on interleukins' expression and only partial, but not significant, effect on interleukins' synthesis. This failure of white wine extract to protect against IL-6 and IL-8 production, in intestinal cells treated with the dietary oxysterol mixture, is in agreement with reports that red wine plays a primary

anti-inflammatory role, thanks to its higher polyphenol content [36,39]. Among different polyphenols present in the both wine extracts used in our experiments (gallic acid, caffeic acid, catechin, epicatechin and quercetin), all except quercetin decreased the synthesis of both IL-6 and IL-8. Thus, the stronger anti-inflammatory effect exerted by the red wine extract might be due to the different amounts of polyphenols present in that extract.

As regards the protective (although moderate) effect displayed by white wine extract on oxysterol-dependent NOX1 activation in differentiated CaCo-2 cells, it could be argued that the limited amount of polyphenolic components of white wine may only partially inhibit NOX1. For example, two polyphenolic compounds found in white wine, namely tyrosol and caffeic acid, have been reported to have strong anti-oxidant and anti-inflammatory properties [40]. Furthermore, there is evidence that white wine exerts a similar antioxidant effect as red wine does, but in much higher doses [16]. A recent study analyzed the capability of different French red and white wines to inhibit the formation of cholesterol oxidation products, especially 7K; the white wines studied exerted anti-oxidant activity, although the capability of red wine to inhibit cholesterol oxidation was much higher than that of white wine [41].

Thus, the NOX1 decrease observed to be caused by white wine is likely due white wine components other than polyphenols, which are released during the wine-making process. Various glycoproteins derived from grapes and yeast during fermentation may have pathophysiological relevance [42]. Sulfur dioxide and ascorbic acid are commonly added to white wine, and might interact with polyphenols, enhancing the antioxidant capacity of white wine [43,44]. Sulfites are present in similar concentrations both in red and white wines, and are also reported to exert antioxidant effects [45].

It is thus likely that the preventive effects of Sardinian wine extracts, against intestinal inflammation induced by dietary oxysterols, are mainly due to the polyphenolic fraction, and to that fraction's ability to down-modulate NOX1 activity.

Although further research is required to elucidate the importance of other wine components, it could be argued that the direct antioxidant effect of polyphenols is predominant over their indirect effect on signaling molecules. Based on this hypothesis, low doses of polyphenols would engage primarily in their immediate function by scavenging oxysterol-dependent ROS production, thus partially preventing oxysterol pro-oxidant insult. Conversely, doses of polyphenols in excess (quantities detectable in red wine) may exert their indirect action, blocking oxysterol-related NOX1 induction, and thus totally preventing the pro-oxidant and pro-inflammatory signals triggered by dietary oxysterols.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1. Evaluation of IL-6 and IL-8 synthesis and expression in differentiated CaCo-2 cells treated with 30 μ M or 60 μ M Oxysterol-mixture.

The synthesis of IL-6 (A) and IL-8 (B) was analyzed by ELISA in the culture medium of CaCo-2 cells treated for 24 h with 30 μ M or 60 μ M Oxysterol-mixture (Oxy-mixture) or solvent alone (Control). The cytokines' concentrations are expressed as pg/mg protein of IL-6 or IL-8 released.

The expression of IL-6 (C) and IL-8 (D) was quantified by RT-PCR, in CaCo-2 cells treated for 4 h with 60 μ M Oxy-mixture, or solvent alone (Control). IL-6 and IL-8 mRNA levels were normalized against the housekeeping gene actin and are expressed as the fold increase in induction versus controls.

Data are shown as means \pm SD of 3 experiments performed in duplicate. Statistical differences within the experimental groups were calculated using ANOVA associated with the Bonferroni post test: ^a $p < 0.001$, ^b $p < 0.05$, ^c $p < 0.01$ significantly different vs. control group; ^{*} $p < 0.001$, [@] $p < 0.05$ significantly different vs. 60 μ M Oxy-mixture

Fig. 2. Evaluation of the colonic NADPH oxidase isoform (NOX1) activity in differentiated CaCo-2 cells treated with 60 μ M Oxysterol-mixture.

