EFFECTS OF POLYPHENOLS AND OMEGA-3 PUFAS ON HEPATIC OXIDATIVE STRESS

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Effects of polyphenols and omega-3 PUFAs on hepatic oxidative stress

Doctoral Thesis

Directed by

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UNIVERSITAT ROVIRA I VIRGILI

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FEM CONSTAR que aquest treball, titulat "Effects of polyphenols and omega-3 PUFAs on hepatic oxidative stress" que presenta Anabel Fernández Iglesias per l'obtenció del títol de Doctor, ha estat realitzat sota la nostra direcció al Departament de Bioquímica i Biotecnologia d'aquesta universitat i que compleix els requisits per poder optar a Menció Europea.

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… a mi **Padre**,

a mi **Madre**,

a mi **Hermano**,

a **Leíto** …

A MI FAMILIA

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"Lo que gran parte de la ciencia moderna no advierte,

es que de poco sirve saber si no se piensa"

G.K. Chesterton

Esta frase la encontré un día de casualidad hace más de cuatro años y decidí escribirla en un *post-it* y engancharla en la pared de enfrente de mi mesa de despacho, de manera que cada día la he podido leer. Ha llegado el momento de resumir estos años en una frase y me he dado cuenta que no podía escoger otra que la que un día escribió *G.K. Chesterton*.

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Y los que siempre van a estar a mi lado son las personas a las que les dedico este trabajo; no sería la persona que soy ahora sin el apoyo incondicional de mi madre Inés

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A todos, muchas gracias.

Abbreviation list

4-HAE	4-hydroxyalkenals
4-HNE	4-hydroxy-2-nonenal
8-OHdG	8-Hydroxy-2'-deoxyguanosine
AA	Arachidonic acid
ARE	Antioxidant response element
AST	Aspartate aminotransferase
CAT	Catalase
Cu,Zn-SOD	Superoxide Dismutase 1
CVD	Cardiovascular diseases
DCFH-DA	2',7'-Dichlorofluorescin diacetate
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
EGCG	Epigallocatechin-3-gallate
EPA	Eicosapentaenoic acid
FFA	Free fatty acids
Gclc	Glutamate-cysteine ligase catalytic subunit
GPx	Glutathione Peroxidase
GR	Glutathione Reductasa
GSH	Reduced glutathione
GSPE	Grape seed proanthocyanidin extract
GSSG	Oxidized glutathione
GST	Glutathione S-transferase
H_2O_2	Hydrogen Peroxide
HO-1	Heme oxygenase-1
LA	Linoleic acid

LDH	Lactate dehydrogenase
LPO	Lipid hydroperoxide
MDA	Malondialdehyde
MetS	Metabolic syndrome
Mn-SOD	Superoxid dismutase 2
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoilum bromide
MUFAs	Monounsaturated fatty acids
n-3	Omega-3
n-6	Omega-6
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
Nrf2	Nuclear factor erythroid 2-related factor 2
NQO1	NADPH quinone oxidoreductase 1
ORAC	Oxygen Radical Absorbance Capacity
PUFAs	Polyunsaturated fatty acids
ROS	Reactive oxygen species
***tert*-BHP**	*tert*-Butylhydroperoxide
TBARS	Thiobarbituric acid reactive substances
TBST-T	Tris-Buffered saline with Tween
TG	Triglyceride

Abstract

Over the past years it has been described the important role of oxidative stress in the metabolic stress related manifestations, specifically in the obesity. It is well known the agents that reduce oxidative stress represent an important tool to also reduce the obesity-induced complications such as hepatic steatosis. In this sense, flavonoids are described as antioxidant molecules, due to their scavenging properties. Additionally, omega-3 PUFAs are considered effective in the treatment and prevention of cardiovascular diseases, although some studies propose that in conditions of oxidative stress, omega-3 PUFAs induce lipid peroxidation increasing the oxidative stress. Hence, the principal aim of this thesis was to study the antioxidant effects of flavonoids and n-3 PUFAs, as well as the possible additive effects of two compounds together. These studies were developed under several oxidative stress conditions (metabolic and chemical) both *in vitro* and *in vivo*. The experimental results obtained *in vivo* suggest that under an oxidative stress environment such as: obesity (genetic and dietary), and lipidic postprandial state; the flavonoids are able to exert a positive modulation on GSH metabolism. When animals were treated with n-3 PUFAs in a postprandial state condition or in diet-induced obesity model, the results also showed an oxidative stress improvement evidenced, and homeostatic redox balance. Interestingly, the combination of polyphenols and n-3 PUFAs modifies hepatic antioxidant status in lipidic postprandial state and could be an interesting option for the prevention of the transient redox unbalance related to a lipidic postprandial state. On the other hand, GSPE and DHA-OR shows different effects on oxidative stress in diet-induced obesity. Otherwise, the *in vitro* results suggest that DHA is not always beneficial for cells and can be considered a double-edged sword in terms of benefits and risks, especially for situations of sustained oxidative stress conditions. However, the combination with EGCG could be an appropriate strategy to reduce the risks related with DHA supplementation.

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1 INTRODUCTION

1.1 Oxidative stress

1.1.1 Reactive Oxygen Species

Reactive oxygen species (ROS) are ubiquitous, highly reactive, short-lived derivatives of oxygen metabolism produced in all biological systems that react with surrounding molecules at the site of formation [1]. ROS are formed from incomplete reduction of oxygen during normal respiration in all aerobic organisms. It includes free radicals containing one or more unpaired electrons and non-radicals species. Additionally, ROS are also generated in metabolic processes by a large number of enzymes such as xanthine oxidase, nitric oxidase synthetase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, cyclooxygenase, lipoxygenases and myeloperoxidase. In addition, mitochondria is the major source of ROS in mammals under physiological conditions [2]. Moreover, exogenous sources of ROS such as ultraviolet light, ionizing radiation (IR), chemotherapeutic, and chemical agents in food and the environment [3, 4] contribute to cellular oxidative damage (Figure 1).

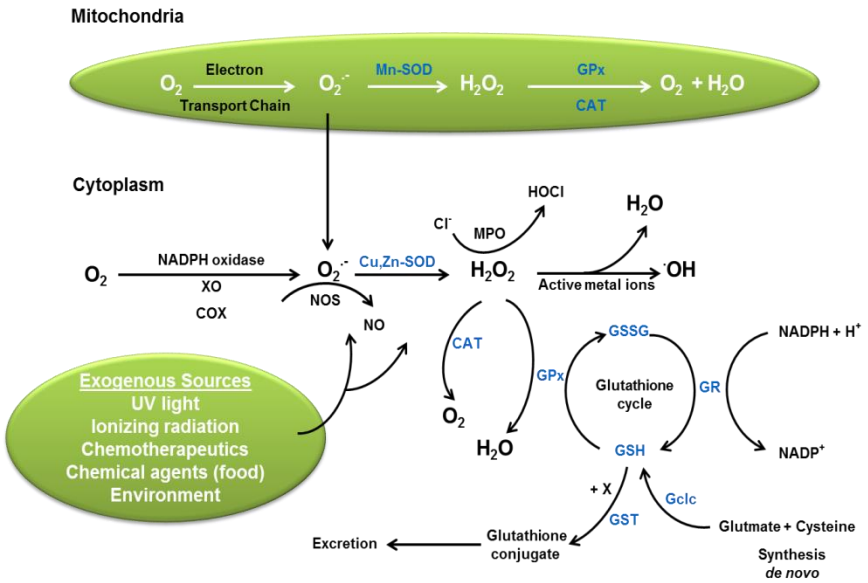

Figure 1. ROS production and cellular antioxidant defense

At low levels, ROS play an important role in human physiological processes, including intracellular messaging, cellular differentiation, growth arrestment, apoptosis, immunity and defense against microorganisms [1, 5]. However, at high concentrations

they react readily with proteins, lipids, carbohydrates and nucleic acids, often inducing irreversible functions or even complete destruction [5].

ROS include molecules with differing degrees of reactivity of such biomolecules. ROS are present in the form of free radicals or non-radicals; however, they all contain oxygen in an activated form (Figure 2).

Reactive Oxygen Species (ROS)			
Radicals		**Non-radicals**	
Superoxide anion	$O_2^{\cdot -}$	Hydrogen peroxide	H_2O_2
Hydroxyl	$^{\cdot}OH$	Ozone	O_3
Alcoxyl	$RO^{\cdot}$	Singlet oxygen	$^1O_2$
Peroxide	$ROO^{\cdot}$	Hypochlorous acid	HClO

Figure 2. Reactive oxygen species molecules

Therefore, oxidative stress is defined as an imbalance between the production of ROS and cellular antioxidant defense [6]. Overproduction of ROS, a depression of the antioxidant systems or both occurs during stimulated disease conditions [5].

1.1.2 Oxidative stress damage

It was originally accepted that ROS exert deleterious effects by oxidizing biologically essential molecules such as lipids, proteins, carbohydrates and deoxyribonucleic acid (DNA).

1.1.2.1 Lipid peroxidation

ROS can react with the unsaturated bonds in membrane lipids, causing lipid peroxidation [7]. Common markers of lipid peroxidation are malondialdehyde (MDA), which is usually measured as thiobarbituric acid reactive substances (TBARS), and 4-hydroxy-2-nonenal (4-HNE).

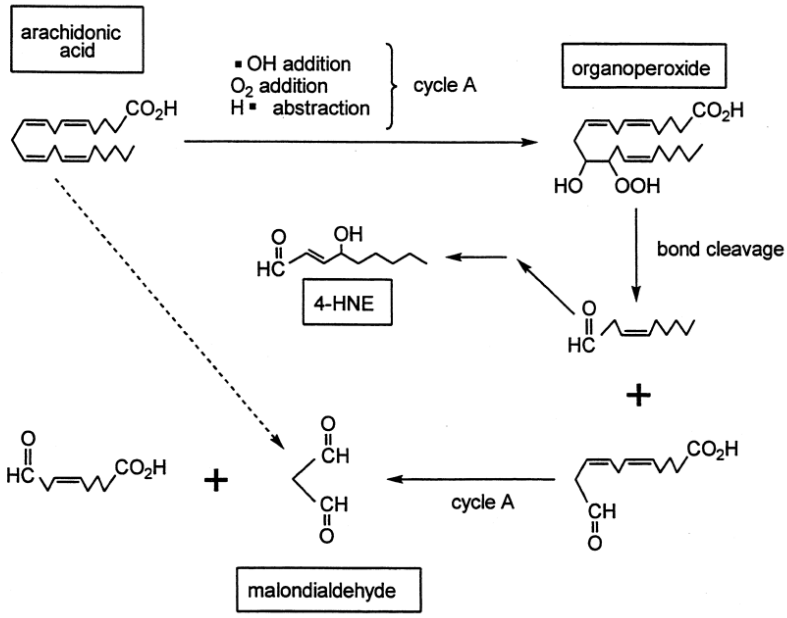

Figure 3. Lipid peroxidation reaction [8]

Lipid hydroperoxides (LPO), the main intermediates of the peroxidative reaction, accumulate in the bilayer and induce changes in the structure and biophysical organization of membrane lipid components. In fact, as a consequence of the oxidation, phospholipid fatty acids in the membrane suffer significant modifications, including the loss or rearrangement of double bonds and, in a later stage, the reductive degradation of lipid acyl chains. It is clear that all these phenomena lead to alterations of membrane physic/chemical properties. In consequence, the loss or modification of important membrane functions could have impact on human health. The main biophysical consequences of lipid membrane peroxidation have been well characterized and include changes in membrane fluidity, permeability, alteration of membrane thermotropic phase properties and membrane protein activity [9, 10]. Furthermore, lipid peroxidation products have been shown to be mutagenic and carcinogenic. The reactive carbonyl compounds, the secondary products of LPO, modify proteins and DNA bases [11]. Thus LPO have been implicated in numerous disorders and diseases such as cardiovascular diseases (CVD), cancer, neurological disorders, and aging [12].

1.1.2.2 Protein oxidation

Proteins that contain sulfur-containing amino acids are the main targets of ROS and LPO, resulting in cross-linking, inactivation, and denaturation of the target molecules [13].

There are numerous different types of protein oxidative modifications. Oxidative attack of amino acyl moieties, such as Lys, Arg, Pro, and Thr, induces formation of carbonyl groups (aldehydes and ketones) on the side chains. To avoid their toxic accumulation, carbonylated proteins are degraded through the action of the 20S proteasome in the cytosol. The two amino acids that are the most prone to oxidative attack are Cys and Met, both of which contain susceptible sulfur atoms [14].

Due to the numerous types of protein oxidative modification, there are at least as many different methods for detection and quantification of those modifications. The most commonly protein oxidation assays in biological samples are the protein carbonyls. These moieties are chemically stable, which is useful for both their detection using dinitrophenylhydrazine (DNPH) coupled assays such as Western blot and ELISA among others [15].

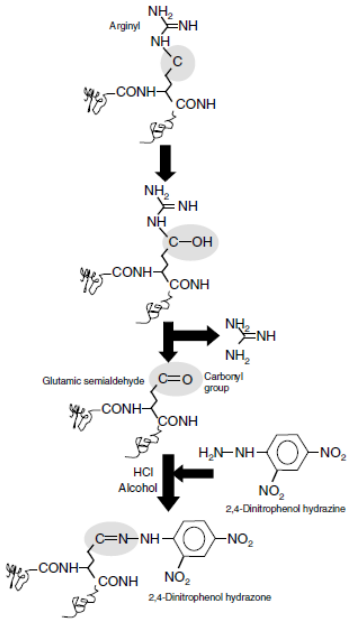

Figure 4. Carbonylation and derivatization of a protein amino-acid side chain. For detection, the carbonyl group, in this case glutamic semialdehyde, is subsequently derivatized by 2,4-dinitrophenol hydrazine. The resulting protein 2,4-dinitrophenol hydrazone can be detected by specific monoclonal or polyclonal antibodies.

1.1.2.3 DNA oxidation

DNA is highly susceptible to ROS and ROS-derived LPO attack, which generate a variety of modified bases, base-free sites, strand breaks and DNA-protein cross-links. The accumulation of such DNA altered products are intimately related with the development of cancer disease as well as ageing process [16].

One of the major forms of oxidative DNA damage is 8-Hydroxy-2'-deoxyguanosine (8-OHdG), and is commonly analyzed as an excellent marker of DNA lesions. It is well known that 8-OHdG is implicated in aging and the development of neurodegenerative disease, such as Alzheimer's disease [17].

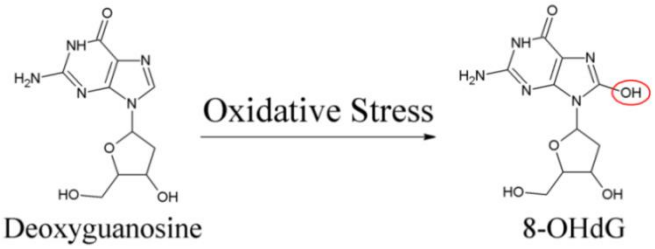

Figure 5. DNA oxidative damage. Formation of 8-OHdG by oxidative stress [17]

High-performance liquid chromatography with electrochemical detection is a highly sensitive and selective method for detecting 8-OHdG. Moreover, the quantitation of 8-OHdG in biological fluids is non-invasive [18].

1.1.3 Oxidative stress molecular pathways

The role played by ROS in transcriptional regulation is of critical importance and is able to affect vital processes such as glucose homeostasis, inflammation, cellular lifespan, and multiple aging-related diseases including cancer. Cells have developed complex signaling cascades to detoxify potentially harmful substances and maintain cellular redox homeostasis. One of these signaling cascades is responsible for the induction of cytoprotective and detoxifying enzymes consisting of phase I (cytochrome P450s) and phase II (detoxifying and antioxidant proteins) enzymes. The coordinated functions of these protective mechanisms are usually able to restore the homeostatic cellular state by the prevention of damage on the main sensitive cellular components (proteins, lipids and DNA). The phase I reaction is mediated by cytochrome P450

monooxygenase systems, such as CYP1A1 and CYP1A2, which modify xenobiotics through oxidation and reduction. The phase II enzymes, such as GST and HO-1, are regulated by the nuclear factor erythroid 2-related factor 2 (Nrf2) [19].

Nrf2 is a transcription factor of the cap ''n'' collar basic region leucine zipper (cnc bZip) family, controlling expression of various cytoprotective antioxidant enzymes. This transcription factor is found in all tissues, but is activated in response to a wide range of oxidative and electrophilic stimulation, including ROS and some chemicals agents. It has been demonstrated that Nrf2 modulates the expression of antioxidant enzymes through interaction with antioxidant response element (ARE). Under normal physiological conditions and a low oxidative stress environment, Nrf2 is confined to the cytoplasm linked with the suppressor protein Keap1, and is degraded by the ubiquitin proteasome pathway. Oxidative and electrophilic stress factors dissociate the Nrf2-Keap1 complex, thereby promoting the release and translocation of Nrf2 into the nucleus [20]. Once in the nucleus, Nrf2 heterodimerizes with a variety of transcriptional regulatory proteins, including members of the activator protein-1 family (Jun and Fos), and the small Maf family of transcription factors. These protein complexes bind to the ARE located upstream of the promoter region of a battery of antioxidant genes such as catalase, heme oxygenase-1 (HO-1), NADPH quinone oxidoreductase (NQO1), glutathione S-transferase (GST), glutamate cysteine ligase catalytic subunit (Gclc), glutathione peroxidase (GPx) and peroxiredoxin I [21].

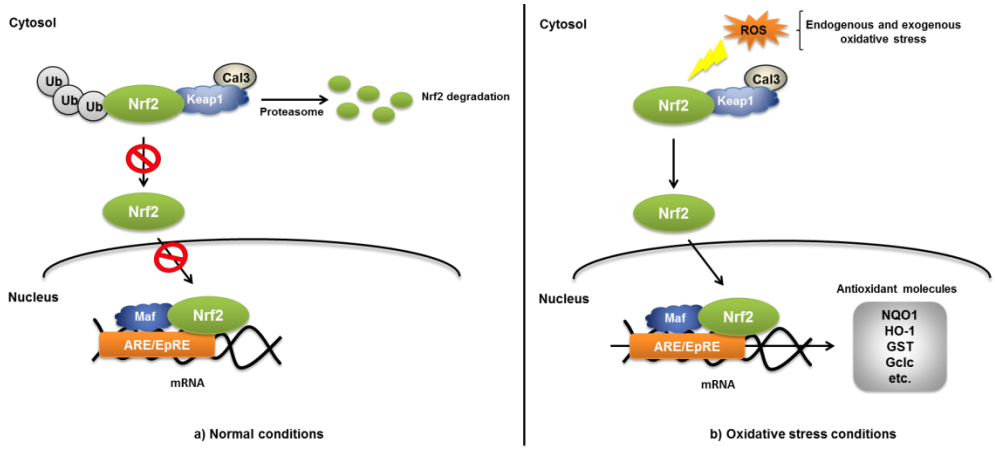

Figure 6. Nrf2 pathway: a)Nrf2 in redox homeostatic balance is linked with Keap1 in the cytosol; b) Nrf2 is translocated to the nucleus under oxidative stress conditions.

1.1.4 Role of oxidative stress in metabolic syndrome/obesity

Obesity is becoming a worldwide epidemic that is being increasingly recognized as a worsening factor in a number of chronic pathologies [22]. Metabolic syndrome (MetS) is defined as a clustering of interconnected factors that directly increase the risk of CVD and diabetes mellitus type 2, such as obesity, atherosclerosis, hypertension, insulin resistance and inflammation [23]. Major reasons of this growing epidemic involve changes in dietary patterns and physical inactivity. In addition, the environmental changes and genetic factors that modify individual susceptibility to these environmental factors, are the major determinants of this epidemic [24].

Emerging evidence suggests that oxidative stress play an important role in the pathophysiology of MetS-related manifestations [25]. In a pathological state such as the MetS, an increased oxidant capacity coupled with decreased antioxidant capacity creates an unbalanced environment that results in oxidative stress [26]. Moreover, obesity in children, without any other MetS components, has been repeatedly correlated with increased oxidative stress and endothelial dysfunction [27].

In obesity several enzymes (NADPH oxidase and xanthine oxidase) that produce ROS are activated. Chronic obesity leads to oxidative damage and stress (Figure 7) [28] .

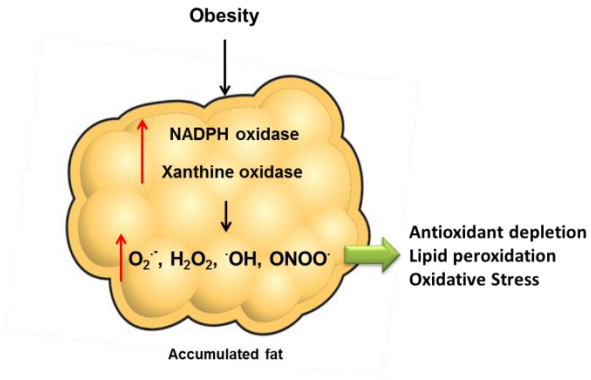

Figure 7. Obesity and oxidative stress relationship [28]

Recently, a role of Nrf2 in obesity has been discovered sing mainly the Nrf2 knockout (Nrf2-KO) mice as a model. It has been shown that deletion of Nrf2 protected mice from diet-induced obesity and insulin resistance [29]. For instance, Furukawa et al. examined the relationship between lipid peroxidation (TBARS and 8-epi-PGF2a) and

obesity in patients with the MetS. Both body mass index and waist circumference were directly correlated with plasma TBARS and urinary 8-epi-PGF2a. Based on these findings and other results developed in obesity animal models, the authors concluded that excessive accumulation of fat leads to enhanced production of ROS in adipocytes and systemic tissues [30]. Therefore, the obesity-induced ROS production stimulates the translocation of Nrf2 into the nucleus as a defense mechanism against oxidative stress.