The effect of 30 min pre-treatment with 5 μ M NADPH inhibitor DPI on the synthesis of IL-6 (A) and IL-8 (B) was analyzed by ELISA, in the culture medium of CaCo-2 cells challenged with 60 μ M Oxy-mixture for 24 h or with solvent alone (Control), likewise in the presence of the chemical inhibitor. The cytokines' concentrations are expressed as pg/mg protein of IL-6 or IL-8 released. Data regarding both interleukin expression and synthesis, and NOX1 activation, are shown as means \pm SD of 3 experiments performed in duplicate.

C: NOX1 activity was evaluated in CaCo-2 cells after 1 h of pre-treatment with Sardinian wine extracts and 30 min of treatment with 60 μ M Oxy-mixture or solvent alone (Control). Protein levels were evaluated by densitometry and are reported as histograms showing percentage increase versus control (taken as 100%). A representative Western blot is shown.

Data regarding NOX1 activation are shown as means \pm SD of 3 experiments.

Statistical differences within the experimental groups were calculated using ANOVA associated with the Bonferroni post test: ^a $p < 0.001$, ^b $p < 0.05$, ^c $p < 0.01$ significantly different vs. control group; * $p < 0.001$, # $p < 0.01$, @ $p < 0.05$ significantly different vs. 60 μ M Oxy-mixture; ^d $p < 0.01$ significantly different vs. Vermentino wine extract alone.

Fig. 3. The effects of Sardinian wine extracts on IL-6 and IL-8 expression and synthesis in CaCo-2 cells treated with 60 μ M Oxysterol-mixture.

The expression of IL-6 (A) and IL-8 (B) was quantified by RT-PCR in differentiated CaCo-2 cells after 1 h of pre-treatment with Sardinian Wine extracts and 4 h of treatment with 60 μ M Oxy-mixture or solvent alone (Control). IL-6 and IL-8 mRNA levels were normalized against the housekeeping gene actin and are expressed as the fold increase in induction versus control.

The synthesis of IL-6 (C) and IL-8 (D) was analyzed by ELISA in the culture medium of CaCo-2 cells after 1 h of pre-treatment with Sardinian Wine extracts and 30 min of treatment with 60 μ M Oxy-mixture, or solvent alone (Control). The cytokines' concentrations are expressed as pg/mg protein of IL-6 or IL-8 released.

Data regarding both expression and synthesis are shown as means \pm SD of 3 experiments performed in duplicate. Statistical differences within the experimental groups were calculated using ANOVA associated with the Bonferroni post test: ^a $p < 0.001$, ^b $p < 0.05$ significantly

different vs. control group; ^c p<0.01 significantly different vs. Vermentino wine extract alone; * p<0.001, [@] p<0.05 significantly different vs. 60 μM Oxy-mixture.

Fig. 4. Effects of the principal polyphenolic components of Sardinian wine extracts on IL-6 and IL-8 production in CaCo-2 cells treated with 60 μM Oxysterols-mixture

The synthesis of IL-6 (A) and IL-8 (B) was analyzed by ELISA in differentiated CaCo-2 cells after 1 hour of pre-treatment with polyphenols and 24 h of treatment with 60 μM Oxy-mixture or solvent alone (Control). The polyphenols used for cell pre-treatment were: gallic acid (10μM), caffeic acid (10μM), catechin, (10μM), epicatechin (EPC) (10μM) and quercetin (1μM).

The cytokines' concentrations are expressed as pg/mg protein of IL-6 or IL-8 released. Data are shown as means ± SD of 3 experiments performed in duplicate. Statistical differences within the experimental groups were calculated using ANOVA associated with the Bonferroni post test: ^a p<0.001, ^b p<0.05 significantly different vs. control group; * p<0.001, [#] p<0.01, [@] p<0.05 significantly different vs. 60 μM Oxy-mixture.