1.1.4.1 Animal models of obesity

1.1.4.1.1 Cafeteria diet

Cafeteria diet is an accurately diet model that reflects the variety of highly energy dense foods that are prevalent in Western society and are associated with the current obesity pandemic. In this model, animals are allowed to free access to standard chow and water while cafeteria diet (bacon, cookies, cheese, milk, sugar and biscuits) is concurrently offered *ad libitum*. This diet promotes rapidly weight gain, obesity, multiorgan dysfunctions and pathologies close to the modern human condition of human obesity [31]. This cafeteria diet is a robust model of MetS that could induce a significant 3-fold increase in body weight gain after 15 weeks compared with standard diet [32], although it is well known that after 3 weeks of cafeteria diet administration the rats body weight gain could be significant different [33].

1.1.4.1.2 Zucker fatty rats (fa/fa)

Zucker fatty rat is the main genetic obese model used for obesity studies. The animals present dyslipidemia, mild glucose intolerance, hyperinsulinemia, hypertension, hepatic steatosis and low-grade inflammation, manifestations similar to those that define human MetS. Additionally Zucker fatty rats develop oxidative stress. Oxidized lipids are elevated in serum, urine and liver and plasma antioxidant defense mechanisms such as glutathione peroxidase are compromised [34, 35].

The fatty Zucker strain has a spontaneous mutant gene (fa or fatty) that affects the action of the adipocyte peptide hormone leptin, a key element in the regulation of food intake through the inhibition of the release of hypothalamic neuropeptide Y [36].

1.1.5 Role of oxidative stress in postprandial state

Western diets, which contain foods high in carbohydrates and saturated fats, are associated with the risk of suffering diseases. The postprandial oxidative stress caused

by the intake of this type of diet may be a key factor in the development of conditions such as diabetes, dyslipidemia, hypertension, inflammation, etc. The postprandial state is a dynamic period, where the absorbed substrates are metabolized and ROS are generated, which may influence long-term health status [37]. Moreover, postprandial hyperlipidemia is a well-defined risk factor for atherosclerosis partially due to the increase of plasma LPO produced in the postprandial phase [38].

In addition, Mediterranean diet has been proposed as healthy dietary model in which the main underlying mechanism for its favorable effects is the modulation of the oxidative stress. Components of this diet include monounsaturated fatty acids (MUFA), α-tocopherol, phenolic compounds, phytosterols, and other antioxidants [39]. Furthermore, it has been studied that some polyphenols present in grape seed extract prevented the plasmatic rise of hydroperoxides produced in a postprandial state and, in consequence, net plasma antioxidant capacity increases [38].

1.1.6 Liver

The liver has a wide range of physiological functions, including detoxification of endogenous and xenobiotic compounds, homeostatic regulation of the plasma concentration of a multitude of metabolites, synthesis of most plasma proteins, bile formation and hormone production. Overload of the liver's metabolic capacity as in drug abuse or inappropriate diets may give rise to long-term silent disease progression such as accumulation of triglycerides in the liver, changes in the plasma level of lipoproteins, enhanced intracellular levels of reactive oxygen species and changes in the bile composition [40].

Liver is the main tissue in controlling the whole body energy homeostasis by its ability to metabolize glucose and fatty acids. When energy intake is abundant, mammals preferentially use carbohydrates to generate adenosine triphosphate (ATP) and glucose, after replenishing glycogen stores, is converted to fatty acids (lipogenesis) for use in the synthesis and storage of TG in white adipose tissue. Although white adipose tissue functions essentially as a limitless reservoir to accumulate TG, the liver is also able to store significant quantities of lipids in conditions associated with prolonged excess energy consumption or impaired fatty acid metabolism manifesting hepatic steatosis. In fasted states, when glucose availability and insulin levels are low, there is a depletion of hepatic glycogen stores and a reduction in fatty acid production. Under these conditions, TGs stored in adipose tissues are hydrolyzed to free fatty acids and mobilized into plasma to reach the liver. In the liver, they undergo oxidation and are converted to ketone bodies to be used as fuel by extrahepatic tissues [41].

1.1.6.1 Hepatocytes

Hepatocytes account for about 60% of the liver in terms of cell number and for 80% of liver volume [42]. They are the principal site of the metabolic conversions underlying the diverse physiological functions of the liver. Accordingly, hepatocyte metabolism integrates a vast array of differentially regulated biochemical pathways and is highly responsive to changes in blood composition evoked by special diets, overnutrition, starvation or enhanced physical activity [43].

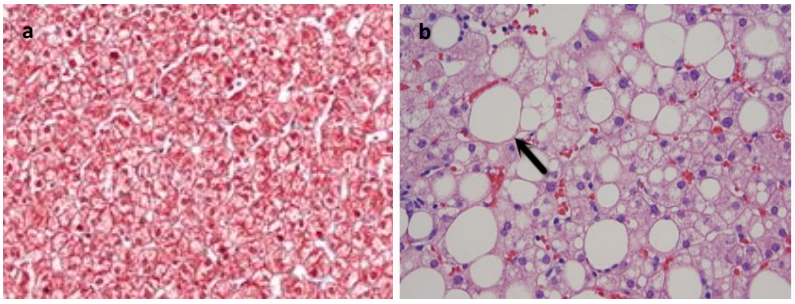

Figure 8. Normal (a) and steatotic (b) hepatocyte cells.
The black arrow illustrates the fat deposited.

1.1.6.2 Oxidative stress and hepatic steatosis

Cellular lipid overload or lipotoxicity has been linked to the dysfunction of multiple organs, including heart [44], endocrine pancreas [45], skeletal muscle, kidney [46] and liver [45]. Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver diseases worldwide and is the result of an imbalance between the hepatic uptake of free fatty acids (FFAs), TG synthesis, and excretion. The causes of NAFLD are varied and include obesity, age, alcoholism and diabetes mellitus [47]. Hepatic steatosis can impair liver function and increase the risk of other diseases. To date, there is a growing body of evidence suggesting that NAFLD is associated with an increased risk of incident CVD [48]. When hepatic steatosis occurs, TG can be transported to the blood in the form of very-low density lipoprotein [49], which is related to CVD. Therefore, NAFLD has been the subject of much attention and research because of the serious health risks of this disease [50].

Intracellular lipid accumulation is the main pathological characteristic of a human liver with NAFLD [51]. FFAs are the main substrates of synthetic TG in hepatocytes. Excessive ingested fat and MetS conditions, such as obesity, insulin resistance, and type 2 diabetes can result in increased FFA levels in the blood. In MetS, the lipolysis of lipids in adipose tissue increases. As a result, the delivery of FFA to the liver is amplified.

NAFLD is a slowly progressive condition and represents a spectrum of varying severity of liver disease, ranging from simple steatosis to co-existent inflammation with hepatocyte ballooning and necrosis, variable grades of fibrosis, and ultimately cirrhosis and an increased risk of hepatocellular carcinoma (Figure 9).

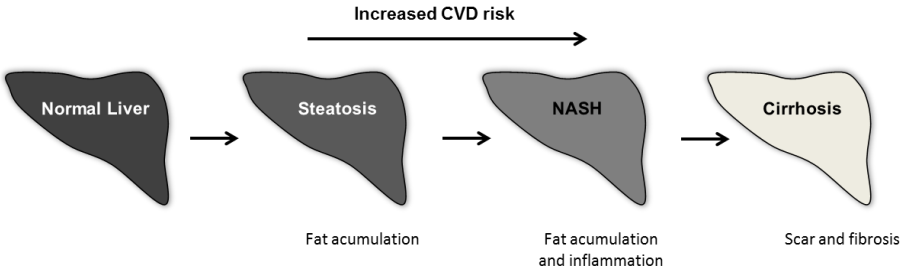

Figure 9. Progressive chronic liver disease [52]

Oxidative stress has often been linked to NAFLD, and may be a final common mechanistic occurrence on the lipotoxicity of free fatty acids (FFAs) [24, 53].

There is considerable evidence that the accumulation of lipids in the liver and the presence of ROS promotes lipid peroxidation, and in consequence produces oxidative stress [35]. Thus, ROS are essential in the development of liver fibrosis. Studies have indicated that high-fat diet resulted in more severe NAFLD in Nrf2-null mice than wild-type mice [54]. This indicates that Nrf2 plays a pivotal role in protection against the development of NAFLD and could be a useful therapeutic target to inhibit this development [55]. Other authors showed that in NAFLD the upregulation of Nrf2 is correlated with hepatic steatosis and inflammation. Hence, activators of Nrf2 gene may be a promising treatment for MetS and its complication such as NAFLD [56].

Importantly, randomized clinical trials have shown promising results to attenuate the progression of NAFLD: (a) using lifestyle modification [57]; and (b) nutritional supplements (antioxidants molecules) [58].

1.1.7 Antioxidant defense

To control the formation of ROS and to repair the cellular oxidative damage, the body's defense mechanisms possess a plethora of antioxidants. The levels of antioxidants in oxidative stress rise to scavenge and inactivate radicals and reactive metabolites. The antioxidants may derive from our diet or are formed endogenously

[59], and the antioxidant defense mechanism can be divided into enzymatic and non-enzymatic.

1.1.7.1 Enzymatic antioxidants

1.1.7.1.1 Superoxide dismutase

Superoxide anion radicals ($O_2^{\cdot-}$) are the major ROS generated in mitochondria, and they are involved in producing several potentially damaging species. The superoxide radical is rapidly dismutated by three types of superoxide dismutase (SOD, EC 1.15.1.1) isoenzymes Cu,Zn-SOD, Mn-SOD and extracellular SOD (EC-SOD). Whereas Mn-SOD and EC-SOD are localized in the mitochondrial matrix and on the cell surface, respectively, Cu,Zn-SOD resides in the cytosol [60].

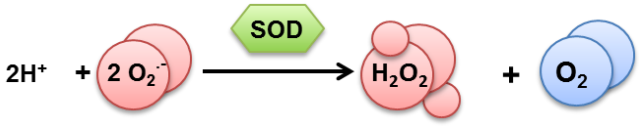

Figure 10. Superoxide dismutase reaction

1.1.7.1.2 Catalase

Catalase (CAT) enzyme (EC 1.11.1.6) is the main regulator of hydrogen peroxide (H_2O_2) metabolism. The enzymatic function of catalase has several unusual features. Its reaction with H_2O_2 is of first order and depends entirely on the concentration of H_2O_2. Hydrogen peroxide is formed by pathways such as superoxide enzymes, ROS, and human tumor cells. H_2O_2 appears to modulate the inflammatory process by regulating the expression of adhesive molecules, controlling cell proliferation and apoptosis and modulating platelet aggregation. However, at high H_2O_2 concentrations, due to a deficiency of catalase, especially when reacted with redox-active metal ions such as iron or copper, yields the highly reactive hydroxyl radical in the Fenton and Haber-Weiss reaction. This radical is responsible for injury in the cell membrane, mitochondrial electron transport, homocysteine metabolism, and DNA [61].

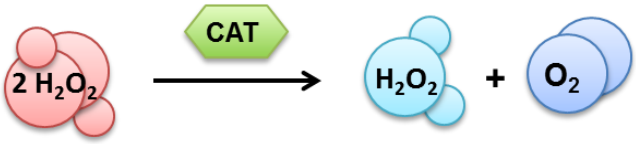

Figure 11. Catalase reaction

1.1.7.1.3 Glutathione Peroxidase

Glutathione peroxidase (GPx, EC 1.11.1.19) catalyzes the reduction of a variety of hydroperoxides such as H_2O_2 and lipid peroxides (ROOH) using reduced glutathione (GSH) as a cofactor that simultaneously is oxidized to GSSG.

Figure 12. Glutathione peroxidase reaction

There are at least five GPx isoenzymes distributed in different cellular compartments. GPx1 is located in cytosol and mitochondria and it is predominantly present in erythrocytes, liver and kidney. Cytosolic GPx2 and extracellular GPx3 are poorly detected in most tissues except for the gastrointestinal tract and kidney respectively. However, cytosolic and membrane GPx4 is highly expressed in renal epithelial cells and testes [62]. Finally, GPx5 is expressed specifically on mouse epididymis as De Haan et al. described [63].

1.1.7.1.4 Glutathione Reductase

Glutathione reductase (GR, EC 1.6.4.2) regenerates GSH from GSSG at the expense of reduced NADPH, thereby forming a closed system (redox cycle) as illustrated in Figure 1. Under normal conditions, GR is quite effective at maintaining most cellular GSH in its reduced state (more than 98%). However, under severe oxidant stress the ability of the cell to reduced GSSG may be overwhelmed, leading to its accumulation within the

cytosol. Nevertheless, GSSG may also react with cellular protein sulfhydryls via a mixed disulfide reaction, a process that can result in impaired protein function [64].

Figure 13. Glutathione reductase reaction

1.1.7.1.5 Glutathione-*S*-Transferase

GSH *S*-transferases (GST, EC 2.5.1.18) are a family of multifunctional enzymes present in the cytosol of most cells as homodimeric or heterodimeric proteins. Four different classes of mammalian cytosolic GST have been identified and named: α, μ, π and τ [65, 66]. These enzymes catalyzes the reaction of xenobiotic compounds with the –SH group of glutathione, thereby neutralizing their electrophilic sites and rendering the products more water-soluble. Glutathione conjugates are further metabolized by cleavage of the glutamate and glycine residues, followed by acetylation of the resultant free amino group of the cysteinyl residue, to produce the final product, the mercapturic acid to be excreted [67].

Figure 14. Glutathione-*S*-transferase reaction

1.1.7.2 Non-enzymatic antioxidants

The non-enzymatic defenses are GSH, metal-binding proteins and several exogenous antioxidants such as vitamins among others.

1.1.7.2.1 Glutathione

Glutathione performs a variety of important physiological and metabolic functions in all mammalian cells, including the detoxification of free radicals, metals, and other electrophilic compounds. Although its antioxidant nature and intrinsic ability of direct ROS removement, the GSH main function is to maintain enzymes and other cellular

compartments in a reduced state. One important detoxification mechanism involves the binding of GSH to electrophilic chemicals and the export of the resulting GSH S-conjugates from the cell. The binding of GSH to these compounds have several important roles: (a) it serves to limit and regulate the reactivity of the chemicals; (b) it facilitates their membrane transport and elimination from the cell and organism; and (c) in some cases, it leads to the formation of essential biological mediators.

GSH is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) and it is the most abundant non-protein thiol in almost all aerobic species, occurring at intracellular concentrations of 0.5 to 10 mM. In contrast, extracellular GSH concentrations are usually 3 to 4 orders of magnitude lower. Under oxidative stress conditions, GSH is transformed to oxidized glutathione (GSSG). Therefore, the GSH/GSSG ratio is often used as a measure of cellular toxicity and oxidative stress and appears to be increased in many diseases such as NAFLD progression, indicating depletion of GSH [64, 68].

Besides the GSH regeneration by the action of the GR enzyme, GSH is also synthesized *de novo* in all mammalian cells and the liver is a major site of biosynthesis [69]. The synthesis takes place in the cytosol in a two-step reaction. The γ-glutamylcysteine ligase (Gcl) catalyzes the first step where an amide linkage is formed between cysteine and glutamate requiring two moles of ATP. Then, GSH synthetase enzyme catalyzes the condensation of gamma-glutamylcysteine and glycine, to form glutathione [64] (Figure 15).

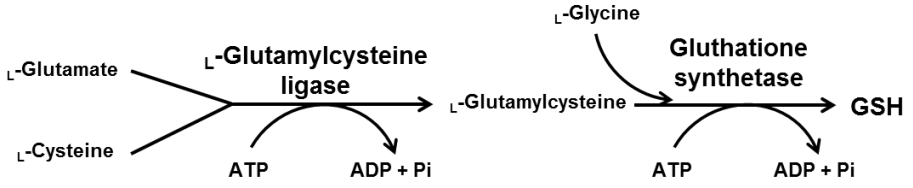

Figure 15.Glutathione synthesis *de novo*.

1.1.7.2.2 Metal-binding proteins

Thioredoxin scavenges oxygen radicals and regulates redox-sensitive molecules. Transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein-1 (AP1) also require thioredoxin reduction for DNA binding. In NASH, thioredoxin serum levels are significantly elevated compared to steatosis and correlated with the severity of hepatic injury [70].

Lactoferrin is a member of the transferrin family of non-heme iron binding glycoproteins. Hereby it protects the liver fromoxygen radicals catalyzed by free iron [71].

1.1.7.2.3 Vitamins

Vitamins as ascorbic acid (C) and α-tocopherol (E) are free-radical scavengers. α-tocopherol is the most active form of vitamin E and a powerful antioxidant, mainly against lipid peroxidation. It cooperates with vitamin C in a cyclic-type reaction. Although α-tocopherol is located in membranes and ascorbic acid in aqueous phases, they meet at the surface of membranes. In the process α-tocopherol gets converted to energetically stable α-tocopheryl radicals by donating hydrogen to lipids or lipid peroxyl radicals. Vitamin C then regenerates the oxidized α-tocopherol from the α-tocopheryl radicals in membranes and lipoproteins. Along with acting as a reducing agent for α-tocopherol, vitamin C is a donor anti-oxidant able to scavenge for reactive radicals and to convert ROS in poorly reactive products as well as to reduce iron [59].

1.2 Antioxidant bioactive compounds

Bioactive compounds present as natural constituents in food provide health benefits beyond the basic nutritional value of the product [72]. Bioactive substances are also referred to nutraceuticals, a term that reflects their existence in the human diet and their biological activity. Moreover, diet is a major source for several essential molecules that act in conjunction with antioxidant enzymes providing protection from the ROS [13].

1.2.1 Polyphenols

Phytochemicals refers to chemical compounds that occurs natural in plants that have been identified as having preventive effect against specific diseases. Phenolic compounds are major phytochemical class, and include the broad term polyphenols, meaning a molecule with one or more phenolic groups [73]. Flavonoids are one of the most ubiquitous groups of compounds among secondary plant products. It has been identified more than 4,000 structural variations of flavonoids, most of which possess multiple physiological functions in humans (antioxidant, anticarcinogenic, anti-inflammatory and, CVD and MetS related diseases prevention [74]). Polyphenols are present in fruits, vegetables and beverages such as tea and red wine [74].

1.2.1.1 Chemistry and classification

Flavonoids are a subclass of phenolic compounds. The structure of the flavonoids consists of three phenolic rings referred to as the A, B and C (Figure 16). The benzene ring A is condensed with a six-member ring (C), which in the 2-position carries a phenyl benzene ring (B). Depending on the oxidation level on the C-ring, flavonoids are divided into subclasses which include anthocyanidins, flavanols, flavones, flavonols, flavanones and isoflavones [75].

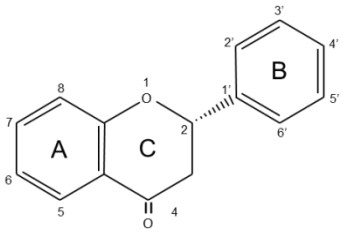

Figure 16. Flavonoid basic structure

Flavanols exist in both the monomer form (catechins) and the polymer form (proanthocyanidins). Catechin and epicatechin form are the main flavanols in fruit, whereas gallocatechin, epigallocatechin and epigallocatechin gallate (EGCG) are found in certain seeds of leguminous plants, in grapes, and more importantly in tea [76].

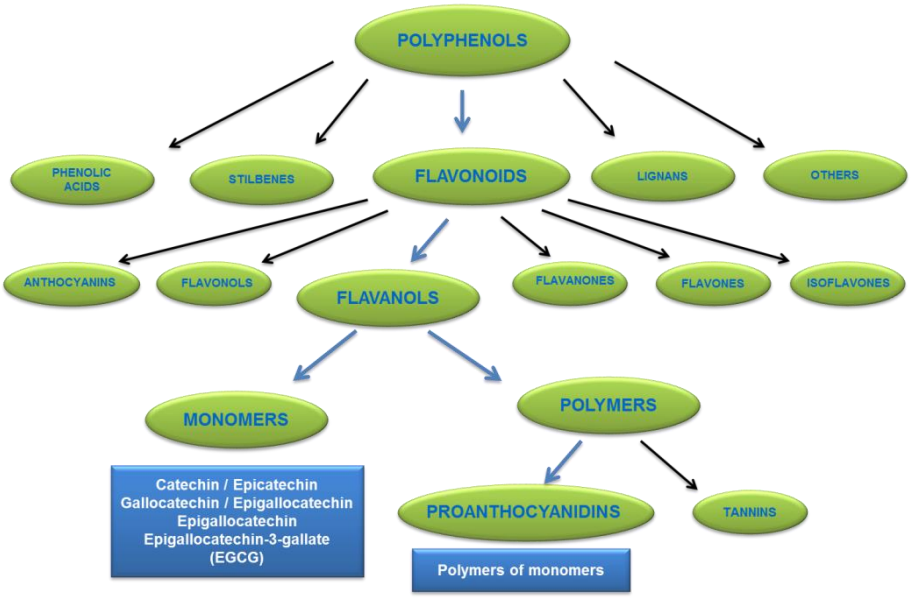

Figure 17. Polyphenols classification

Proanthocyanidins are the oligomeric or polymeric forms of favanols. The different oligomers ranging between 2 and 10 units are considered proanthocyanidins, while further polymerized forms are named tannins [77].

1.2.1.2 Polyphenols bioavailability and metabolism

Only partial information is available on the quantities of polyphenols that are consumed daily throughout the world [76]. One reason is because the phenolic content of fruit, vegetables and other plant foods varies considerably, not only between different types but also between cultivars of the same type and even depend on growing conditions and the time of harvest [77]. Various authors have noted a high variability in polyphenol intake, for example, in Spain the total consumption of catechins and proanthocyanidins dimers and trimmers has been estimated at 18-31 mg/day while the intake of monomer flavonols in Holland is significantly higher (50mg/day) [76]. It has been reported that the level of intake of flavonoids from diet is considerably high as compared with those of vitamin C (70 mg/day), vitamin E (7–10 mg/day), and carotenoids (β-carotene: 2–3 mg/day) [78].

Biological properties of polyphenols depend on their bioavailability. Indirect evidence of their absorption through the gut barrier is the increase in the antioxidant capacity of the plasma after the consumption of polyphenol-rich foods [79, 80], which can be experimentally measured by the ORAC assay. However, polyphenols have a lower intrinsic activity and are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated. In addition, the metabolites that are found in blood and target organs and that result from digestive or hepatic activity may differ from the native substances in terms of biological activity [76].

The metabolism of several polyphenols is now well understood. Generally, in foods, all flavonoids except flavanols are found in glycosylated forms, and glycosylation influences absorption. However, only aglycones and some glycosides can be absorbed from the small intestine, and other forms such as esters and polymers must be hydrolyzed by intestinal enzymes or by the colonic microflora. During the course of the absorption, polyphenols undergo extensive modification; in fact they are conjugated in the intestinal cells and later in the liver by methylation, sulfation and/or glucuronidation. As a consequence, the forms reaching the blood and tissues are different from those present in food and it is very difficult to identify all the metabolites and to evaluate their biological activity [81].

It has been reported that flavanols and their metabolites present in grape seed proanthocyanidin extract (GSPE) are specifically accumulated in different organs such

as liver, adipose tissue and muscle [82]. Moreover, rat plasma obtained after GSPE ingestion was analyzed using a liquid chromatography-tandem mass spectrometry method. Conjugated forms were identified and quantified, founding different concentrations of catechin and epicatechin glucuronide, epicatechin methyl glucuronide and epicatechin methyl-sulfate. Furthermore, monomers, dimers and trimmers in their native form also were detected and quantified in plasma samples. Thus, flavanols predominantly exist in their modified form in plasma even though the intact molecules have been found at a micromolar level [83].

Polyphenols and their derivatives are eliminated mainly in urine and bile. Extensively conjugated metabolites are more likely to be eliminated in the bile, whereas small conjugates, such as monosulfates, are preferentially excreted in urine [81].

1.2.1.1 Polyphenols and Oxidative stress

The best-described property of almost every group of flavonoids is their capacity to act as antioxidants. Flavonoids may have an additive effect to the endogenous scavenging compounds, interfering with different free radical-producing systems, but they can also increase the function of the endogenous antioxidants.

Flavonoids can prevent injury caused by free radicals in various ways. One way is the direct scavenging of free radicals. Flavonoids are oxidized by radicals, resulting in a more stable, less-reactive radical. In other words, flavonoids stabilize the reactive oxygen species by reacting with the reactive compound of the radical. Because of the high reactivity of the hydroxyl group of the flavonoids, radicals are made inactive.

Another possible mechanism by which flavonoids act is through interaction with various enzyme systems. Furthermore, some effects may be a result of a combination of radical scavenging and an interaction with enzyme functions. A direct inhibition of lipid peroxidation, chelating iron, is another protective measure, as well as selected flavonoids can reduce complement activation and inhibit the metabolism of arachidonic acid giving to the flavonoids anti-inflammatory and antithrombogenic properties [84].

Specifically, it has been reported by Murakami et al. that EGCG and epicatechin, had an antioxidant activity and suppressed lipid peroxidation in HepG2 cells, in a CYP2E1-independent mechanism [85]. In rats fed ethanol and EGCG, fatty livers were reduced, not by the reduction of CYP2E1 levels, a member of cytochrome P450, but by increasing fatty acid oxidation [86]. EGCG decreased free radical adduct formation

including 4-HNE adduct formation and hepatocyte necrosis, although had no effect on fatty liver and inflammation [87].

In our research group, it had been described the protective effect of procyanidins against hydrogen peroxide-induced oxidative stress in hepatocyte cells [88]. Moreover, it has been reported that GSPE modifies the activity and mRNA expression of several antioxidant enzymes [89].

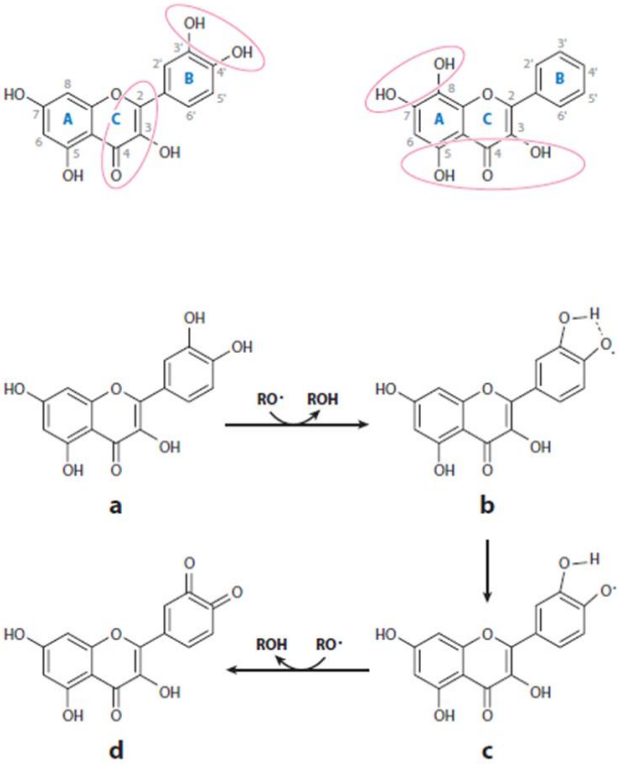

Figure 18. Basic structural features that characterize antioxidant functionality in polyphenols (top). Areas circled show features that are critical for efficient antioxidant function. (a,d) Structures depict oxidation-dependent changes in the structure of flavonoids. (b) Structure depicted shows a flavonoid radical stabilized by a hydrogen bond. (d) Further oxidation of the flavonoid radical leads to the formation of an orthodiquinone [13].

1.2.1.2 Polyphenols and Nrf2

Accumulating reports indicate that the antioxidant properties of phenolic compounds are mainly due to their stimulation of phase II detoxifying and antioxidant defense systems through Nrf2 signaling pathway [13, 90]. Polyphenols, including flavonoids, are able to stimulate the transcription of genes encoding antioxidant enzymes through the Keap1/Nrf2/ARE pathway and thereby enhance detoxification. They may influence pathways mediated through ARE binding, either by modifying the ability of Keap1 in sequestering Nrf2 and/or by its activation of MAPK proteins (ERK, JNK, and p38) that are involved in the stabilization of Nrf2 [91]. Specifically, EGCG has been proposed to conjugate with GSH, causing the disruption of the cellular redox status. This in turn can activate protein kinase pathways that trigger Nrf2 phosphorylation on serine/threonine residues and enable enhanced nuclear migration as well as binding to the ARE. Promoting, in consequence, transcriptional activity of antioxidant and phase II enzymes [92]. However, it has been reported that in liver, most antioxidant genes were down-regulated or not affected by intervention with phenolic apple juices, concomitantly to an absence of Nrf2 induction. The downregulation of genes involved in antioxidant defense, could be a consequence of cellular ROS scavenging by polyphenols leading to decreased oxidant status. Moreover, the extent of polyphenol absorption in the small intestine, largely determined by chemical structure and nature/amount of metabolites formed by microflora, is a major influential factor [93]. Furthermore, such dual effects (upregulation and downregulation) of polyphenols on Nrf2-mediated ARE activation and the target expression could be associated with the used polyphenols concentration [94].

Particularly, in the case of EGCG, it has been reported that EGCG could directly and/or indirectly interact with cysteine residue present in Keap1, thereby inducing Nrf2 nuclear translocation. Moreover, EGCG can be oxidized to form semiquinone radical which would react with the sulfydryl group of GSH. Just in this form, it may modify the cysteine thiols on Keap1 and facilitate the release of Nrf2 for nuclear translocation. Another most plausible mechanism responsible for EGCG-induced activation of Nrf2 involves phosphorylation of serine/threonine residues of Nrf2 via protein Kinases [94].

1.2.1.3 Polyphenols and hepatic steatosis

Several studies have reported the protective effects of flavonoids on steatosis. Kuzu et al., showed the preventive role of EGCG in the development of experimental NASH in non-alcoholic steatohepatitis induced by high-fat diet. This experimental effect of EGCG could be explained by the effect on lipid metabolism, as well as by its antioxidant properties [95]. Furthermore, it has recently been described that the green tea polyphenols could represent a useful supplement in the treatment of NAFLD [96]. Some evidences from *in vitro* systems and animal models suggest that green tea

catechins are likely to prevent steatosis by: (a) decreasing intestinal lipid and carbohydrate absorption; (b) decreasing adipose lipolysis and both adipose and hepatic *de novo* lipogenesis; (c) stimulating hepatic β-oxidation and thermogenesis; and (d) improving insulin sensitivity. Furthermore, catechins are likely to prevent the progression from liver steatosis to NASH through their antioxidant and anti-inflammatory properties [97].

1.2.2 Polyunsaturated Fatty Acids

1.2.2.1 Chemistry and classification

The fatty acyl structure represents the major lipid building block of practically all lipids and therefore is one of the most fundamental categories of these molecules. Fatty acids (FAs), which consist of three fatty acids esterified to a glycerol backbone to form a TG, particularly differ in their chain length, number of double bonds and position of the bonds in the chain. Saturated fatty acids are straight chain fatty acids of variable carbon chain length with no double bonds, monounsaturated fatty acids (MUFAs) have one double bond along the fatty acid chain and polyunsaturated fatty acids (PUFAs) contain more than one double bond in the fatty acid chain. Omega-6 (n-6) PUFAs have their first double bound on the sixth carbon atom from the methyl end, whereas n-3 (n-3) PUFAs have their first double bond on the third carbon atom [98].

Linoleic acid (LA) and α-linolenic acid (ALA) are necessary for growth and reproduction and are considered essential fatty acids due to the lack of the enzymes necessary to insert a *cis* double bond at the n-3 or the n-6 position of a FA [99]. Moreover, LA and ALA are the precursor of the long chain PUFA n-6 group and n-3 group respectively. ALA can be converted endogenously to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), although EPA and DHA can be consumed directly through the diet.

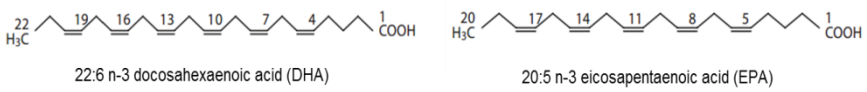

Figure 19. Molecular structure of EPA and DHA [100]

Dietary sources of LA include all major plant and vegetable oils such as sunflower oil, grape seed oil and walnut oil among others. ALA is abundant in several vegetable oils, such as chia, perilla and linseed oil, but is also found in walnut oil, albeit in smaller amounts.

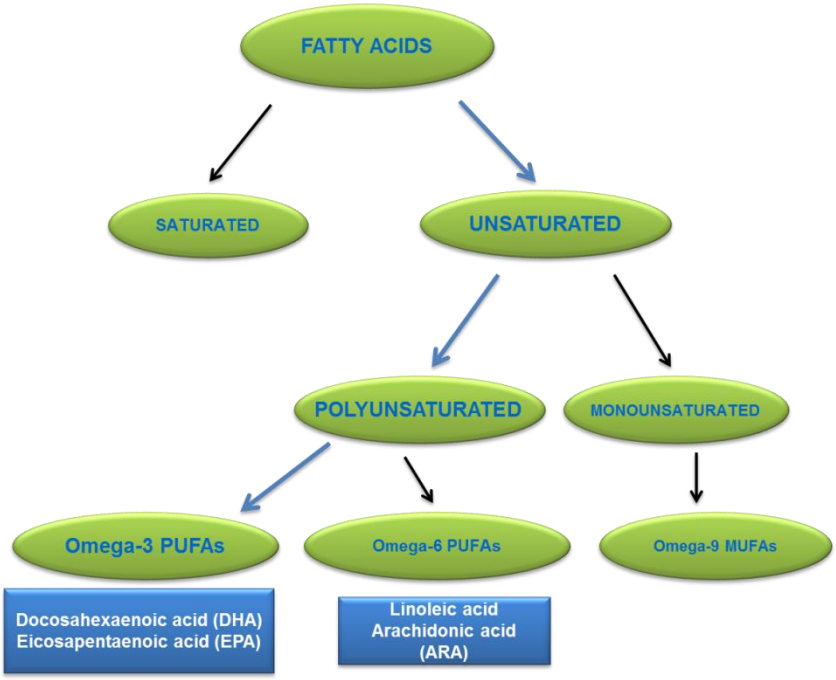

Figure 20. Classification of Fatty Acids.

1.2.2.2 Omega-3 PUFAs bioavailability and metabolism

ALA is the precursor of EPA and DHA. For this reason the intake of ALA could be sufficient to ensure an adequate supply of n-3 PUFAs. However, it has been reported that the ALA conversion to n-3 PUFAs is very limited and could not be sufficient to provide the preventive and therapeutic benefits associated with EPA and DHA. Therefore, additional intake of n-3 PUFAs is indispensable [101].

The sources of n−3 PUFAs that have by far the greatest quantitative significance for humans are cold-water fish, such as salmon, mackerel, herring, and tuna. Microalgae (e.g. *Schizochytrium*) are of minor importance. Natural fish oils contain approximately 18% EPA and 12% DHA, which after the process of re-esterification can reach up to 90% EPA and DHA [102].

n-3 PUFAs can be detected in the blood in various forms. These include FFAs, as well as bound to TG and phospholipids, which in turn are included in lipoproteins. The blood transports n-3 PUFAs to the target tissues, where they are primarily incorporated in

membranes [103]. The bioavailability of n-3 PUFAs depends on numerous factors. Besides the type of chemical bond, the concomitant intake of food (especially its fat content) and the presence of other components affect the uptake of n−3 PUFAs. Normally, the amount of substance absorbed by the body is measured by its concentration in blood serum or plasma after administration of a single dose. However, n-3 PUFAs is incorporated into membranes. Consequently, determining the levels of n−3 PUFAs that are not incorporated in membranes does not allow conclusions to be drawn regarding their availability.

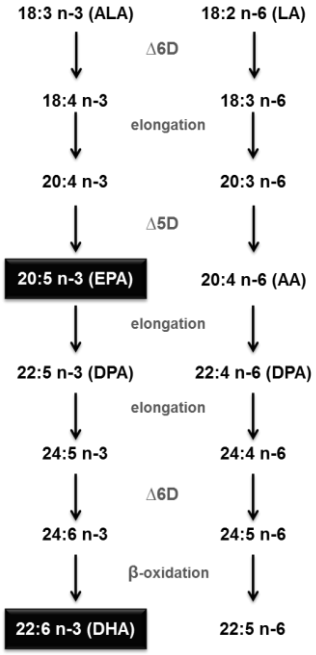

Figure 21. Desaturation and elongation of n-3 and n-6 fatty acids. Δ6D, Δ6 desaturase; Δ5D, Δ5 desaturase; ALA, α-linolenic acid; LA, linoleic acid; EPA, eicosapentaenoic acid; AA, arachidonic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

The synthesis of the n-3 PUFAs EPA and DHA involves the formation of polyunsaturated C_{24} intermediates by sequential elongation and desaturation in the endoplasmic reticulum, followed by a β-oxidation step of 24:6 n-3 to 22:6 n-3 in the peroxisomes [104].

1.2.2.3 Omega-3 PUFAs and Oxidative stress

Diets rich in n-3 PUFAs are considered effective in the prevention of many chronic diseases [105]. For example, one of the intriguing and promising therapeutic potentials for n-3 PUFAs is in the treatment and prevention of heart failure, improving cardiac function efficiency [106]. Some studies proposed that the n-3 PUFAs might indirectly act as anti- rather than pro-oxidant, hence diminishing inflammation and, in turn, the risk of atherosclerosis and CVD [107]. Furthermore, some results reported support a role for n-3 PUFAs dietary supplementation in reducing hepatic lipid content in mice subjected to high fat diet, asserting the potential therapeutic use of n-3 PUFAs supplementation in the treatment of human liver steatosis induced by nutritional factors or other etiologies [108]. In all these cases, it is assumed that there is no concomitant oxidative stress, but the importance of such a phenomenon must not be neglected. Paradoxically, oxidative stress is a common factor in the etiology of the diseases impacted by n-3 PUFAs treatments. Accordingly, there remains a theoretical concern that these fatty acids may increase lipid peroxidation [109]. The theoretical oxidation of PUFA is directly proportional to their degree of unsaturation, and supplementation with highly unsaturated n-3 PUFAs has been reported to increase the oxidative stress in both animals and humans [110]. Concretely, it has been suggested that DHA is able to exert a beneficial effect with regard to reducing the risk of atherosclerosis, only if anti-oxidative protection against oxidative stress is sufficient [111]. However, the impact of n-3 PUFAs supplementation on lipid peroxidation is controversial. Several clinical studies have reported no significant change in the lipid peroxidation following increased consumption of n-3 PUFAs [112, 113].

1.2.2.4 Omega-3 and hepatic steatosis

n-3 PUFAs represent a potentially viable pharmacological treatment option in NAFLD. This group of fatty acids has an excellent side-effect profile and in high dose is effective in reducing plasma TGs and FFA levels, both increased in NAFLD and associated with increased CV risk [52]. It has been reported that n-3 PUFAs selectively incorporate into hepatic phospholipids, inhibit *de novo* lipogenesis and change the hepatic fatty acid profile via reduced desaturases activity in the non-steatotic liver [114].

The first meta-analysis of studies investigating the effect of n-3 PUFA on liver fat in humans revealed that marine n-3 fatty acid supplementation in humans is associated with a positive effect on liver fat [115].

It has been reported that the hepatic fatty acid composition can influence the degree of liver injury and disease progression. NASH and NAFLD associated with obesity were linked to the depletion of n-3 PUFAs. The n-6/n-3 PUFA ratios of both steatosis and

steatohepatitis patients are increased compared with healthy individuals [116, 117]. Mensik et al. reported that the decreasing total fat intake and increasing the intake of fish oils may be beneficial on the treatment of NASH [118]. It was demonstrated that the fish-oil containing 46% of DHA, ameliorated the progression of NASH in a rat model of the disease by lowering oxidative stress and the n-6/n-3 PUFAs ratio in the liver. These findings suggested that a decrease in n-3 PUFAs contributes to the progression of NASH via the associated oxidative stress-induced damage and inflammation [117].

1.2.2.5 Omega-3 PUFAs and Nrf2

Some studies revealed that the n-3 PUFA DHA induces the anti-oxidative enzyme HO-1 through Nrf2 pathway in adipocytes [119] and also in endothelial cells [120]. This could be represent a positive factor against oxidative stress. However, the mechanism to activate the signaling pathways that lead to Nrf2 nuclear translocation and eventual HO-1 induction is related with the transient generation of ROS by the DHA treatment. Therefore, the Nrf2 pathway could be induced to counteract the increased ROS production due to the n-3 PUFA oxidation. Additionally, in cell culture model systems, beneficial effects of DHA supplementation were mainly observed when DHA was supplied in conjunction with an antioxidant molecule such as α-tocopherol [120].

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2 HYPOTHESIS & OBJECTIVES

Over the past years it has been described the important role of oxidative stress in the MetS-related manifestations, specifically in the obesity. It is well known that agents that reduce oxidative stress represent an important tool to reduce the obesity-induced complications such as hepatic steatosis. In this sense, flavonoids are described as antioxidant molecules, due to their scavenging properties. Moreover, n-3 PUFAs are considered effective in the treatment and prevention of cardiovascular diseases, although some studies propose that in conditions of oxidative stress, n-3 PUFAs induce lipid peroxidation increasing the oxidative stress.

On the other hand, the liver, a tissue implicated in detoxification of compounds and in homeostasis regulation, is an important tissue affected in the obesity and can develop NAFLD, which is the most common liver disease. The lipid accumulation in hepatocytes and the presence of ROS promotes oxidative stress and, consequently, impairs liver function increasing, in consequence, the risk of other diseases.

Accordingly, it has been demonstrated that polyphenols and n-3 PUFAs ameliorate obesity-related effects, such as hepatic steatosis and oxidative stress. Therefore, we hypothesize that the combination of these two bioactive compounds results in an additive beneficial effect against obesity and related metabolic disorders including oxidative stress.

Hence, the principal aim of this thesis was to study the antioxidant effects of flavonoids and n-3 PUFAs, as well as the possible additive effects of the two compounds together. These studies were developed under several oxidative stress conditions (metabolic, chemical) both *in vitro* and *in vivo*. For this, four objectives were proposed:

1) To assess the GSPE effect on oxidative stress alterations related to a genetically obesity model rats.

2) To *in vitro* explore if the flavonoid EGCG is able to revert the oxidative damage induced by DHA, one of the omega-3 PUFAs, in an oxidative stress environment.

3) To investigate the additive effect of a natural compound extract such as GSPE and DHA oil rich on oxidative stress produced in a lipidic postprandial state.

4) To study the additive effects of GSPE and DHA oil rich on the oxidative stress in diet-induced obesity in rats.

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3 RESULTS

3.1 Grape seed proanthocyanidin extract improves the hepatic glutathione metabolism in obese Zucker rats

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Abbreviations: 4-HAE, 4-hydroxyalkenal; **AST,** aspartate aminotransferase; **Cu,Zn-Sod**, superoxide dismutase 1; **DCFH-DA**, 2',7'-Ddchlorofluorescin diacetate; **Gclc**, glutamate-cysteine ligase catalytic subunit; **GPx**, glutathione peroxidase; **GR**, glutathione reductase; **GSH**, reduced glutathione; **GSPE**, grape seed proanthocyanidin extract; **GSSG**, oxidized glutathione; **GST**, glutathione S-transferase; **MDA**, malondialdehyde; **ORAC**, oxygen radical absorbance capacity; **ROS**, reactive oxygen species; **TBARS**, thiobarbituric acid reactive substances; **TG**, triglycerides.