Graphical Abstract

Dietary oxysterols induce intestinal inflammation through NOX1 activation. Low polyphenol doses protect from oxysterol-dependent ROS production as direct free radical scavengers; only high doses completely prevent oxysterol-induced inflammation targeting NOX1.

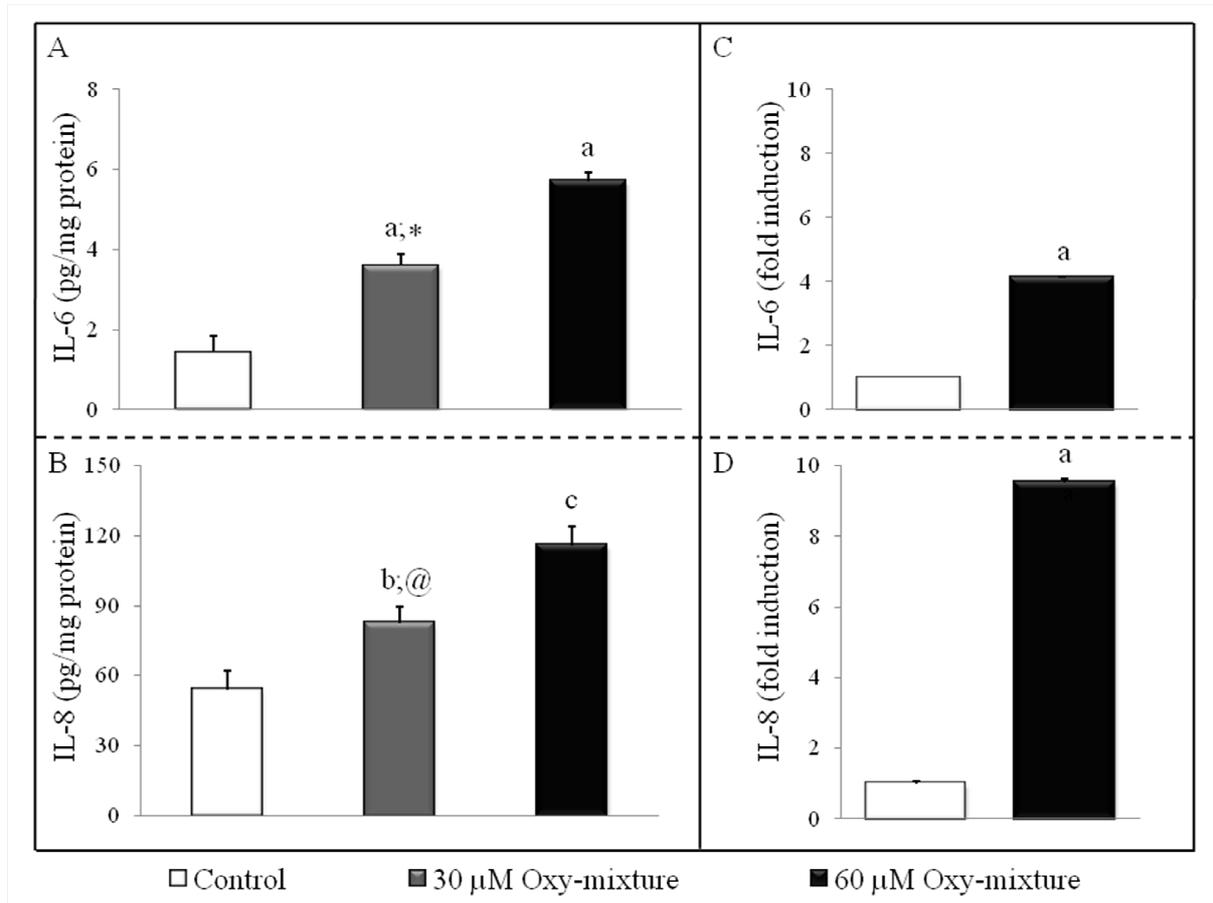


Figure 1

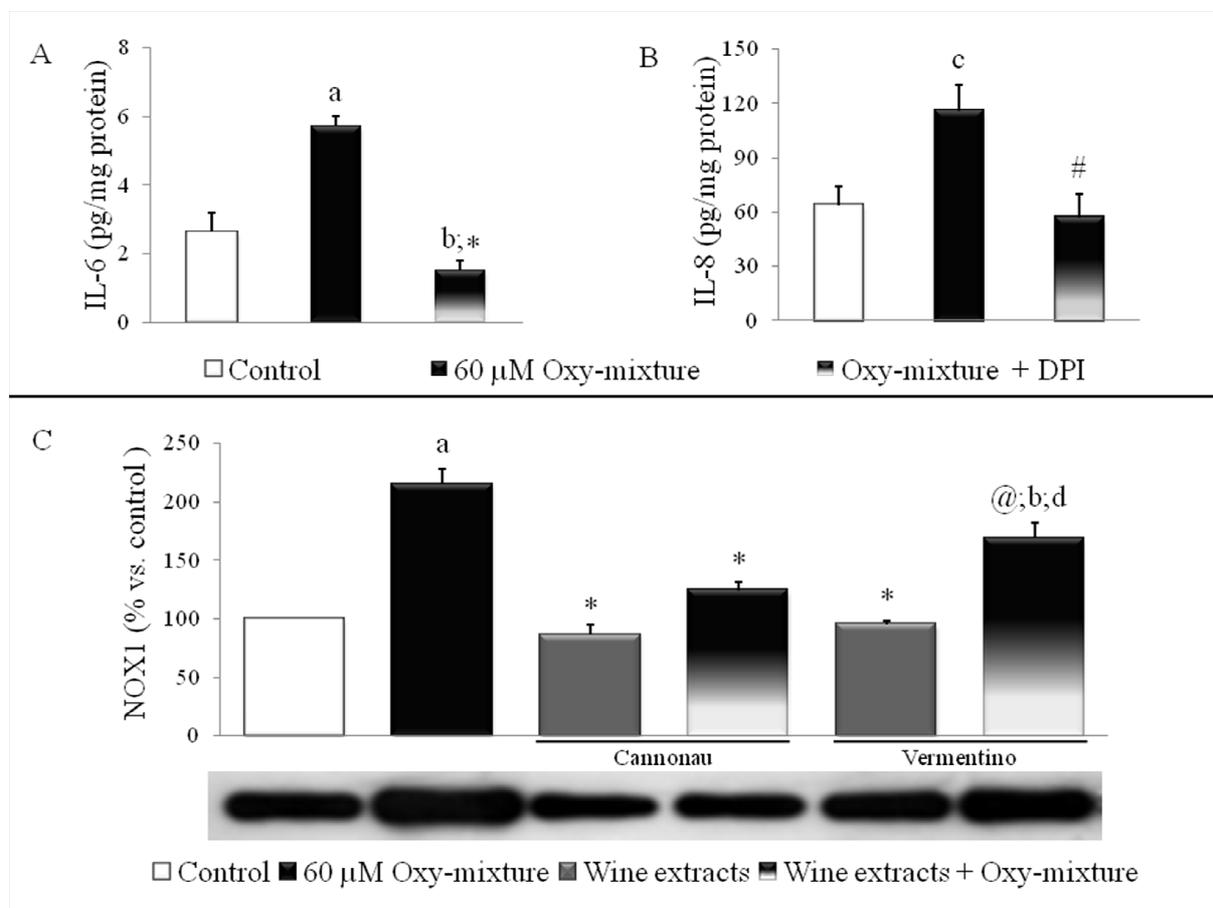


Figure 2

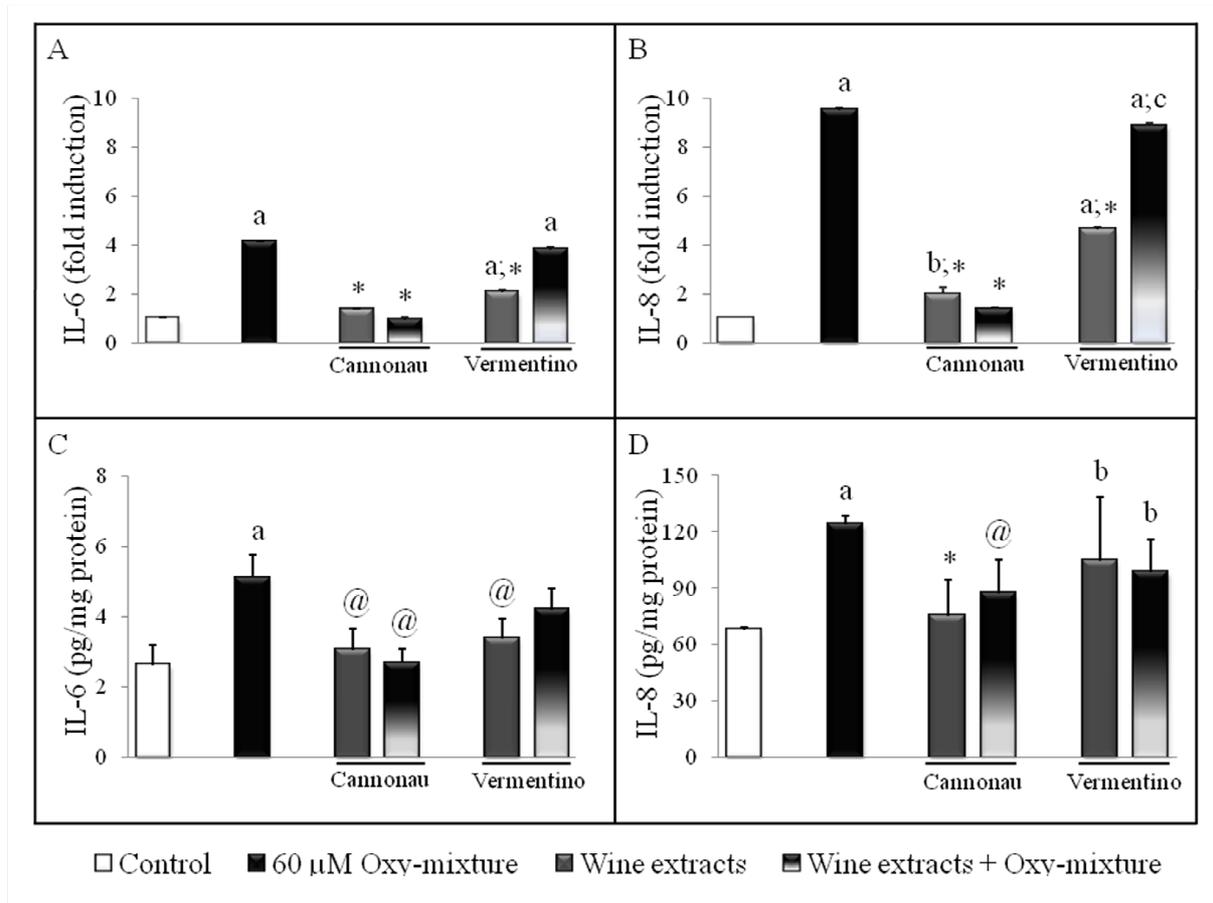