Keywords: Liver / Glutathione / Oxidative Stress / Proanthocyanidins / Zucker fatty rats

ABSTRACT

Scope: Increased oxidative stress may play an important role in metabolic syndrome and related manifestations, including obesity, atherosclerosis, hypertension and insulin resistance. Its relation to obesity is due to increased reactive oxygen species and/or decreased glutathione (GSH) antioxidant metabolism. Consequently, the activation of glutathione metabolism appears to be a central defense response to prevent oxidative stress. In this sense, dietary supplements with natural antioxidant molecules, including proanthocyanidins, may present a useful strategy of controlling and reducing complications of obesity, including hepatic steatosis. **Materials and results:** We assess the grape seed proanthocyanidin extract (GSPE) effect on oxidative

alterations related to genetically obese rats (Zucker rats) and, more specifically, to hepatic GSH metabolism. We demonstrate that the administration of GSPE reduced the oxidized glutathione (GSSG) accumulation increasing the total GSH/GSSG hepatic ratio and consequently decreasing the activation of antioxidant enzymes, including glutathione peroxidase, glutathione reductase and glutathione-S-transferase, and increasing the total antioxidant capacity of the cell. **Conclusion:** In Zucker rats, the obesity-induced oxidative stress related to liver glutathione alteration was mitigated by GSPE administration.

1 Introduction

Ideas about the potential role of oxidative stress in metabolic syndrome are rapidly evolving. Reported results support the idea that increased oxidative stress may play an important role in metabolic syndrome related manifestations, including atherosclerosis, hypertension and insulin resistance [1].

Oxidative stress, a condition in which an imbalance occurs between the production and inactivation of reactive oxygen species (ROS) [1], is known to trigger cytotoxic reactions that damage membrane lipids, proteins, nucleic acids and carbohydrates. A number of studies have revealed the link between oxidative stress and obesity [2].

Hepatic steatosis is characterized by the accumulation of triglycerides (TG) in the liver. There is considerable evidence that the accumulation of lipids in the liver and the presence of ROS promotes lipid peroxidation and consequently produces oxidative stress [3]. However, some studies in diabetic, fatty rats have indicated that is not clear whether an increase in oxidative stress related to obesity is due to increased ROS or decreased glutathione (GSH) antioxidant metabolism [4]. GSH is a major component of the non-enzymatic cellular antioxidant defense. GSH from the diet can be partly absorbed from the small intestine, but additionally it can be synthesized *de novo* in cells. It has been reported that GSH can react with a variety of xenobiotic electrophilic compounds in catalytic reactions of glutathione-S-transferase (GST) and effectively scavenge ROS directly or indirectly through enzymatic reactions. Because GSH is rapidly oxidized to GSSG by radicals and other reactive species and GSSG is exported from cells, the intracellular GSH/GSSG ratio can provide a valid index of oxidative stress [5].

Factors that reduce oxidative stress could provide an important tool to reduce the burden associated with obesity and related chronic disease, including diabetes and CVD. Dietary supplements with natural antioxidant molecules, including phenolic compounds, may present a strategy of controlling and reducing the complications of obesity. It has been demonstrated that the administration of polyphenols, including resveratrol, to Zucker fatty rats was able to reduce the oxidative damage in liver, measured by lipid peroxidation (TBARS) and the amount of GSSG, and limit the progression of liver steatosis. These results were related to the decrease in fatty acid availability in the liver by resveratrol [6].

Proanthocyanidins are the most abundant polyphenols in human diets, and they are present in fruits, beans, nuts, cocoa, tea and wine [7]. In previous studies, it was reported that grape seed proanthocyanidin extract (GSPE) presents anti-inflammatory and antioxidant properties in LPS-induced acute inflammation experiments.

Specifically, the administration of GSPE for 15 days before the LPS injection increased the liver total GSH /GSSG ratio and reduced oxidative stress [8]. Furthermore, GSPE administration to obese Zucker rats resulted in a decrease in muscle lipid peroxidation [9]. Additionally, it has been demonstrated that other polyphenols, including methylxanthines, present in cocoa seeds may protect against lipid peroxidation and enhance the antioxidant defense system [10].

Obese Zucker rats are the most widely used animal model of obesity because they develop dyslipidemia, hyperinsulinemia, hypertension and low-grade inflammation, and these are manifestations similar to those that define human metabolic syndrome [11]. Additionally, it has been reported that obese Zucker rats exhibit hepatic steatosis, increased oxidative stress [12, 13] and elevated oxidized lipids in serum and liver; consequently, the plasma antioxidant defense mechanisms, including glutathione peroxidase, are compromised [14].

One important task for cellular GSH is to scavenge free radicals and peroxides produced during normal cellular respiration and in metabolic disorders such as obesity-induced oxidative stress [15]. Hence, the activation of glutathione metabolism appears to be a central defense response to prevent oxidative stress. Accordingly, this study aimed to assess the GSPE effect on oxidative alterations related to a genetic obesity model (Zucker rats) and, more specifically, on hepatic GSH metabolism.

2 Materials and methods

2.1. Proanthocyanidin extract

GSPE was provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the previous analyses [16], the GSPE contained catechin (58 μmol/g), epicatechin (52 μmol/g), epigallocatechin (5.50 μmol/g), epicatechin gallate (89 μmol/g), epigallocatechin gallate (1.40 μmol/g), dimeric procyanidins (250 μmol/g), trimeric procyanidins (15.68 μmol/g), tetrameric procyanidins (8.8 μmol/g), pentameric procyanidins (0.73 μmol/g), and hexameric procyanidins (0.38 μmol/g).

The GSPE was dissolved in sweetened condensed milk purchased from La Lechera. Its composition per 100 g was: 8.9 g protein, 0.4 g fat, 60.5 g carbohydrates and 281 kcal.

2.2. Animals and experimental design

Five-week-old obese (fa/fa) female Zucker rats (n=20) and heterozygous lean rats (Fa/fa) (n=10) (Charles River Laboratories, Barcelona, Spain) were housed in cages by pairs at 22ºC with a 12 h light/dark cycle and were fed ad libitum with standard chow (Panlab A-04, Barcelona, Spain) and water. After a week of adaptation, the animals were divided into three experimental groups (n=10/group): lean Zucker rats (LZ) and obese Zucker rats (OZ) treated with the vehicle (sweetened condensed milk diluted 1:6 in water), and obese Zucker rats treated with GSPE (OZ+GSPE) (vehicle + 35 mg GSPE/kg body weight/day). The treatments were administered daily by controlled oral intake with a syringe for 10 weeks.

After the treatment period, the rats were fasted overnight and were anaesthetized with 75 mg/kg of sodium pentobarbital and then sacrificed by abdominal aorta exsanguination. Blood was collected, and plasma was obtained by centrifugation. Liver samples were excised, weighed and frozen immediately in liquid nitrogen. All the samples were stored at -80ºC until analysis.

The Animal Ethics Committee of our University approved all procedures, with the permission number 4250 of the Catalonia Government (Generalitat de Catalunya. Departament de Medi Ambient i Habitatge).

2.3. Hepatic function assay

Plasma aspartate aminotransferase (AST) was determined using commercial kits purchased from QCA (Barcelona, Spain) to assess liver function. This assay is based on the spectrophotometric measurement of NADH disappearance at λ=334 nm.

2.4. Liver content of triglycerides

The content of triglycerides (TG) in liver was determined in alcoholic KOH solution prepared as previously described [17] using an enzymatic colorimetric kit in accordance with the manufacturer's instructions (QCA, Barcelona, Spain).

2.5. GSH metabolism

GSH is required to maintain the normal reduced state and to counteract the deleterious effects of oxidative stress. Therefore, it is commonly recognized that reductions in total GSH/GSSG ratios denote the presence of oxidative stress [6]. For this assay, the total and oxidized glutathione levels in liver homogenates were determined by an enzymatic method reported by Griffith, et al. [18]. The assay is based on the reaction of GSH with DTNB (5,5′-dithiobis(2-nitrobenzoic acid) D8130,

Sigma-Aldrich, Madrid, Spain) to generate 2-nitro-5-thiobenzoic acid, a yellow compound that absorbs at λ=412 nm, and GSSG. GSH is regenerated from GSSG in the presence of excess glutathione reductase. Under these conditions, total GSH in the sample is determined. GSSG is measured by the reaction of GSH and 2-vinylpyridine, a thiol-scavenging reagent, to form a pyridinium salt. For this assay, liver was homogenized and deproteinized with metaphosphoric acid 6% and then centrifuged at 5000 g for 15 min at 4 °C. The supernatants were used for the analysis.

2.6. Antioxidant enzyme activities

Glutathione peroxidase (GPx) activity was assessed as Flohe and Günzler described previously [19]. Briefly, liver homogenates in phosphate buffer were incubated in a mixture containing 50 mM phosphate buffer, 10 mM GSH, 2.4 U/mL GR and 1.5 mM NADPH for 3 min at 37 °C. After the addition of 2 mM H_2O_2, NADPH oxidation was measured at λ=340 nm for 2 min. The results were expressed in nmol NADPH oxidized/min * mg liver homogenate protein.

Glutathione reductase (GR) activity was measured according to the Carlberg and Mannervik [20] method. Briefly, liver homogenates were mixed with 3 mM GSSG and 1.5 mM NADPH, and the disappearance of NADPH was measured spectrophotometrically at λ=340 nm for 2 min. The results were expressed in nmol NADPH oxidized/min * mg liver homogenate protein.

GST activity was determined spectrophotometrically according to the method described by Habig [21], which measures the reaction of 1-chloro-2,4-dinitrobenzene (CDNB, Sigma-Aldrich, Madrid, Spain) with GSH. The reaction mixture contained 5 mM GSH, 25 mM CDNB and liver homogenate in phosphate buffer. The CDNB-GSH product was measured at λ=340 nm for 2 min. The results were expressed as nmol GDNB-GSH/min * mg liver homogenate protein.

2.7. Oxygen Radical Absorbance Capacity (ORAC) assay

2.7.1. Preparation of plasma samples

Plasma samples were prepared as previously described by Prior, et al. [22] with some modifications [23]. Briefly, 50 µL of plasma stored at -80 °C was defrosted and mixed with 100 µL of ethanol and 50 µL of distilled water. After the solution was vortexed, 200 µL of metaphosphoric acid (0.75 M) was added and the mixture was vortexed once again. The solution was centrifuged at 2000 g for 5 min at 10 °C. For the ORAC assay, 80 µL of the supernatant was diluted with 420 µL of phosphate buffer.

2.7.2. ORAC assay

The ORAC assay was carried out using the method described by Huang, et al. [24]. Briefly, 25 µL of plasma solution and 150 µL of 59.8 nM fluorescein (FL) (Sigma-Aldrich, Madrid, Spain) were added to each well of a 96-well microplate. The fluorescence was measured at λex=485 nm and λem=520 nm every 1 min for 90 min in the FLx800 Multi-Detection Microplate Reader (Biotek, Winooski, USA) after the addition by injector of 25 µL of 73 mM of the radical generator 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH, Acros Organics, Belgium). As a standard, Trolox (Sigma-Aldrich, Madrid, Spain) solution was used at different concentrations (0, 3.125, 6.25, 12.5, 25, 50, and 100 µM). The final ORAC values were calculated by a regression equation between the Trolox concentration and the net area under the FL decay curve and were expressed as µmol Trolox equivalents per liter.

2.8. Lipid peroxidation

Lipid peroxidation was determined from the malondialdehyde (MDA) and 4-hydroxyalkenal (4-HAE) content by colorimetric assays based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA and 4-HAE to yield a stable chromophore with maximal absorbance at λ=586 nm. For this assay, liver was homogenized in Tris buffer at pH=7.4; 10 µL of 0.5 M butylated hydroxytoluene (Sigma-Aldrich, Madrid, Spain) stock solution per 1 mL of homogenate was added to prevent sample oxidation. The homogenates were centrifuged at 3000 g for 10 min at 4 °C. Subsequently, the MDA+4-HAE levels were assessed using Lipid Peroxidation Microplate Assay Kits FR-22 (Oxford Biomedical Research, Barcelona, Spain) in accordance with the manufacturer's instructions. The results were expressed as mM MDA+4-HAE/ mg protein.

2.9. Hepatic reactive oxygen species content

ROS formation was estimated in liver homogenates using the 2',7'-dichlorfluorescein-diacetate (DCFH-DA) method according to previous reports [12, 25] with some modifications. Briefly, liver was homogenized on ice with 1 mM EDTA–50 mM sodium phosphate buffer (pH 7.4), and DCFH-DA was added to the homogenates to a final concentration of 20 µM. The reactions were incubated for 1 h at 37 °C, and the changes in fluorescence were determined at λex=485 nm and λem=530 nm using the FLx800 Multi-Detection Microplate Reader (Biotek, USA). Cellular ROS production was expressed as relative fluorescence units (%)/mg protein.

2.10. Real-time PCR of liver antioxidant genes

2.10.1 mRNA isolation

Total RNA was obtained from liver using RNeasy Mini Kits (Qiagen, Valencia, CA) according to the manufacturer's protocol. DNase I, RNAase-free kits (High Capacity complementary DNA Reverse transcription kits, Applied Biosystems, Madrid, Spain) were used to remove the genomic DNA from RNA preparations. RNA was quantified by spectrophotometer (Nanodrop 1000 Spectrophotometer, Thermo Scientific) at an absorbance of 260 nm and tested for purity (by the A260/280 ratio) and integrity (by denaturing gel electrophoresis). The cDNA was subsequently amplified by PCR using specific TaqMan Assay-on-Demand Probes for Gclc (Rn00563101_m1), GR (Rn00588153_m1), GPx (Rn00577994_g1), GST (Rn01757146_m1) and for cyclophilin peptidylprolyl isomerase A (PPIA) (Rn00690933_m1).

2.10.2 Real-time RT-PCR

Quantitative PCR for different genes was performed using TaqMan PCR Core Reagent Kits according to the manufacturer's protocol and analyses on a Real-Time 7300 PCR System, all from Applied Biosystems. The thermal cycling comprised an initial step at 50 °C for 2 min, followed by a polymerase activation step at 95 °C for 10 min and a cycling step with the following conditions: 40 cycles of denaturing at 95 °C for 15 s and annealing at 60 °C for 1 min. The relative levels of expression of the target genes were measured using PPIA mRNA as an internal control according to the $2^{-\Delta\Delta Ct}$ method.

2.11. Protein assay

Bradford reagent (Sigma-Aldrich, Madrid, Spain) was used to determine the protein content of liver homogenates.

3 Results

3.1. Liver function: weight, AST and TG

The TG content increased in the OZ rats compared with the LZ rats and was not reverted by the GSPE treatment (Figure 1A). This result was related to the significant liver weight differences reported previously (DOI:10.1016/j.foodchem.2013.02.104) and to the significant changes in body weight, although in this case GSPE was able to significantly decrease the animal body weight compared with the OZ group (data not shown). In this case, plasma levels of AST were assessed as an indicator of liver damage. The AST levels were moderately but not significantly increased in the OZ

group compared to the LZ group (11.6 ± 0.4 for LZ vs 14.8 ± 1.2 for OZ). Moreover, GSPE treatment did not alter the AST plasma levels of the OZ rats (Figure 1B).

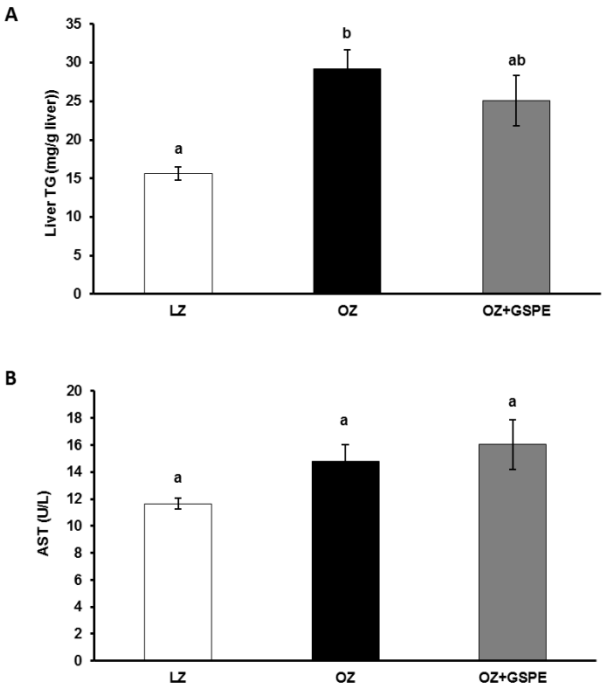

Figure 1. Liver function. Liver TG content (A) and plasmatic AST activity (B) was assessed in experimental animals. The results are expressed as the mean±SEM for each group (n=10): lean Zucker rats (LZ), obese Zucker rats (OZ) and obese Zucker rats treated with GSPE (35 mg/kg) (OZ+GSPE). Different letters indicate statistically significant differences ($p \leq 0.05$) among different groups.

3.2. GSH metabolism in liver

A decrease in the total GSH/GSSG ratio in OZ rats was observed compared with the LZ group as shown in Figure 2A (25.5 ± 3.9 for LZ vs 3.8 ± 1.3 for OZ; $p \leq 0.05$). This GSH metabolism alteration was prevented when rats were treated with GSPE. The GSSG content was significantly decreased by GSPE administration, and consequently, the glutathione levels were significantly increased. In contrast, in the OZ rats, the GSSG levels were significantly increased compared with the LZ rats (Figure 2B).

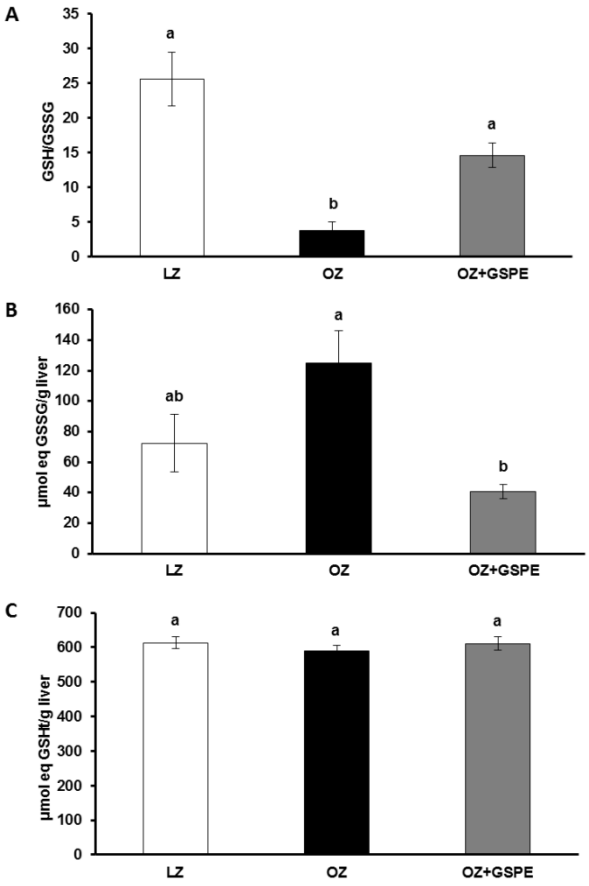

Figure 2. GSH metabolism. Total GSH/GSSG (A) was estimated by assessing total GSH (C) levels and GSSG (B) in liver homogenates. The results are expressed as the mean±SEM for each group (n=10): lean Zucker rats (LZ), obese Zucker rats (OZ) and obese Zucker rats treated with GSPE (35 mg/kg) (OZ+GSPE). Different letters indicate statistically significant differences ($p \leq 0.05$) among different groups.

The glutamate-cysteine ligase catalytic subunit (Gclc) is the enzyme responsible for GSH synthesis de novo. In the OZ group, the Gclc mRNA expression was upregulated, although the GSH levels were not modified. However, the Gclc mRNA expression was not significantly changed by the GSPE treatment compared with the OZ group, and the GSH content also did not differ (Figure 3A).

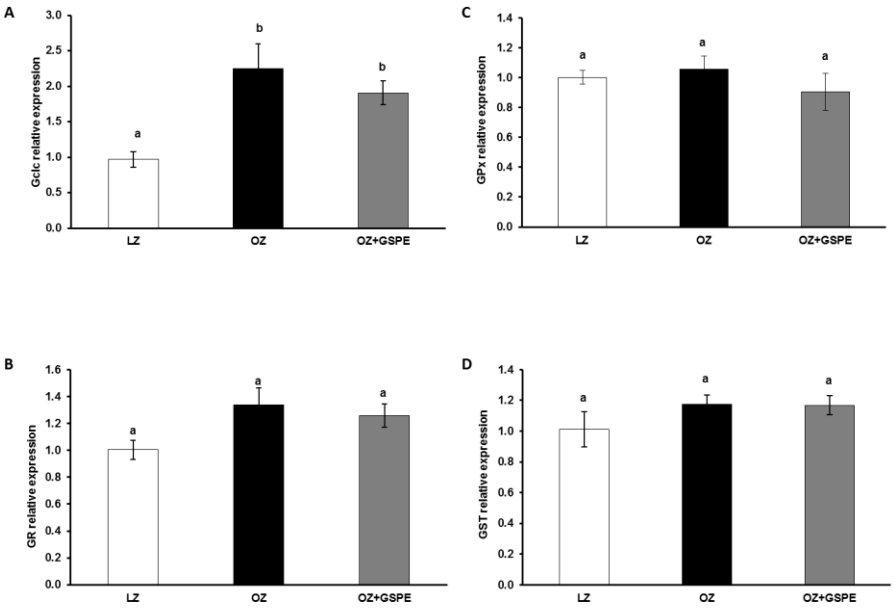

Figure 3. The mRNA expression of liver antioxidant enzymes. The mRNA expression of liver Gclc (A), GR (B), GPx (C) and GST (D) was analyzed by real-time RT-PCR. The relative levels of expression of the target genes were measured using PPIA mRNA as an internal control according to the $2^{-\Delta\Delta Ct}$ method. The results are expressed as the mean±SEM for each group (n=10): lean Zucker rats (LZ), obese Zucker rats (OZ) and obese Zucker rats treated with GSPE (35 mg/kg) (OZ+GSPE). Different letters indicate statistically significant differences ($p \leq 0.05$) among different groups.

Moreover, the activity of GR, which is the enzyme responsible for reducing GSSG to GSH, was slightly increased in the OZ group although the mRNA expression was not modified. However, in the OZ+GSPE group, even though the mRNA expression did not decrease, the GR activity significantly decreased (Figure 3B, 4A)

GPx is the enzyme responsible for the GSH conversion to GSSG, whereas the GST enzyme is involved in detoxifing the cellular environment. As shown in Figure 4B, hepatic GPx activity was markedly increased in the OZ group compared with the LZ rats (112.3 ± 10.7 for LZ vs 148.9 ± 14.7 for OZ; $p \leq 0.05$). However, the GST activity did not change in the OZ group (Figure 4C). In contrast, the GSPE treatment slightly decreased the GPx activity of OZ animals (148.9 ± 14.7 for OZ+GSPE vs 118.5 ± 3.5 for OZ). Nevertheless, the GSPE treatment significantly decreased the GST activity (230.1 ± 14.7 for OZ+GSPE vs 281 ± 8.4 for OZ; $p \leq 0.05$) (Figure 4B, 4C). However, neither the

mRNA expression of GPx nor that of GST showed any significant alteration (Figure 3C, 3D).

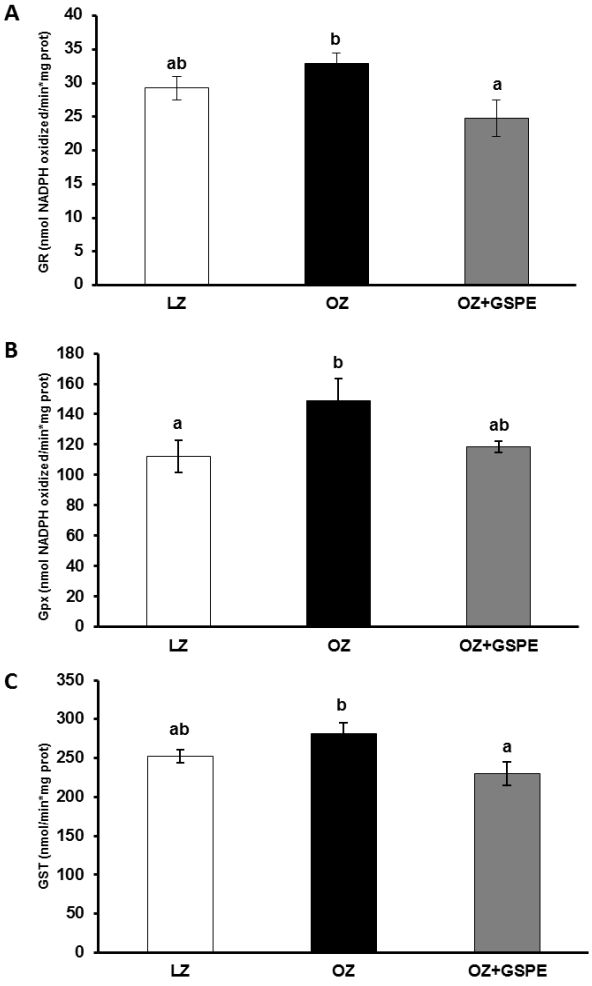

Figure 4. **Antioxidant enzyme activity.** Liver GR (A), GPx (B) and GST (C) antioxidant enzyme activities were assessed. The results are expressed as the mean±SEM for each group (n=10): lean Zucker rats (LZ), obese Zucker rats (OZ) and obese Zucker rats treated with GSPE (35 mg/kg) (OZ+GSPE). Different letters indicate statistically significant differences ($p \leq 0.05$) among different groups.

3.3. ORAC

Plasma total antioxidant status, assessed as oxygen radical absorbance capacity (ORAC), was significantly lower in obese Zucker rats than in control lean rats (3.6 ± 1.7 for OZ vs 18.5 ± 1.0 for LZ; p≤0.001). When the obese rats were treated with GSPE, ORAC values significantly increased compared with obese rats not treated (3.6 ± 1.7 for OZ vs 12.6 ± 0.6 for OZ+GSPE; p≤0.001), although the values did not reach the ORAC levels of the lean group (Figure 5).

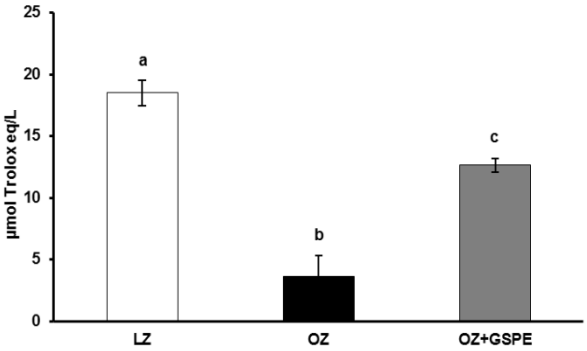

Figure 5. Total antioxidant capacity (ORAC). Plasmatic total antioxidant capacity was assessed using the ORAC method. The results are expressed as the mean±SEM for each group (n=10): lean Zucker rats (LZ), obese Zucker rats (OZ) and obese Zucker rats treated with GSPE (35 mg/kg) (OZ+GSPE). Different letters indicate statistically significant differences (p≤0.05) among different groups.

3.4. Hepatic lipid peroxidation and reactive oxygen species

Liver lipid peroxidation as a marker of oxidative stress damage was determined in the different animal groups by measuring the MDA+4-HAE content. Surprisingly, in the OZ and the OZ+GSPE group, significant decreases were observed in the MDA+4-HAE levels compared to those of the LZ group (Figure 6A). Lipid peroxidation is mediated by ROS production. Therefore, the liver ROS content was assessed by the DFCH method. Interestingly, neither the OZ group nor the OZ+GSPE group showed any difference from the LZ group in liver ROS content (Figure 6B).

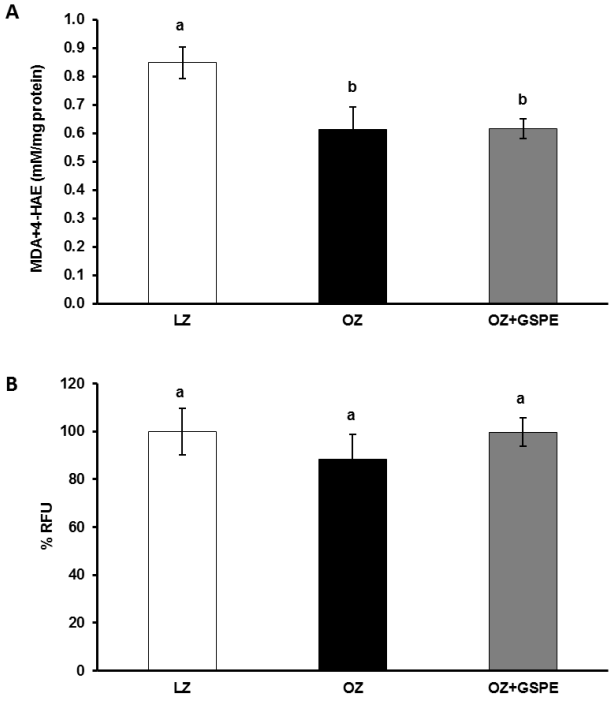

Figure 6. Hepatic lipid peroxidation and ROS content. Lipid peroxidation was determined by measuring MDA+4-HAE levels in liver homogenates (A). Liver ROS production was assessed by the DCFH method (B). The results are expressed as the mean±SEM for each group (n=10): lean Zucker rats (LZ), obese Zucker rats (OZ) and obese Zucker rats treated with GSPE (35 mg/kg) (OZ+GSPE). Different letters indicate statistically significant differences ($p \leq 0.05$) among different groups.

3.5. Cu,Zn-SOD mRNA expression

Superoxide anion radicals ($O_2^{\cdot-}$) are the major ROS generated in mitochondria and are rapidly altered by Cu,Zn-SOD to H_2O_2. Therefore, Cu,Zn-SOD mRNA expression was analyzed in the liver as an antioxidant enzyme. As Figure 7 shows, neither the OZ group nor the OZ+GSPE group displayed altered antioxidant enzyme mRNA expression compared with the LZ group.

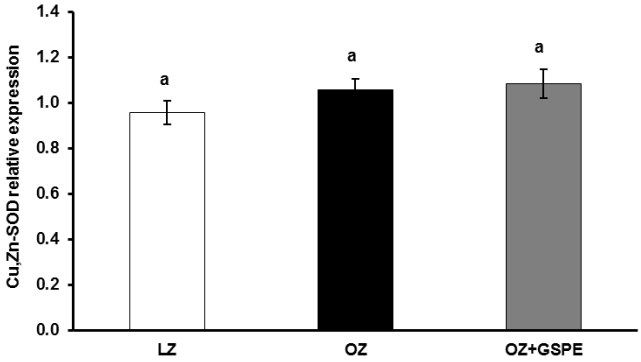

Figure 7. Hepatic Cu,Zn-SOD mRNA expression. Liver Cu,Zn-SOD mRNA expression was analyzed by real-time RT-PCR. The relative levels of expression of the target gene were measured using PPIA mRNA as an internal control according to the 2−ΔΔCt method. The results are expressed as the mean±SEM for each group (n=10): lean Zucker rats (LZ), obese Zucker rats (OZ) and obese Zucker rats treated with GSPE (35 mg/kg) (OZ+GSPE). Different letters indicate statistically significant differences (p≤0.05) among different groups.

4 Discussion

The present study demonstrated that GSPE administration improves the liver GSH metabolism, and in consequence, increase the total antioxidant capacity of the cell and decrease antioxidant enzymes activities. These results indicated an improvement in the redox homeostasis by the polyphenol content in GSPE in a rat model of genetic obesity.

Previously, it was confirmed that the experimental OZ rats showed increased body weight (DOI:10.1016/j.foodchem.2013.02.104), hyperinsulinemia [26] and hypertriglyceridemia [9] compared with lean Zucker rats. Therefore, the OZ rat was used as an experimental model for metabolic syndrome.

Moreover, the results obtained in this study suggested a development of hepatic steatosis in the OZ rats, due to the increase of liver weight and liver TG content, in spite of the fact that the plasma AST levels, assessed as a hepatic injury biomarker, were not increased in the OZ rats. Previously, it was reported that the levels of plasma free fatty acids (FFA) were increased in OZ rats compared with LZ rats (data not shown) [26]. It has been known that plasma FFAs enter the hepatocytes and are either oxidized or esterified. An enhanced esterification is accompanied by increased TG

production, secretion and accumulation in the liver [27]. It has been reported that dislipidemic changes occur in obesity and may be due to the increased TG content of the liver due to increased influx of excess FFAs into the liver [28].

It is commonly recognized that the GSH/GSSG ratio is the most important redox couple and plays crucial roles in antioxidant defense, nutrient metabolism, and the regulation of pathways essential for whole body homeostasis [29]. The results obtained revealed that the liver GSH/GSSG ratio decrease was due to GSSG accumulation and not to the modification of the GSH content. GSH effectively scavenges free radicals and other ROS, and it is oxidized to form GSSG, which is then reduced to GSH by the NADPH-dependent GR enzyme. In this case, GR mRNA expression and enzyme activity results were correlated with the GSH levels obtained. In contrast, the gene expression of the enzyme responsible for GSH synthesis de novo, Gclc, was significantly upregulated in the OZ group. This increase is necessary to reestablish the GSH content, which can be decreased during the detoxification of some metabolites produced within the cell by oxidative stress by the action of the GST enzyme. In agreement with this idea, the enzymatic activity of GST was slightly increased in the OZ animals. Accordingly, OZ animals did not show an increase in liver ROS production. This could be the result of an effective ROS inactivation by GSH oxidation. Furthermore, it is well known that the liver is able to store significant quantities of lipids in conditions associated with prolonged excess energy consumption or impaired fatty acid metabolism manifested as steatosis [30]. Therefore, the liver TG accumulation is not necessarily reflected in an increase in ROS production. In agreement with our results, it has been reported that the increased TG accumulation in human hepatoblastoma cells does not perturb ROS formation [31].

Liver MDA+4-HAE levels in LZ rats were unexpectedly higher than in the OZ group. It is well known that Zucker fatty rats are genetically obese due to a mutation in the leptin receptor [32]. Therefore, as Richards, et al. corroborated, the leptin plasma levels were markedly increased in obese Zucker rats compared with lean rats [33]. In agreement, Sailaja, et al. [34] reported that leptin administration to mice fed a high fat diet significantly reduced the level of TBARs in the liver. In addition, they showed that an increase in leptin content elevated levels of glutathione related enzymes in the liver. Activation of these enzymes may be a compensatory mechanism and may be one of the reasons for the lowered lipid peroxidation observed in the liver. In this context, studies have shown that decreased lipid peroxidation in biological membranes is associated with increased GPx activity [35]. Therefore, the activation of cellular antioxidant defenses could be the reason for the decreased lipid peroxidation in obese Zucker rats. In our study, the increase in the GPx activity of OZ rats could be

interpreted as a positive feedback mechanism reflecting a favorable response of the organism to oxidative stress [36].

Several studies of natural compounds revealed positive effects on obesity-related hyperlipidemia, hypertension and hyperinsulinemia, as well as reductions in vascular, adipose and plasmatic proinflammatory and prooxidant biomarkers [37, 38]. Additionally, it has been reported that phenolic compounds, including resveratrol, can reduce fatty acid availability in the liver and protect this organ from hepatic steatosis-induced oxidative stress [6].

Various authors have noted a high variability in polyphenol intake, for example, in Spain the total consumption of catechins and proanthocyanidins dimers and trimmers has been estimated at 18-31 mg/day while the intake of monomer flavonols in Holland is significantly higher (50mg/day) [39].

In this sense, a moderate dose of GSPE (≈17.5 mg/day) was assessed as an antioxidant agent using a model of genetic obesity. It has been reported that flavanols and their metabolites provided from the grape seed proanthocyanidin extract (GSPE) are specifically accumulated in different organs such as liver, adipose tissue and muscle [16]. Moreover, rat plasma obtained after GSPE ingestion was analyzed using a liquid chromatography-tandem mass spectrometry method. Conjugated forms were identified and quantified, founding different concentrations of catechin and epicatechin glucuronide, epicatechin methyl glucuronide and epicatechin methyl-sulfate. Furthermore, monomers, dimers and trimmers in their native form also were detected and quantified in plasma samples. Thus, flavanols predominantly exist in their modified form in plasma even though the intact molecules have been found at micromolar level [40].

In this context, dietary antioxidants have long been suspected to scavenge reactive ROS and thereby avert deleterious effects on proteins, lipids, and nucleic acids in cells. This has been put forward as one of the major mechanisms for the disease-preventing effects of fruits and vegetables [15]. It has been reported that modulation of intracellular GSH concentrations is a possible disease preventing effect of polyphenols, with the implication that they modulate GSH-dependent cellular processes, including detoxification of xenobiotics, glutathionylation of proteins, and regulation of redox switching of protein functions in major cellular processes [41]. The liver is the largest gGlutathione reservoir. The parenchymal cells synthesize GSH for P450 conjugation and numerous other metabolic requirements—then export GSH as a systemic source of SH-reducing power [42].

Polyphenols may be regarded as xenobiotics by animal cells and are to some extent treated as such, ie, they interact with phase I and phase II enzyme systems. In this sense, despite GSPE contains several xenobiotic compounds and polyphenols are known to interact with oxidative cytochrome P450, it has been also shown that GSPE has the ability to inhibit several cytochrome P450 enzymes [43]. In consequence, the absence of utilization of GSH pool could also be produced due to the minor GSH conjugation to the xenobitoitcs due to the polyphenol inhibitory effect our results were no decrease on GSH content was observed.

Interestingly, the GSSG increase in OZ animals was abrogated by GSPE administration. Our results showed that GSPE treatment significantly reduced the GSSG levels to the LZ group levels. This reduction resulted in a total GSH/GSSG ratio increase compared with OZ rats. Furthermore, GSPE did not modify the GSH content and did not change the expression of GR mRNA, although the GR activity was significantly decreased. The GST activity was significantly decreased by the GSPE treatment compared with OZ animals, and the Gclc mRNA levels were slightly downregulated. In this context, it seems that the de novo GSH synthesis as well as GSSG increases are counteracted by the GSPE effect, suggesting a “glutathione-like” effect of the polyphenol extract.

In addition, the slight decrease in the enzyme activity caused by the treatment with GSPE could be considered to be an indicator of an improvement in the redox homeostasis by the polyphenol content in GSPE. It has been reported that GSPE can act as a signaling compound by regulating the antioxidant enzyme system [44, 45].

Despite the beneficial GSPE effect on the GSH cycle, the OZ-induced hepatic steatosis was not reversed by the GSPE treatment. Nevertheless, the plasma AST levels were not changed in the OZ+GSPE animals. In this regard, it has been reported that GSPE administration was not able to decrease the liver C-reactive protein (CRP) after 10 weeks of treatment [46] and that the chronic inflammation induced by obesity [47] was not reduced.

The GSPE antioxidant capacity was confirmed by assessing the total antioxidant capacity in rat plasma. Our results revealed that the total antioxidant capacity was decreased in the plasma of the OZ rats, resulting in oxidative stress in these animals. It has been reported in the ATTICA study that obesity is critically correlated with decreased antioxidant capacity irrespective of age, metabolic and various lifestyle variables, and therefore, lifestyle or pharmaceutical actions that modify the redox state can be potentially useful therapeutic agents for obesity associated metabolic syndrome or other clinical conditions, including diabetes mellitus type II, that lead to atherosclerosis [48]. The reduction of ORAC in the OZ group was related to increased oxidative stress markers, including increased GSSG levels and the resulting decreased

GSH/GSSG ratios and increased GPx activity. On the contrary, the total antioxidant capacity was significantly enhanced in the OZ+GSPE rat plasma in accord with the decrease in GPx activity and the improvement in the GSH metabolism. Accordingly, Kesh, et al. showed that the total antioxidant capacity in the livers of obese mice was increased after supplementation with ferulic acid, an abundant phenolic compound present in the seeds of many plants, which ameliorates oxidative stress related to obesity [49].

In general, the antioxidant defense system maintains or restores redox homeostasis. It was reported that to cope with higher oxidative stress, the activities of antioxidant enzymes, including catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase, were significantly increased in animals fed with high fat diets. However, the animals treated with antioxidant compounds showed lower antioxidant enzyme activities, which might be due to the oxidative stress-reducing capacity and inhibition of the generation of superoxide anions and hydroxyl free radicals [50].

The reduction of these enzymes could be a consequence of this attempt to regulate the body's redox balance [51]. Some studies also reported that consuming a polyphenol-rich diet decreased scavenger enzymes such as Cu,Zn-Sod and GPx. The implication is that a regular diet, rich in exogenous antioxidants, could reduce the dependence on endogenous antioxidants [52, 53]. In our study, in agreement with Shrivastava, et al. [50], the activity of some antioxidant enzymes was increased in the obese Zucker rats to counteract the GSSG increase. Furthermore, the GSPE treatment lowered the enzyme activities, including GST, GR and GPx, due to the direct antioxidant effect of the polyphenol extract, which made it unnecessary to activate the cellular antioxidant defense.

In conclusion, in Zucker rats the obesity-induced oxidative stress related to liver glutathione alteration was mitigated by GSPE administration.

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Manuscript # mnfr.201300455 entitled "Grape seed proanthocyanidin extract improves the hepatic glutathione metabolism in obese Zucker rats" which you submitted to Molecular Nutrition & Food Research has been reviewed. The comments of the referees are included at the end of this email.

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Once again, thank you for submitting your work to Molecular Nutrition & Food Research. I look forward to receiving your revision.

Sincerely,

Prof. Hans-Ulrich Humpf

Editor-in-Chief, Molecular Nutrition and Food Research

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** Referee(s)' and Editors Comments to Author. (Please note that these comments may have been delivered to us as files. We attach a description at the bottom how to access them.)

Reviewer: 1

Comments to the Author

This is a nice work, performed by a top group in the field of procyanidins and health in animal models. The work is sound, despite its limited focus on GSH. The evidence of a significant effect obtained after the administration of procyanidins is clear, but the authors fail to hypothesize relevant mechanisms which keep into account the fact that intact prcyanidins are too big to be absorbed in quantity and act at systemic level or at hepatic level. The sole "antioxidant hypothesis is not sufficient. A paragraph discussing their metabolites (microbial and hepatic) must be added to face this conundrum. Several recent work have investigated the transformations undergone by procyanidins in detail. A very complete review on phenolics and health, also summarizing these aspects, has recently become a reference for the topic.

The first sentence of the discussion is misleading (antioxidant capacity and activity?).

In some figures the letters are missing from above the bars. This would be OK if the authors assume that there is no significant difference among the values, but then, in some cases, three "a" are reported, making the whole thing mysterious.

Reviewer: 2

Comments to the Author

This manuscript describes results regarding hepatic glutathione metabolism and liver function in response to treatment of rats with an extract from grape seeds, GSPE. The focus is on the possible GSPE mediated strengthening of cellular antioxidant defense against reactive oxygen species. Several observations are possibly interesting, however, the manuscript appears premature and key information is lacking. There is no information on how GSPE was administered, per os? Gavage? Injections?

No information on the actual intake (if per os) or levels of GSPE polyphenols in plasma or liver tissue.

LZ rats should have been treated with extract, not only OZ rats, to relate observations specifically to oxidative stress in obesity.

GSPE contains several xenobiotic compounds and polyphenols are known to interact with oxidative cytochrome P450 enzymes and may in fact be metabolized by these enzymes and subsequently conjugated to glutathione. This is not adequately discussed.

Several figures show statistically non-significant differences and are redundant.

Information on how RT-PCR was performed is inadequate, no primer sequences or cycling parameters listed.

Discussion is lengthy and unfocused,

Editor: Stevens, Jan Frederik

3.2 DHA sensitizes FaO cells to *tert*-BHP-induced oxidative effects. Protective role of EGCG.

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(Submitted to Food Chemical Toxicology)

DHA sensitizes FaO cells to *tert*-BHP-induced oxidative effects. Protective role of EGCG.