Figure 3

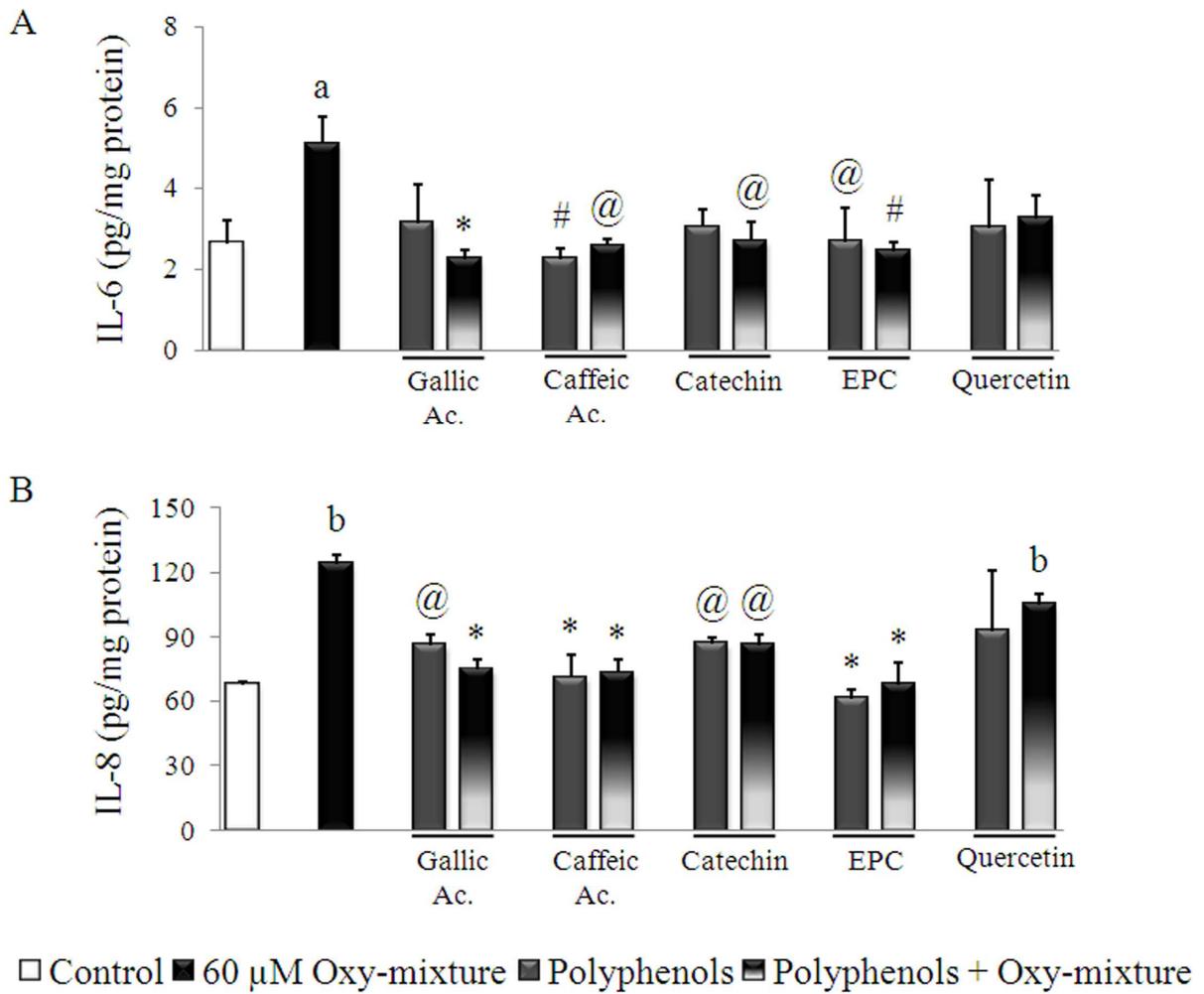
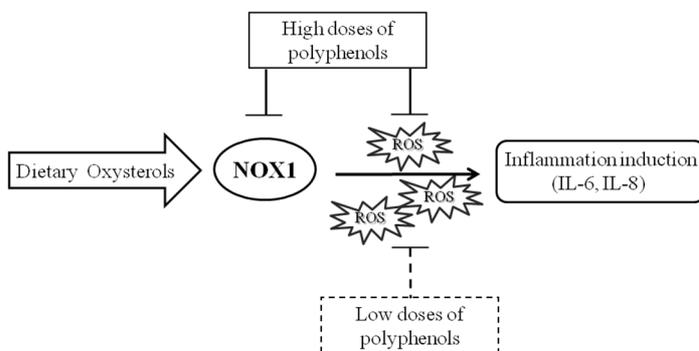


Figure 4



Graphical abstract