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Keywords: Docosahexaenoic acid/ Epigallocatechin-3-gallate/ Heme oxygenase-1/ Lipid peroxidation/ Nrf2 / Oxidative stress

ABSTRACT

The excessive production of reactive oxygen species has been implicated in several pathologies, such as atherosclerosis, obesity, hypertension and insulin resistance. Docosahexaenoic acid (DHA) may protect against the abovementioned diseases, but paradoxically the main DHA treated pathologies are also associated with increased ROS levels. Therefore, the aim of this study was to explore if in vitro DHA supplementation may increase the sensitivity of cells to tert-BHP induced oxidative stress, and if the green tea polyphenol epigallocatechin-3-gallate (EGCG) is able to correct such detrimental effect. We found that DHA-enriched cells exacerbate ROS generation, decrease cell viability and increase Nrf2 nuclear translocation and HO-1 expression. Interestingly, cellular EGCG is able to counteract oxidative damage from either tert-BHP or DHA-enriched cells. In consequence, our results suggest that in a ROS enriched environment DHA could not always be beneficial for cells and can be considered a double-edged sword in terms of its benefits versus risks. In this sense, our results propose that the supplementation with potent antioxidant molecules could be an appropriate strategy to reduce the risks related with the DHA supplementation in an oxidative stress-associated condition.

Abbreviations: DCFH-DA, 2',7'-Dichlorofluorescin diacetate; **DHA,** Docosahexaenoic acid; **EGCG,** Epigallocatechin-3-gallate; **GSH,** Reduced glutathione; **GSSG,** Oxidized glutathione; **H_2O_2**, Hydrogen Peroxide; **HO-1**, Heme oxygenase-1; **LDH**, Lactate dehydrogenase; **MTT,** 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoilum bromide; **Nrf2,** Nuclear factor erythroid 2; **ROS,** Reactive oxygen species; ***tert*-BHP,** *tert*-Butylhydroperoxide; **TBST-T,** Tris-Buffered saline with Tween.

1 INTRODUCTION

Oxygen free radicals are generated continuously within mammalian cells as a direct consequence of aerobic metabolism and respiration (Llopiz et al., 2004). The excessive production of reactive oxygen species (ROS) is associated with cellular damage and the development of chronic diseases in humans. In this sense, oxidative stress has been implicated in metabolic syndrome-related conditions, including atherosclerosis, obesity, hypertension and insulin resistance. Therefore, compounds with antioxidant properties may be useful for preventing diseases related to oxidative stress (Yang et al., 2011).

One of these compounds are flavonoids, which are naturally occurring polyphenolic compounds that are abundant in fruits, vegetables, nuts, medical herbs, and beverages such as tea, coffee, and red wine. Epigallocatechin-3-gallate (EGCG) is the most abundant polyphenol in green tea and present antioxidant and antinflammatory capacity (Na and Surh, 2008; Yang et al., 2011).

Furthermore, EGCG is an effective ROS scavenger in vitro and may also function indirectly as an antioxidant through its effects on transcription factors, such as the redox-sensitive transcription factor nuclear factor erythroid 2 p45 (NF-E2)-related factor (Nrf2) (Na and Surh, 2008), and its effects on enzyme activities (Sahin et al., 2010).

On the other hand, it is well known that the long-chain omega-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic (EPA), and docosahexaenoic (DHA) acid from fish oil (Kang et al., 2010; Mozaffarian and Wu, 2011) have a beneficial effect on lowering mortality due to cardiovascular issues or all general causes.

The prevention of ischemia/reperfusion liver injury by omega-3 PUFAs has also been established. Furthermore, recent studies suggest that the anti-steatotic effects of omega-3 PUFAs in the liver include directing fatty acids away from triglyceride storage with the promotion of their oxidation as well as an enhanced glucose flux to glycogen synthesis (Valenzuela et al., 2012).

Despite the benefits associated with increased omega-3 fatty acid consumption, it had been shown that fish could be a potential source of human exposure to toxic contaminants (Domingo et al., 2007). Moreover, there remains a theoretical concern that these fatty acids may increase lipid peroxidation (Mas et al., 2010). The peroxidation reaction is a degenerative process that mainly affects unsaturated membrane lipids, such as an omega-3 enriched membrane, under oxidative stress conditions. In consequence, non-enzymatic free-radical peroxidation of DHA is

predicted to generate reactive species analogous to those formed from arachidonic acid (Gao et al., 2007). In this sense, it has been shown that DHA is rapidly incorporated into membrane phospholipids by a variety of cell. Interestingly, oxidative stress is a common factor in the etiology of the diseases impacted by DHA and EPA. Furthermore, it has been demonstrated that the early-stage and transient generation of ROS by DHA might activate the signaling pathway that lead to Nrf2 nuclear translocation and eventual HO-1 induction (Yang et al., 2013).

In this sense, the redox-sensitive transcription factor Nrf2 plays a key role in the upregulation of many antioxidant/detoxifying enzymes and it is necessary for the induction of numerous phase II enzymes (Yang et al., 2011), achieved through its interaction with antioxidant-response element (ARE) or the electrophile-responsive element . Under basal conditions, Nrf2 is retained in the cytosol by association with the ubiquitin ligase adaptor Keap1. The Keap1-Nrf2 complex is disrupted in response to several electrophilic antioxidants, and free Nrf2 is translocated to the nucleus. There, it binds to the ARE, increasing gene expression of antioxidant/detoxifying enzymes including glutamate-cysteine ligase (GCL), glutathione S-transferase (GST), and heme oxygenase-1 (HO-1) (Kong et al., 2001). In addition to inducing several phase II detoxifying enzymes, Nrf2 is also involved in the *de novo* synthesis of various antioxidant enzymes responsible for protection against cytotoxicity caused by oxidative stress, and pro-inflammatory insults (Itoh et al., 2004). It has been recently reported that non-enzymatic DHA oxidation induces the formation of cyclopentenone-containing J-ring isoprostanes that could stabilize Nrf2 levels and induce Nrf2-directed gene expression (Gao et al., 2007).

HO-1, a key target of Nrf2, belongs to the family of heme oxygenases that catalyzes the oxidative degradation of heme to biliverdin, which is subsequently converted to bilirubin and equimolar amounts of carbon monoxide and iron (Brand et al., 2010). HO-1 is known for its cytoprotective effects against oxidative stress. The upregulation of HO-1 by DHA supplementation (Lu et al., 2010) and the down or upregulation of HO-1 by EGCG, depending on the concentration of this polyphenol (Na and Surh, 2008), has been reported.

Therefore, the aim of this study was to explore if *in vitro* DHA supplementation may increase the sensitivity of cells to *tert*-BHP induced oxidative stress, and if the green tea polyphenol EGCG is able to correct such detrimental effect.

2 MATERIALS AND METHODS

2.1 Chemicals

(-)-Epigallocatechin-3-gallate from green tea, *cis*-4,7,10,13,16,19-docosahexaenoic acid , fatty acid free bovine serum albumin (BSA), cell culture Nutrient Mixture F-12 Coon's Modification medium, *tert*-butylhydroperoxide solution (*tert*-BHP), 2',7'-dichlorofluorescin diacetate (DCFH-DA), dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoilum bromide (MTT), protease/phosphatase inhibitors and Bradford reagent were obtained from Sigma-Aldrich (Madrid, Spain). Cell culture reagents were obtained from BioWhittaker (Verviers, Belgium).

2.2 Cell culture

FaO cells are a subclone of the H4-11-E-C3 rat hepatoma obtained from Reuber H35 (Sigma-Aldrich). The cells were routinely cultured in Nutrient Mixture F-12 Coon's Modification medium containing 10% (v/v) Fetal Bovine Serum (FBS), 1% (v/v) penicillin (100 U/ml) / streptomycin (100 µg/ml) and 0.1% (v/v) of fungizone. Cells were grown in a 5% CO_2 humidified atmosphere at 37 °C. The growth medium was replaced every 2 days. On the day of the experiment, FaO cells were preincubated with EGCG, DHA or EGCG + DHA in serum-free medium conjugated with BSA (molar ratio 4:1) at final concentration of 50 µM for 1 h. Control cells were treated with only the natural products vehicle (ethanol) at a nontoxic final concentration, ≤0.1%. After removal of the medium, oxidative stress was induced by incubating the cells with 100 µM *tert*-BHP for 3 h, except for the control group, which was treated with the vehicle (DMSO) at a nontoxic concentration. Cells were lysed with lysis buffer (0.1% Triton X-100 and 25 mM Hepes, pH 7 in PBS) for subsequent LDH, TBARS, GSH/GSSG and catalase assays.

2.3 Lactate dehydrogenase assay

Cell toxicity produced by different *tert*-BHP concentrations and by EGCG and DHA treatments was assessed by relative LDH medium content. For this assay, FaO cells were seeded in 12-well plates ($2.5x10^5$ cells/well). At 90% confluence, cells were treated as described previously, and the cell toxicity was determined using an LDH liquid kit (QCA, Spain) according to the manufacturer's instructions. The percentage of LDH present in the medium was calculated using the following formula:

% LDH = ((mU medium) / (mU medium*mU cells))*100

2.4 Viability assay

Cell viability was assessed using MTT assay. For this assay, FaO cells were seeded in 96-well plates ($5x10^4$ cells/well) for 48 h. The cells were treated as described previously, and cell viability was determined by the MTT assay (Alley et al., 1988). The yellow water-soluble MTT was added to each well at 1 mg/ml final concentration and incubated at 37 °C for 2 h. Formazan crystals were dissolved in DMSO, and absorbance was determined at λ=570 nm in an Eon Microplate Spectrophotometer (Biotek, USA). The results were expressed as relative cell viability (%).

2. 5 Determination of intracellular ROS generation by DCFH assay

The intracellular ROS formation was detected using the fluorescent DCFH-DA (Wang and Joseph, 1999). For this assay, the cells were seeded in a 96 well-black plate (5 x 10^4 cells/well) for 48 h. Cells were preincubated with 50 µM of EGCG, DHA and/or EGCG + DHA for 1 h before being incubated with 20 µM DCFH-DA for 30 min at 37 °C. Thereafter, the cells were treated with 100 µM *tert*-BHP, and ROS production was measured over 3 h using a FLx800 Multi-Detection Microplate Reader (Biotek, USA) at λ_{ex}=485 nm and λ_{em}=530 nm. Cellular ROS levels were expressed as relative fluorescence units (%).

2.6 Thiobarbituric acid-reactive substances assay

The TBARS method was used to determine malondialdehyde (MDA) levels (Buege and Aust, 1978). Briefly, cell lysates were mixed with 6 volumes of phosphoric acid and 2 volumes of thiobarbituric acid, mixed and heated for 45 min in boiling water. After cooling, the chromogen was extracted in 8 volumes of butanol. The absorbance of the organic phase was determined at λ=535 nm. The MDA levels were calculated using a 1,1,3,3-tetraethoxypropane (Bradford, Sigma) standard curve and normalized by protein content (Bradford, Sigma). The results were expressed as nmols MDA / mg protein.

2.7 Glutathione ratio measurement

The determination of the GSH/GSSG ratio and the GSSG levels are useful non-enzymatic indicators of oxidative stress in cells and tissues (Tietze, 1969). Briefly, the reaction of GSH with Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid (DTNB)) generates a product that can be quantified spectrophotometrically at λ=412 nm. This reaction is used to measure the reduction of GSSG to GSH. The rate of the reaction is proportional to the concentration of GSH and GSSG. For this assay, a GSH/GSSG GT-40

kit was used according to the manufacturer's instructions (Oxford Biomedical Research, Barcelona, Spain).

2.8 Catalase Activity

The determination of CAT activity is a useful enzymatic indicator of oxidative stress in cells and tissues. Briefly, cell lysates, 30 mM H_20_2 (Panreac, Spain) and 50 mM of phosphate buffer were mixed in a quartz cuvette, and the absorbance was measured for 30 s. The enzymatic activity was calculated using the H_20_2 molar extinction coefficient (ε=0.0394 $mM^{-1}cm^{-1}$).

2.9 Nuclear and cytoplasmic isolation

Nuclear and cytoplasmic fractions were isolated from treated cells using previously described method (Martinez-Micaelo et al., 2011) with some modifications. Briefly, the cells were washed twice with cold PBS. Then, the cells were lysed for 10 min using 300 µl of lysis buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 0.4% NP-40, protease inhibitor (1/1000) and phosphatase inhibitor (1/100)). The lysates were centrifuged at 22000 g for 3 min. The resulting supernatant was collected and stored at -80 °C as the cytoplasmic fraction. The nuclear pellet was resuspended in 50 µl of lysis buffer B (20 mM Hepes, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, protease inhibitor (1/1000) and phosphatase inhibitor (1/100)), and incubated on ice in a shaker for 1 h. Finally, the nuclear extract was obtained by centrifugation at 22000 g for 5 min, and the resulting supernatant was stored at -80 °C. The protein concentrations of the cytoplasmic and nuclear fractions were determined by the Bradford assay.

2.10 Western Blot analyses

Aliquots of the cytoplasmic (20 µg protein) and nuclear fractions (15 µg protein) were analyzed by Western blot (WB). Briefly, the samples were placed in sample buffer (0.5 M Tris–HCl pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% (v/v) 2-β-mercaptoethanol, 0.05% bromophenol blue) and denatured by boiling at 95-100 °C for 5 min. The samples were then separated by electrophoresis on 10% SDS polyacrylamide gels. The proteins were subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) using a transblot apparatus (Bio-Rad). The membranes were blocked for 1 h with 5% non-fat milk dissolved in TBST buffer (50 mM Tris, 1.5% NaCl, 0.2% Tween 20, pH 7.5). They were then incubated overnight at 4 °C with primary monoclonal antibodies against HO-1 (ADI-OSA-150-F, Enzo Life Sciencies), Nrf2 (ADI-KAP-TF125-F, Enzo Life Sciencies), α-Tubulin (T3526, Sigma) and LaminA/C (L1293, Sigma). The blots were

washed thoroughly in TBST buffer and incubated for 1 h with an anti-rabbit peroxidase-conjugated IgG antibody (Amersham Biosciences, GE Healthcare). Immunoreactive proteins were visualized using an enhanced chemiluminescence substrate kit (ECL plus; Amersham Biosciences, GE Healthcare) according to the manufacturer's instructions. Digital images were taken with a GBOX Chemi XL 1.4 image system (Syngene). Band quantification was performed with Image J software (NIH, USA). The results were expressed as relative intensity (HO-1/α-Tubulin and Nrf2/LaminA/C).

2.11 RNA isolation and cDNA synthesis

Total RNA was obtained from FaO cells using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. A DNase I/RNAase-free kit (High Capacity complementary DNA Reverse transcription kit, Applied Biosystems, Madrid, Spain) was used to remove the genomic DNA from the RNA preparations. RNA was quantified by spectrophotometry (Nanodrop 1000 Spectrophotometer, Thermo Scientific) at an absorbance of λ=260 nm and tested for purity (by A260/280 ratio) and integrity (by denaturing gel electrophoresis). The cDNA was subsequently amplified by PCR using specific TaqMan Assay-on-Demand Probes for HO-1 (Rn01536933_m1) and cyclophilin peptidylprolyl isomerase A (PPIA) (Rn00690933_m1).

2.12 Real-time RT-PCR

Quantitative PCR for HO-1 and PPIA was performed using the TaqMan PCR Core Reagent Kit according to the manufacturer's protocol and analyzed on a Real-Time 7300 PCR System, both from Applied Biosystems. The thermal cycling comprised an initial step at 50 °C for 2 min, followed by a polymerase activation step at 95 °C for 10 min and a cycling step with the following conditions: 40 cycles of denaturing at 95 °C for 15 s and annealing at 60 °C for 1 min. The relative expression levels of the target gene were measured using PPIA mRNA as an internal control according to the $2^{-\Delta\Delta}$Ct method.

2.13 Statistical analysis

The results are expressed as the mean±SEM of triplicate measurements representative of three independent experiments. SPSS Statistics version 19 software (Inc., Chicago, IL) was used for the statistical analysis. Significant differences were analyzed by one-way ANOVA followed by the Tukey post-hoc test. Different letters indicate statistically significant differences among different groups when p values were less than 0.05.

3 RESULTS

3.1 EGCG prevents DHA-associated toxicity and viability

To investigate the effect of *tert*-BHP on cell death, FaO cells were treated with increasing concentrations of *tert*-BHP (10, 25, 50, 75, 100 and 200 µM) for 3 h. Figure 1A shows the LDH leakage related to *tert*-BHP concentrations. The 100 µM *tert*-BHP concentration was chosen for the rest of the experiments (~40% cell death in the LDH assay). Furthermore, the toxicity of EGCG and DHA controls were also tested. Neither EGCG nor DHA showed any significant toxicity as assessed by LDH leakage (2.8±0.26 for vehicle vs. 2.9±0.1 for EGCG + DHA), shown in Figure 1B, or by the MTT assay (100±2.7 for vehicle vs. 110.4±4.4 for EGCG + DHA), shown in Figure 1C.

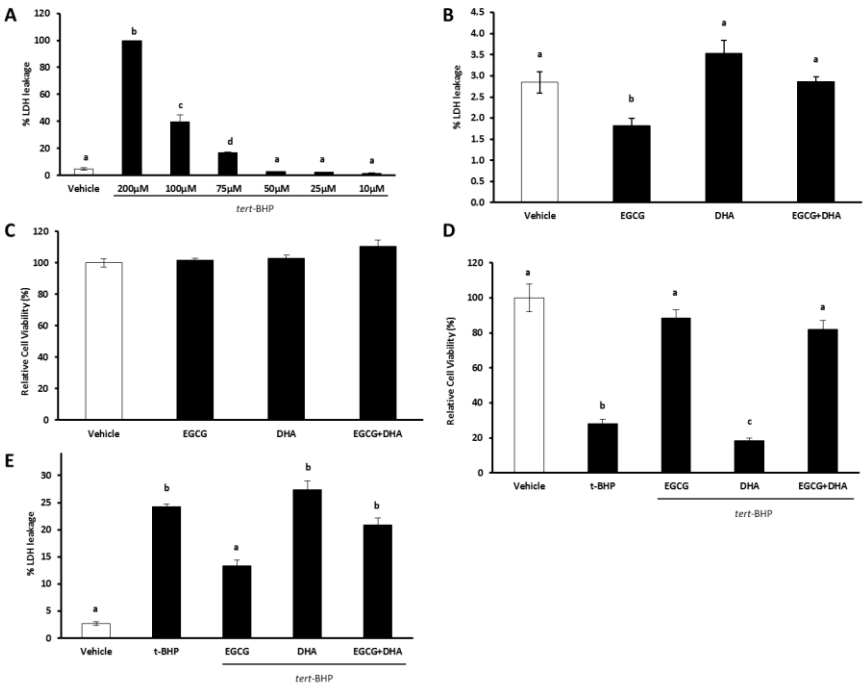

Figure 1. EGCG prevents DHA-associated toxicity and viability. A. Cells were incubated with increasing concentrations (10, 25, 50, 75, 100 and 200 µM) of tert-BHP for 3 h, and cell toxicity was assessed by the LDH method. B,C. Cells were treated with 50 µM of EGCG, DHA and/or EGCG + DHA for 24 h, and cell death and viability was assessed by the LDH and MTT methods, respectively. D, E. Cells were preincubated with 50 µM of EGCG and DHA and EGCG + DHA for 1 h and then treated with 100 µM of tert-BHP for an additional 3 h. Viability and cell death was

assessed by the MTT and LDH methods, respectively. The results are expressed as the mean±SEM from triplicate measurements representative of three independent experiments. Different letters indicate statistically significant differences ($p<0.05$) among different groups.

We also investigated the protective effect of EGCG against *tert*-BHP-induced oxidative damage on DHA-enriched FaO cells. As shown in Figure 1D, pretreatment with 50 µM EGCG significantly prevented the *tert*-BHP-induced viability decrease (28.1±2.7 for *tert*-BHP vs. 88.6±4.7 for EGCG; $p<0.05$). Fifty micromolar DHA was not able to prevent the *tert*-BHP-induced viability decrease and elicited an increase in the deleterious effect on the cell compared to *tert*-BHP alone (28.1±2.7 for *tert*-BHP vs. 18.6±1.3 for DHA; $p<0.05$). Interestingly, when cells were pretreated with EGCG + DHA, the oxidative insult (*tert*-BHP) did not induce any significant oxidative damage (28.1±2.7 for *tert*-BHP vs. 82.2±4.8 for EGCG + DHA; $p<0.05$), preventing the DHA-associated decrease in cellular viability. The LDH results followed the same pattern in the EGCG and DHA pretreatments as those observed in the MTT assay, and the EGCG+DHA pretreatment decreased the LDH content but not significantly. (Figure 1E).

3.2 EGCG reverts DHA-associated ROS production

Oxidative stress is largely mediated by intracellular ROS production. Cellular ROS were measured by the DCFH assay. Figure 2A shows the increase in fluorescence produced by the ROS inducer *tert*-BHP over 3 h.

Moreover, the assayed cell pretreatments had different effects on ROS production over 3 h (Figure 2A). After 3 h of the oxidative damage insult (Figure 2B), 50 µM EGCG pretreatment reduced the *tert*-BHP-induced ROS production by 23% (100 for *tert*-BHP vs. 77.5±3.5 for EGCG; $p<0.05$). In contrast, 50 µM DHA significantly induced ROS production at higher levels than that produced by *tert*-BHP treatment (100 for *tert*-BHP vs. 123.4±4.3 for DHA; $p<0.05$). Nevertheless, when cells were pretreated with EGCG + DHA, ROS production was decreased compared to treatment with *tert*-BHP alone (100 for *tert*-BHP vs. 81.3±3.6 for EGCG + DHA; $p<0.05$).

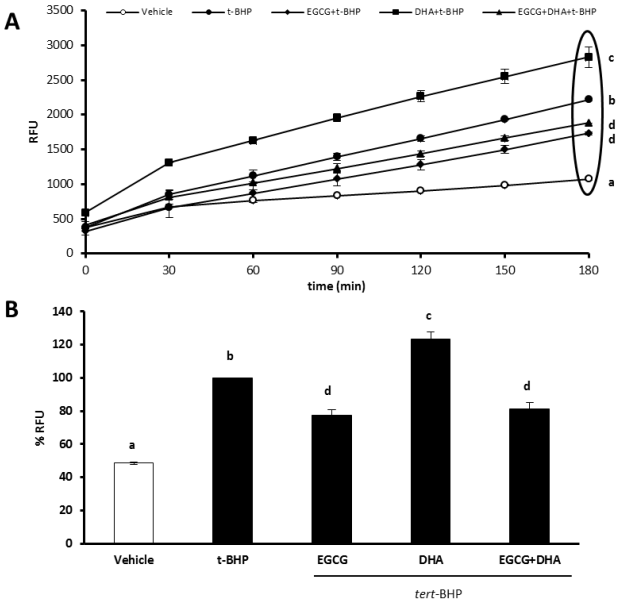

Figure 2. EGCG reverts DHA-associated ROS production. A. Cells were preincubated with 50 µM of EGCG, DHA and/or EGCG + DHA for 1 h and then exposed to 100 µM of *tert*-BHP for 3 h while ROS production was measured. B. ROS production is represented after 3 h of *tert*-BHP treatment. The results are expressed as the mean±SEM from triplicate measurements representative of three independent experiments. Different letters indicate statistically significant differences ($p < 0.05$) among different groups.

3.3 EGCG reduces DHA-related MDA increase

To study the effect of EGCG on lipid peroxidation on DHA-enriched FaO cells, the MDA level was assessed. At 3 h, cells treated with 100 µM *tert*-BHP presented an increase in the MDA cellular content, indicating oxidative damage to cell lipids. Interestingly, when cells were pretreated with EGCG for 1 h, MDA levels were decreased (81.7±8.5 for *tert*-BHP vs. 50.5±2.2 for EGCG; $p<0.05$). Conversely, DHA pretreatment promoted MDA production (36.7±3.1 for vehicle vs. 83.6±8 for DHA). In addition, EGCG + DHA pretreatment slightly decreased MDA levels (81.7±8.5 for *tert*-BHP vs. 58.9±2.9 for EGCG + DHA; $p<0.05$) (Figure 3A).

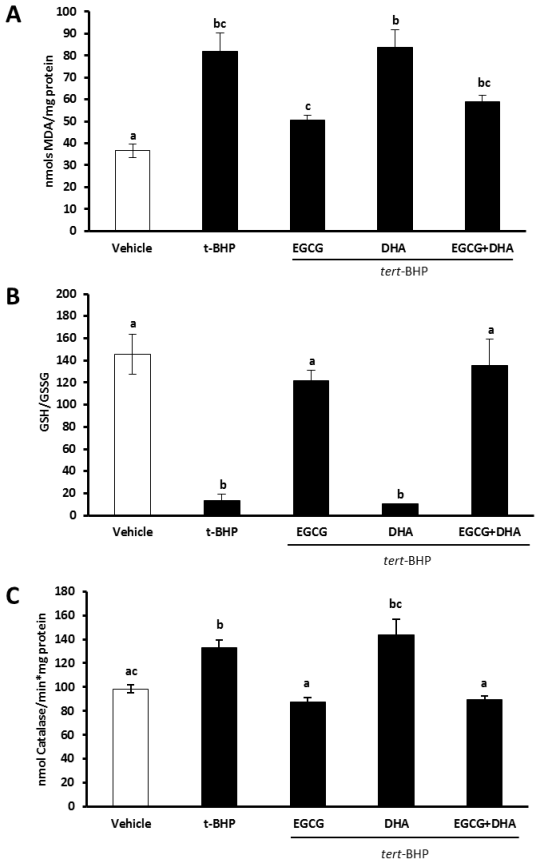

Figure 3. EGCG avoids the DHA related oxidative damage. Cells were preincubated with 50 µM of EGCG, DHA and/or EGCG + DHA for 1 h and then treated with 100 µM *tert*-BHP for 3 h. MDA levels were measured by TBARS assay (A), the GSH/GSSG ratio was assessed using a GSH/GSSG specific kit (B) and the catalase activity was assessed by an enzymatic method (C). The results are expressed as the mean±SEM of triplicate measurements representative of three independent experiments. Different letters indicate statistically significant differences ($p < 0.05$) among different groups.

3.4 EGCG improves the DHA-related GSH/GSSG ratio

We investigated changes in the GSH/GSSG ratio as indicator of non-enzymatic antioxidant defense. As expected, hepatocytes treated with 100 µM *tert*-BHP had a significantly decreased GSH/GSSG ratio compared to the untreated cells (145.5±17.9

for vehicle vs. 13.2±6.3 for *tert*-BHP; p<0.05). Moreover, 50 µM DHA presented the GSH/GSSG ratio similar result to that obtained by *tert*-BHP treatment. In contrast, 50 µM EGCG counteracted the *tert*-BHP effect with regard to the GSH/GSSG ratio (13.2±6.3 for *tert*-BHP vs. 121.9±9.5 for EGCG; p<0.05). Interestingly, pretreatment with EGCG + DHA rescued the GSH/GSSG ratio levels compared with DHA pretreatment (10.3±0.1 for DHA vs. 135.6±23.5 for EGCG+DHA; p<0.05) (Figure 3B).

3.5 EGCG avoids increased catalase activity associated with DHA

The catalase activity was measured as an indicator of the enzymatic antioxidant defense of cells treated with EGGC and/or DHA followed by *tert*-BHP treatment. Treatment with 100 µM *tert*-BHP (98.5±3.4 for vehicle vs. 133±6.7 for *tert*-BHP; p<0.05) and pretreatment with 50 µM DHA (98.5±3.4 for vehicle vs. 143.9±13.2 for DHA) slightly increased catalase activity compared with vehicle, although was not differences versus tert-BHP group. As previously observed, EGCG was able to protect against the pro-oxidant effect of DHA. When cells were pretreated with EGCG + DHA, the catalase activity significantly decreased compared to DHA treatment (143.9±13.2 for DHA vs. 89.7±2.5 for EGCG + DHA; p<0.05), suggesting a potent protective effect of EGCG against *tert*-BHP-induced DHA oxidation and the concomitant increase in damage induced by oxidized PUFAs (Figure 3C).

3.6 EGCG impairs DHA-related Nrf2 nuclear translocation and decreases HO-1 protein levels

To study the Nrf2 signaling pathway, we studied the nuclear translocation of this transcription factor by WB of isolated nuclear fractions, as well as the HO-1 (one key target of Nrf2) mRNA and protein levels of cytoplasmic fraction by Q-PCR and WB, respectively. *tert*-BHP treatment (100 µM) significantly induced the protein expression of HO-1 in response to oxidative damage (0.64±0.09 for vehicle vs. 1.00±0.07 for *tert*-BHP, p<0.05); and compared to *tert*-BHP alone, DHA pretreatment induced an increase in HO-1 protein content, although the increase was not significant (1.00±0.07 for *tert*-BHP vs. 1.12±0.07 for DHA). Interestingly, compared with DHA pretreatment, the addition of EGCG slightly decreased the HO-1 protein level (1.12±0.07 for DHA vs. 0.86±0.05 for EGCG and DHA) (Figure 4A).

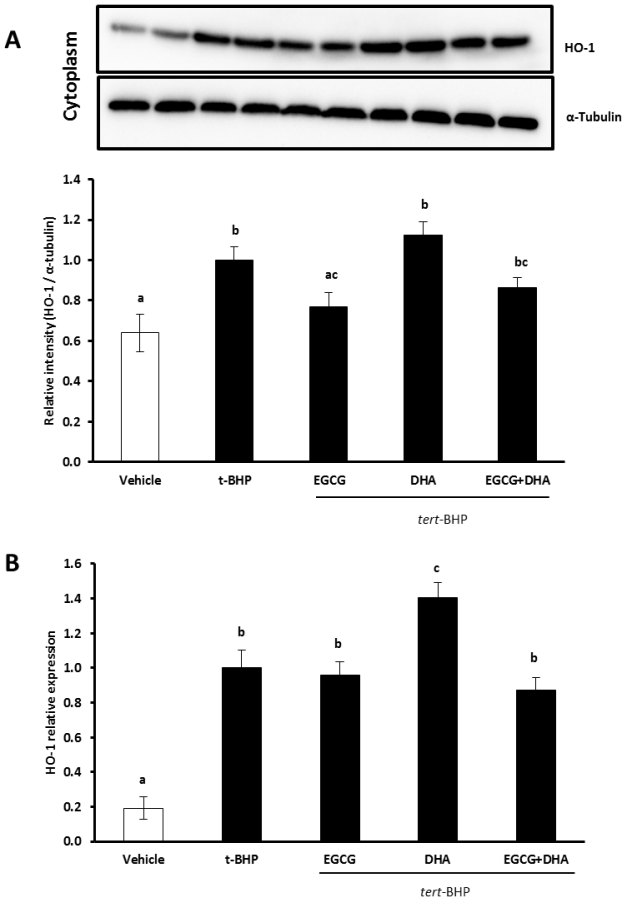

Figure 4. EGCG decreases the induced HO-1 protein level associated with DHA. Cells were preincubated with 50 µM of EGCG, DHA and/or EGCG+DHA for 1 h and then treated with 100 µM *tert*-BHP for additional 3 h. A. The cytoplasm fraction was isolated, and the HO-1 protein content was analyzed by Western blot with a specific antibody (upper panel: Western blot image; lower graph: relative image quantification). B. The HO-1 mRNA level was analyzed by real-time RT-PCR. The results are expressed as the mean±SEM from triplicate measurements representative of three independent experiments. Different letters indicate statistically significant differences ($p < 0.05$) among different groups.

Additionally, mRNA levels of HO-1 were also studied (Figure 4B). The HO-1 mRNA level was increased by the oxidant treatment compared with the vehicle treatment (0.19±0.06 for vehicle vs. 1.00±0.1 for *tert*-BHP, $p<0.05$). As expected, DHA pretreatment significantly upregulated the HO-1 expression compared to *tert*-BHP

alone (1.00±0.1 for *tert*-BHP vs. 1.41±0.08 for DHA, p<0.05). On the other hand, pretreatment with EGCG alone was not capable of counteracting the *tert*-BHP effect (1.00±0.1 for *tert*-BHP vs. 0.96±0.07 for EGCG). In contrast, treatment with EGCG + DHA partially abrogated the non-enzymatic oxidation of DHA relative to the HO-1 mRNA levels (0.87±0.07 for EGCG + DHA vs. 1.41±0.08 for DHA, p<0.05).

The Nrf2 nuclear translocation was assessed by WB of the nuclear fraction. As shown in Figure 5, *tert*-BHP induced Nrf2 translocation to the nucleus (0.42±0.14 for vehicle vs. 1.00±0.16 for *tert*-BHP, p<0.05). Additionally, DHA pretreatment significantly activated Nrf2 nuclear translocation comapred with control group (0.42±0.14 for vehicle vs. 0.73±0.07 for DHA, p<0.05).although presented similar results those obtained by *tert*-BHP treatment. In agreement with the abovementioned HO-1 results, when cells were preincubated with EGCG + DHA, a small decrease in Nrf2 nuclear translocation appeared, although the oxidative insult (*tert*-BHP) was still present (0.76±0.01 for EGCG + DHA vs. 1.00±0.16 for *tert*-BHP).

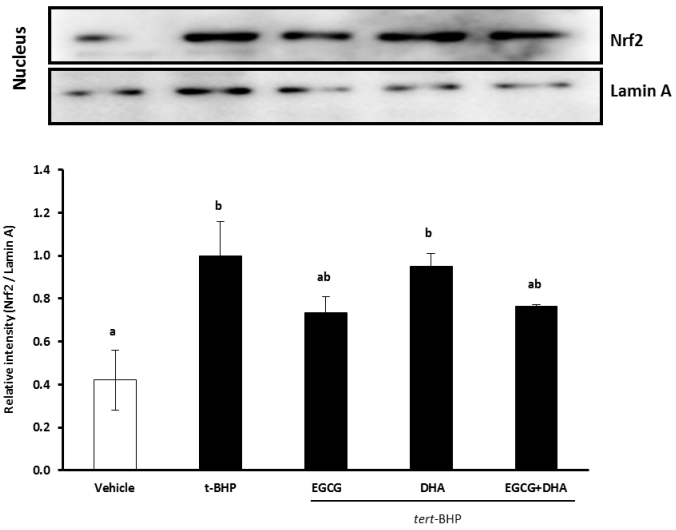

Figure 5. EGCG impairs Nrf2 nuclear translocation related to DHA. Cells were preincubated with 50 µM of EGCG, DHA and/or EGCG + DHA for 1 h and then treated with 100 µM *tert*-BHP for additional 3 h. The nuclear fraction was isolated, and Nrf2 protein content was analyzed by Western blot with specific antibody (upper panel: Western blot image; lower graph: relative image quantification). The results are expressed as the mean±SEM from triplicate measurements representative of three independent experiments. Different letters indicate statistically significant differences (p< 0.05) among different groups.

4 DISCUSSION

The major finding of this study is that a potent oxidative insult will affect FaO cells differently depending on their DHA content. We found that DHA-enriched cells are more susceptible to oxidative insults, resulting in an exacerbation of ROS generation and related damage and increasing Nrf2 nuclear translocation and HO-1 expression. Interestingly, we also demonstrated that the polyphenol EGCG is able to avoid cellular sensitivity to *tert*-BHP-induced oxidative damage related with DHA enrichment.

DHA has been postulated as a therapeutic option for several important pathologies such as obesity or cardiovascular diseases (Calder, 2012). Additionally, the importance and beneficial health effects of the enrichment of PUFAs on occidental diets have been addressed in several studies (Shek et al., 2012; Urquiaga et al., 2004). In all these cases, it is assumed that there is no concomitant oxidative stress, but the importance of such a phenomenon must not be neglected. Paradoxically, oxidative stress is a common factor in the etiology of the diseases impacted by DHA treatment.

Polyphenols have been shown to exhibit a variety of biological properties. In these sense, it has been shown that EGCG presents several biological and pharmacological properties including free radical scavenging activity, antioxidant actions, iron-chelating capabilities and attenuation of lipid peroxidation due to various forms of radicals (Guo et al., 1996).

As previously mentioned, the first objective was to study the effects of an oxidative cellular environment on DHA-enriched hepatic cells. We observed that omega-3 pretreatment was not able to revert the oxidative damage induced by *tert*-BHP, and interestingly, DHA pretreatment exacerbated the oxidative damage as shown in in cell viability, ROS and HO-1 results compared with tert-BHP group. Moreover, DHA pretreatment presented oxidative stress production compared with control group as shown in TBARS, GSH/GSSG ratio, catalase activity and Nrf2 nuclear translocation. In this sense, it has been previously reported that DHA is rapidly incorporated into membrane phospholipids, particularly into phosphatidylcholine and phosphatidylethanolamine (Vericel et al., 2003), and it enhances the liver susceptibility to lipid peroxidation. This susceptibility is thought to be attributable to the substitution of membrane fatty acids with potentially unstable omega-3 PUFAs, especially DHA (Kubo et al., 1998). Additionally, several clinical studies have reported marked increases in blood antioxidant enzymes, such as catalase, in patients taking omega-3 PUFAs, and in some cases, lipid peroxidation levels increased in parallel with the elevation in these enzymes to mitigate the pro-oxidant effect (Allard et al., 1997).

Interestingly, Palozza and coauthors (Palozza et al., 1996) showed that the substitution of omega-6 PUFAs with potentially unstable omega-3 PUFAs may shift the membrane fatty acid composition toward a higher degree of unsaturation and enhance membrane susceptibility to lipid peroxidation. The consequential increased production of lipid peroxides may be deleterious to membrane integrity and may also result in the accumulation of degradation products of peroxidized lipids.

Additionally, Maheo and coauthors (Maheo et al., 2005) showed that DHA induced a potent sensitizing effect to doxorubicin cytotoxicity on breast cancer cells. Although the anticancer drug doxorubicin is able to exert its cytotoxic effect by intercalation into DNA and initiation of DNA damage via the inhibition of topoisomerase II, it is also effective due to its ability to strongly induce ROS generation. Interestingly, DHA enrichment increased lipoperoxidation and strengthened the effect of other mechanisms involved in doxorubicin cytotoxicity. Although *tert*-BHP could not be compared to doxorubicin in terms of cytotoxic potential, they share the ability to generate ROS, as well as its ability to generate peroxyl, alkoxyl and methyl radicals that catalyze the peroxidation of membrane lipids (Deiana et al., 2010). Therefore, the fact that a DHA-rich cellular environment is detrimental in the presence of elevated ROS levels is not exclusive of an anticancer drug therapy and could be relevant in non-homeostatic situations, such as pathologic states, where disruption of the antioxidant balance has occurred.

Numerous compounds with antioxidant properties have the ability to neutralize the oxidative stress that accompanies several pathologies and therefore have been proposed as therapeutic agents to counteract cellular damage. The hypothetical protection of a polyphenol, such as EGCG, against the observed cellular DHA sensitivity to *tert*-BHP-induced oxidative effects became interesting due to its hypothetical ability of avoiding, or at least decreasing, the activation of the antioxidant machinery that fights oxidative damage.

Interestingly, as reflected by the results of cells pretreated with EGCG + DHA, the polyphenol is able to mitigate the cellular DHA-related oxidant effects. EGCG + DHA pretreatment prevented the decrease in cell viability as well as abrogated the *tert*-BHP-induced oxidative damage on omega-3-enriched cells through restoration of the antioxidant cell defenses and the avoidance of pro-oxidant damage. As far as we know, this is the first report that demonstrates a positive effect of EGCG on DHA-pre-enriched cells exposed to an oxidative stressor. Nevertheless, other authors have previously described the concomitant effects of other antioxidants related to the final beneficial output of DHA. Brand et al. (Brand et al., 2010) demonstrated that in cell culture model systems, the beneficial effects of DHA supplementation were primarily

observed when DHA was supplied in conjunction with the liposoluble antioxidant vitamin E which will mainly affect membrane oxidative status.

In addition, it has been reported that EGCG binds to the interface of membranes and that the protective effects are either due to its scavenging activity against peroxides before attacking membranes and/or to the in situ avoidance of the oxidation of the membrane lipids (Saffari and Sadrzadeh, 2004). EGCG interaction with peroxy radicals and inhibition of lipid peroxidation was also suggested by Katiyar and Mukhtar (Katiyar and Mukhtar, 1997). We hypothesize that this membrane context, EGCG blocks TBARS formation by acting as a ‘‘chain blocker’’, interrupting the propagation of lipid peroxidation in the membranes, and that a part of the EGCG effect could also be due to its scavenging activity, which would be expected to remove a portion of *tert*-BHP before its interaction with membrane lipids. Furthermore, we must emphasize that, due to the absence of EGCG co-incubation with *tert*-BHP, a polyphenol effect is produced by the EGCG molecules previously located in either the cellular membrane compartment or the intracellular compartment.

Nrf2 is considered a master regulator of antioxidant defense mechanisms. Some authors have shown that the increased Nrf2 nuclear translocation could be a beneficial strategy due to the concomitant upregulation of phase II enzymes and, consequently, the opportunity for enhanced antioxidant protection (Reuland et al., 2012). Therefore, decrease on Nrf2 nuclear translocation could be an indicator of ROS clearance.

The cellular DHA supplementation promoted an increased sensitivity to *tert*-BHP induced oxidative stress, as reflected by the upregulation of HO-1. On the other hand, Nrf2 nuclear translocation was slightly inhibited when cells were pretreated with EGCG + DHA. Interestingly, HO-1 protein expression followed the same pattern as that observed in the Nrf2 Western blot. In this regard, the HO-1 mRNA levels confirmed the protective effect of EGCG. In agreement with our results, it has been demonstrated that when oligodendroglial cells were treated with DHA in combination with the antioxidant vitamin E, HO-1 upregulation was inhibited. This protective effect was not only due to its antioxidant capacity but also to its direct influence on the fatty acids profile of cell membranes, possibly by upregulation of specific elongases and desaturases, which are enzymes that are involved in omega-3 biosynthesis (Brand et al., 2010).

Based on these results, we propose the following model (schematic overview in Figure 6). A) First, in the absence of pretreatments (EGCG and/or DHA), the oxidative insult (*tert*-BHP) increases cellular ROS production, activates the lipid peroxidation reaction (MDA levels) and decreases the non-enzymatic antioxidant defense (GSH/GSSG). These ROS act as secondary metabolites and activate Nrf2 nuclear translocation,

inducing HO-1 and CAT expression. These antioxidant enzymes are activated as adaptive response to oxidative stress produced by *tert*-BHP. B) In omega-3 PUFA-pretreated cells, DHA is oxidized by *tert*-BHP-produced ROS. In an oxidative stress environment (*tert*-BHP), DHA is able to produce more oxidative damage. These oxidative inputs strongly induce Nrf2 nuclear translocation, which activates the antioxidant cell defense (HO-1 and CAT). C) In EGCG + DHA-pretreated cells, DHA is preserved due to the polyphenol effect, and fewer oxidative signaling mediators are generated; as a result, there is a decrease of the Nrf2 nuclear translocation and HO-1 expression compared to DHA pretreatment. EGCG is also able to decrease lipid peroxidation (MDA) and restore the GSH/GSSG ratio.

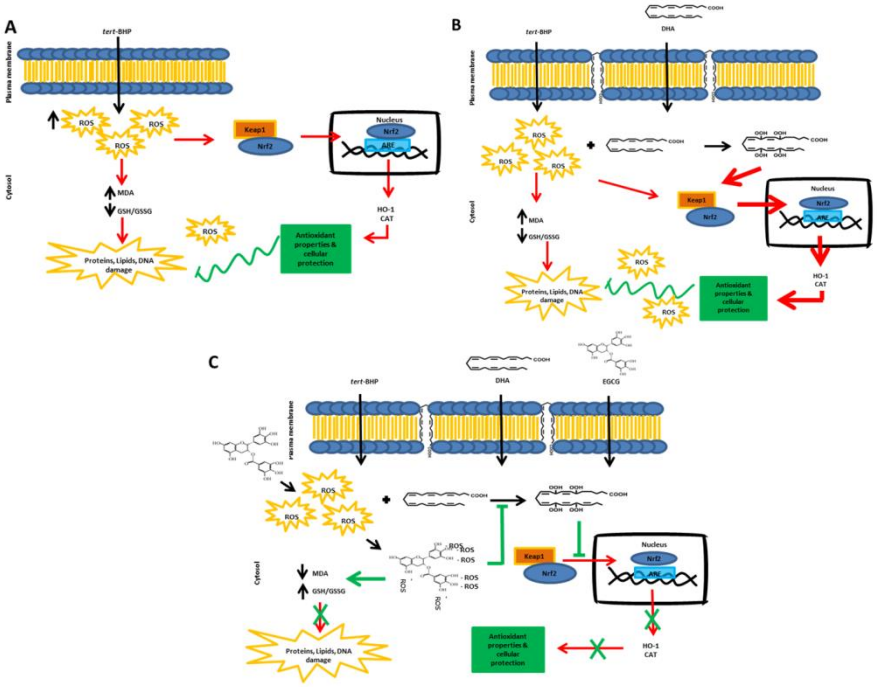

Figure 6. Schematic overview of EGCG protection against non-enzymatic oxidation DHA.

With regard to the doses used in this study, it has been reported that subjects consuming fish 1-2 times a month had a detectable plasma DHA level of 182 µM (Saw et al., 2010). The DHA concentration tested in this study (50 µM) is a bioavailable and nontoxic cellular concentration. On the other hand, several studies in human and rats are in controversy in respect to the bioavailability of flavanols in blood plasma. It can

vary depending on the molecules they joined to, the source of food used (apples, cocoa, or grapes), and the doses administrated (Manach et al., 2004). In fact, it has been reported that the levels of EGCG may reach concentrations of up to 1.5 µM in human plasma following 4 weeks of a single 800 mg oral dose (Chow et al., 2001). Furthermore, absorbed flavonoids are widely distributed and can be detected in the liver (Martin et al., 2013).

In consequence, the tested DHA concentration closes to the bioavailability levels found in plasma. In contrast, EGCG concentration could be approached by a supranutritional diet or a pharmacological intake, although it has been reported that EGCG is non-genotoxic when it was administered to animals at doses which are significantly higher than those intended for humans (Isbrucker et al., 2006). Moreover, one methodological limitation of the study was not to determine the range concentration where EGCG presented protective effect against DHA oxidative stress damage, as well as the exposition time and the period.

Finally, our results suggest that DHA molecules are not always beneficial for cells and can be considered a double-edged sword in terms of benefits and risks, especially for situations of sustained oxidative stress conditions. More specifically, in a ROS environment, pretreatment with potent antioxidant molecules in pure form such EGCG could be an appropriate strategy to reduce the risks related with the DHA supplementation. In conclusion, the presented results suggest that it would also be useful to consider the oxidative status when PUFAs will be administered.

However, despite of these results, it is important consider that new and complementary studies in other cell lines should be carried out to clarify the nature of the DHA effects on cellular oxidative stress.

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The authors declare no conflicts of interest.

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Food and Chemical Toxicology- Decision on Manuscript:

The review of your manuscript submitted to Food and Chemical Toxicology is complete. The reviewers have made an important number of suggestions that will improve the quality and significance of your manuscript. I encourage you to consider these comments and make an appropriate revision of your manuscript. The reviewers' comments are below.

Thank you, and we look forward to receiving your revised manuscript, within 45 days of this letter.

With best regards,

Jose L. Domingo, PhD

Editor Food and Chemical Toxicology

Reviewers' comments:

Reviewer #1: The study investigate the role of DHA in the sensibilization of FaO cells to tert-BHP-induced oxidative effects as well as if the EGCG supplementation could presents a protective role. Despite the relevance of the study, the authors neeed to clarify some methodological aspects as well as to review some structural aspects of the manuscrits.

In the methodology is not clear why the authors choose just one EGCG concentration (50 <mu>M)? Some antioxidant and other bioactive molecules could to present hormesis effect, so the "affirmative of protective effect related to determined chemical" need to consider de dose, period (pre-treatment, concomitantly treatment, etc) and time of exposition. For this reason, I suggest that authors include a sentence in the Discussion section comment this methodological limitation of the study that was not determined the "range concentration that EGCG presented protective role agains DHA oxidative stress sensibilization!

The increase of ROS levels per se is not necessarily a toxic effect. However, when some product decrease the cell viability this fact is important to be considered. In the Figure 1D the citotoxic effect of cells treated just with DHA and exposed to tert-BHP is well observed. So, please add this relevant information in the abstract.

The Figures are poor and need to be improved

Reviewer #3: Fernandez-Iglesias et al. studied the effect of DHA supplementation on the sensitivity of FaO cells to tert-BHP induced oxidative stress and if the supplementation of EGCG is able to counteract these effects.

One of the main conclusions is that DHA pretreatment exacerbated the oxidative damage induced by tert-BHP, as showed in TBARS, ROS, GSH/GSSG ratio and catalase activity results. However, only in the case of the production of ROS we observe a greater effect with regard to the treatment with tert-BHP alone. In other cases the effect is similar, with no significant differences between both groups. In different parts of the paper the DHA inducing effect is mentioned without considering that the proper comparison is to the group treated with tert-BHP and not with controls. So phrases like those mentioned below should be revised:

Results, section 3.1. "These results confirm the protective effect of EGCG on DHA oxidation and subsequent decrease in toxicity (Figure 1E)". This can be right for Figure 1D (higher decrease of cell viability with DHA pretreatment compared with tert-BHP alone), but not for Figure 1E.

Results, section 3.4. "Moreover, 50 µM DHA induced a significant decrease of the GSH/GSSG ratio similar to that obtained by tert-BHP treatment". DHA did not induce a further decrease compared with tert-BHP treated cells, so DHA has no effect on this parameter.

Results, section 3.5. "Pretreatment with 50 µM DHA slightly increased catalase activity". There are no significant differences between DHA+tert-BHP and tert-BHP treated cells.

Results, section 3.6. "Additionally, DHA pretreatment significantly activated Nrf2 nuclear translocation". The adequate comparison is with tert-BHP treated cells, not with controls.

The manuscript is potentially sound and interesting, but some of the Results and all the Discussion section should be revised to adequate the conclusions. There is a certain oxidative effect induced by DHA, as shown in figures 1D, 2 and 4B (when compared with tert-BHP treated cells), but not in the other figures. This differential effect of DHA should also be discussed.

Minor points: Abbreviations. There are some abbreviations as AA, ARE, BSA, EpRE, WB,... included in the text but not in the Abbreviations page; also, the meaning of the abbreviation is not, in all cases, explained in the text the first time it appears

mentioned (in some cases the meaning is not included at all, in other cases it is explained in a second or posterior apparition). This referee suggests the elimination of all the unnecessary abbreviations.

The text is too long. Materials and Methods, Results and the Discussion section should be considerably shortened.

Figure 6 is unnecessary and should be deleted.

All the figure legends and Materials and Methods, section 2.5. "Cells were preincubated with 50 µM of EGCG, DHA and or EGCG+DHA".

Reviewer #4: For a best comprehenssion by readers, could be very good include an up-to-date of references and discuss them.

3.3 Combination of grape seed proanthocyanidin extract and docosahexaenoic acid oil rich modifies hepatic antioxidant status in lipidic postpandrial state.

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(Submitted to British Journal of Nutrition. Under review)

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Keywords: GSH/ GST / Nrf2 / Docosahexaenoic acid omega-3 / Oxidative Stress / Postprandial / Proanthocyanidins

Abbreviations: ARE, Antioxidant response element; **AST,** Aspartate aminotransferase; **CVD**, Cardiovascular diseases; **DCFH-DA,** 2',7'-Dichlorofluorescin diacetate; **DHA,** Docosahexaenoic acid; **DHA-OR,** Oil rich in DHA; **EPA,** Eicosapentaenoic acid; **GSH,** Reduced glutathione; **GSPE,** Grape seed proanthocyanidin extract; **GSSG,** Oxidized glutathione; **HO-1,** Heme oxygenase-1; **MDA,** Malondialdehyde; **n-3,** Omega-3; **Nrf2,** Nuclear factor erythroid 2; **PUFAs,** Polyunsaturated fatty acids; **ROS,** Reactive oxygen species; **TG,** Triglycerides.

Abstract

The ingestion of dietary lipids leads to oxidative stress. This postprandial oxidative stress may potentiate the adverse effects of postprandial hyperlipidemia. The consumption of antioxidant compounds protects against oxidative stress. Proanthocyanidins have been shown to alleviate oxidative stress and hypertriglyceridemia associated with the postprandial state. Additionally, omega-3

polyunsaturated fatty acids (PUFAs) also have beneficial effects on lipoprotein metabolism and oxidative stress. The present study was designed to investigate the possible additive effects of an acute dose of grape seed proanthocyanidins extract (GSPE) and oil rich in docosahexaenoic acid (DHA-OR) on postprandial oxidative stress induced by the oral intake of lard oil in Wistar rats. GSPE+DHA-OR influenced GSH metabolism in the blood and liver, as well as increased Cu/Zn-SOD mRNA expression. It also abrogated Nrf2 nuclear translocation and modified several antioxidant enzymatic activities, such as GPx (decreased) and GST (increased). In conclusion, this study provides evidence that the combination of GSPE and DHA-OR modifies hepatic antioxidant status in the context of a single high-fat meal, ameliorating the transient imbalance between the lipid hydroperoxide level and antioxidant status related to a lipidic postprandial state.

1. Introduction

Postprandial oxidative stress is characterized by the increased susceptibility of the organism towards oxidative damage after the consumption of a meal rich in lipids and/or carbohydrates [1]. Accordingly, postprandial hyperlipidemia, with the associated elevated levels of triglycerides, chylomicron remnants, and free fatty acids, results in oxidative stress and inflammation and may independently potentiate the adverse effects of postprandial hyperglycemia [2]. Therefore, postprandial hyperlipidemia is a well-defined risk factor for atherosclerosis due to the increase in plasma lipid hydroperoxides produced in the postprandial phase [3].

Some studies have shown increases in postprandial oxidative stress through multiple markers [4; 5]. However, the postprandial plasma antioxidant status depends on factors such as the intake and consumption of compounds that are either oxidants or antioxidants [6]. Therefore, consumption of antioxidant compounds compliments the endogenous defense system in protecting against oxidative stress [7]. Consequently, proanthocyanidin (PA)-rich foods may alleviate the oxidative stress and hypertriglyceridemia associated with the postprandial state and may therefore improve tolerance of dietary lipids [8]. PAs are the oligomeric and polymeric forms of flavan-3-ol or flavanols. They are the most structurally complex subclass of flavonoids, one of the main constituents of phenolic intake in the diet, and are mainly provided by fruits, beans, nuts, cocoa, tea and wine [9]. It has been reported that some polyphenols present in grape seed extract prevent the plasma level increase of hydroperoxides produced in the postprandial state and, consequently, increase the plasma antioxidant capacity [6]. Moreover, grape seed proanthocyanidin extract (GSPE) protects rat hepatocytes from oxidative injury [10] and exerts anti-inflammatory effects [11]. Additionally, GSPE represses hepatic triglyceride (TG) secretion due to its hypotriglyceridemic action [8], reduces the plasma levels of TG-rich lipoproteins and improves the serum cholesterol profile in normolipidemic rats after 5 h of proanthocyanidin extract treatment [12].

On the other hand, it has been reported that the consumption of fish or fish oil rich in omega-3 (n-3) polyunsaturated fatty acids (PUFA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid reduces postprandial serum TG and is inversely associated with cardiovascular diseases (CVD) risk [13]. Recent investigations have suggested that both DHA and fish oil have beneficial effects on lipoprotein metabolism and oxidative stress [14].

To restore the cellular redox imbalance caused by an oxidative stress state, the activation of endogenous antioxidant enzymes is required. The transcription of these cytoprotective proteins is largely under the control of the nuclear factor erythroid 2-

related factor 2 (Nrf2), which plays a central role in the regulation of the cellular redox status [15]. Under normal cellular conditions, the cytosolic Nrf2-Keap1 complex is constantly degrading Nrf2. When the cell is exposed to oxidative stress, Nrf2 dissociates from the Nrf2-Keap1 complex, becomes stable, and translocates into the nucleus, leading to the activation of antioxidant response element (ARE)-mediated gene expression [16] of antioxidant enzymes, including heme oxygenase-1 (HO-1).

HO-1 belongs to the heat shock protein family and protects cells from oxidative stress by degrading toxic heme into the antioxidants biliverdin, free iron, and carbon monoxide. Subsequently, biliverdin is converted by biliverdin reductase into bilirubin [15]. HO-1 is present in the liver parenchyma, which filters blood to remove toxins [17].

Therefore, taking into account the antioxidant postprandial effect of flavonoids and n-3 PUFAs, the purpose of this study was to investigate the possible additive effect of GSPE and oil rich in DHA (DHA-OR) on the response to postprandial oxidative stress.

2. Materials and Methods

2.1 Chemicals

2',7'-dichlorofluorescin diacetate (DCFH-DA), reduced glutathione (GSH), Bradford, butylated hydroxytoluene (BHT), 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB), 2-vinylpyridine, metaphosphoric acid, glutathione reductase (GR), β-Nicotinamide adenine dinucleotide phosphate (NADPH), aprotinin, phenylmethanesulfonyl fluoride (PMSF), SODium deoxycholate, and SODium dodecyl sulfate-polyacrilamide (SDS) was purchased from Sigma-Aldrich (Madrid, Spain). Hydrogen peroxide (H_2O_2) was purchased from Panreac (Barcelona, Spain). The anesthetics ketamine and xylacine were obtained from Parke-Davis, Pfizer (Madrid, Sapin) and Bayer (Barcelona, Spain) respectively.

2.2 Grape seed proanthocyanidin extract

GSPE was provided by *Les Dérives Résiniques et Terpéniques* (Dax, France). According to the previous analyses [18], the GSPE contained catechin (58 µmol/g), epicatechin (52 µmol/g), epigallocatechin (5.50 µmol/g), epicatechin gallate (89 µmol/g), epigallocatechin gallate (1.40 µmol/g), dimeric procyanidins (250 µmol/g), trimeric procyanidins (15.68 µmol/g), tetrameric procyanidins (8.8 µmol/g), pentameric procyanidins (0.73 µmol/g), and hexameric procyanidins (0.38 µmol/g).

2.3 DHA Oil rich

Oil rich in n-3 DHA PUFA (DHA-OR) (Martek DHA^{TM}-S) was nutritional oil derived from marine algae *Schizochytrium* sp., that contained 38.8% of DHA and 1.1% of EPA. In addition the PUFAs n-6/n-3 ratio is 0.45.

2.4 Animals and experimental design

Healthy males Wistar rats weighting 300-350 g (Charles River Laboratories, Barcelona, Spain) were housed in cages by pairs at 22ºC with 12 h light/dark cycle and were fed ad libitum with standard chow diet (Panlab A-04, Barcelona, Spain) and water. After a week of adaptation, rats were divided in 4 experimental groups: vehicle, GSPE, DHA-OR and GSPE+DHA-OR (n=6). The animals were fasted for 14 h, and they were administrated with oral gavage lard oil (2.5 ml/kg body weight (bw)) to induce a postprandial state, with or without (control) an acute non-toxic dose of GSPE [19], DHA-OR [20] and GSPE+DHA-OR (250 mg/kg bw). After 5 h of administration, rats were anesthetized by ketamine (70 mg/kg bw) – xylazine (5 mg/kg bw) mixture and sacrificed by exsanguination (Figure 1). Blood was collected from abdominal aorta and plasma was obtained by centrifugation and stored at -80 °C until analyses. Liver was excised, weighed and frozen immediately in liquid nitrogen and stored at -80 °C until assays. The Animal Ethics Committee of our University approved all procedures.

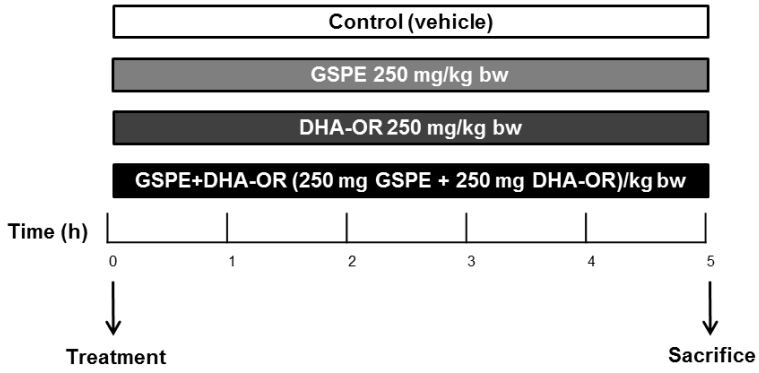

Figure 1. Schematic experimental design. Rats were fasted for 14 h, and they were administrated with oral gavage lard oil (2.5 ml/kg body weight (bw)) to induce a lipidic postprandial state, with or without (control) an acute non-toxic dose of GSPE, DHA-OR, and GSPE+DHA-OR (250 mg/kg bw). After 5 h, rats were sacrificed.

2.5 Liver lipid analysis

Liver lipids were extracted using the method described by Folch et al. [21]. An extract aliquot was subjected to gravimetry to determine total lipids. The TG and total cholesterol was assessed using enzymatic colorimetric kits (QCA, Tarragona, Spain) in the remaining extract, previously evaporated under a nitrogen draft and redissolved in a 2:1 mixture of chloroform and methanol and further diluted with NaCl (0.29% w/v) .

2.6 Assessment of liver function (AST)

Serum aspartate aminotransferase (AST) is an important liver function marker. The AST activity is based in the reaction of aspartate and α-ketoglutarate to oxaloacetate and glutamate and was measured according to the manufacturer's instructions of GOT/AST UV kit (QCA, Tarragona, Spain).

2.7 Lipid peroxidation in liver

Lipid peroxidation was determined in liver tissue quantifying malondialdehyde (MDA) content by colorimetric assay based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA to yield a stable chromophore with maximal absorbance at 586 nm [22]. For this assay, liver was homogenized in Tris-Buffer pH=7.4, and 10 µl of 0.5M BHT stock solution per 1 ml of homogenate was added to prevent sample oxidation. The homogenates were centrifuged at 3000 g for 10 min at 4 °C. Subsequently, MDA level was assessed using Lipid Peroxidation Microplate Assay Kit FR-22 according to the manufacturer's instructions (Oxford Biomedical Research, Barcelona, Spain).

2.8 GSH/GSSG ratio in plasma and liver

When cells are exposed to increased levels of oxidative stress, GSSG will accumulate and the ratio of reduced/oxidized glutathione (GSH/GSSG) will decrease. Therefore, the determination of the GSH/GSSG ratio is useful non-enzymatic oxidative stress marker. This parameter was assessed in whole blood and in liver homogenate. In the case of whole blood the GSH/GSSG ratio was assessed using the GSH/GSSG GT-40 kit according to the manufacturer's instructions (Oxford Biomedical Research, Barcelona, Spain). For the glutathione analyses present in liver homogenate, the enzymatic method reported by Griffith [23] was used. GSH was determined by following the rate of reduction of DTNB by GSH at λ=412 nm. On the other hand, the quantification of "GSSG only" could be measured due to the GSH scavenge by 2-vinylpyridine, a thiol-scavenging reagent to form a pyridinium salt. For this assay, liver was homogenized

and deproteinized with metaphosphoric acid 6% and centrifuged at 5000 g for 15 min at 4 °C. The supernatants were used to the total glutathione and GSSG analysis independently. A standard curve was conducted using increasing concentrations of GSH and the results were expressed as equivalents/g liver. The GSH/GSSG ratio was also calculated as an oxidative stress marker.

2.9 Glutathione peroxidase enzyme activity

GPx enzyme activity of liver was quantified spectrophotometrically by the method of Flohe and Gunzler [24]. Briefly, 50 mM potassium phosphate (pH 7.0), 1 mM EDTA, 2 mM SODium azide, liver homogenate, 10 mM GSH, 2.4 U ml $^{-1}$ GR and 1.5 mM NADPH was incubated at 37°C for 3 min. After the addition of 2 mM H_2O_2, the rate of NADPH consumption was monitored at at λ=340 nm, 37°C for 5 min. GPx activity was calculated using the extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ and expressed as µmol NADPH/min* mg protein.

2.10 Glutathione-*S*-transferase enzyme activity

Glutathione-*S*-transferase (GST) activity was spectrophotometrically determined [25] measuring the conjunction of 1-Chloro-2,4-dinitrobenzene (CDNB, Sigma-Aldrich, Madrid, Spain) with GSH. The CDNB-GSH conjunction was measured at λ=340 nm for 2 min. The results were expressed in nmol GDNB-GSH/min * mg protein.

2.11 Measurement of CRP levels

Acute phase proteins are plasma proteins which increase in concentration following infection, inflammation or trauma. One of these proteins is C-reactive protein (CRP) and the levels in serum rises following acute tissue damage. The CRP quantification in plasma can provide valuable information about tissue damage. Plasma CRP levels were determined using a specific enzyme immunoassay (ELISA) according to the manufacturer's instructions (Immunology Consultants Laboratory, Inc., USA).

2.12 Liver mRNA levels

2.13.1 mRNA isolation

Total RNA from liver was isolated using RNeasy Protect Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA was resuspended in 50 µL of RNase free water. DNase I RNAase free kit (High Capacity complementary DNA Reverse transcription kit (Applied Biosystems, Madrid, Spain) was used to remove the genomic DNA from RNA preparations. RNA was quantified by spectrophotometer (Nanodrop 1000 Spectrophotometer, Thermo Scientific) at at λ=260 nm, and tested for purity (by

A260/A280 ratio) and integrity (by denaturing gel electrophoresis). The cDNA was subsequently amplified by PCR using specific TaqMan Assay-on-Demand Probes for GPx (Rn00577994_g1), GST (Rn01757146_m1), Cu,Zn-SOD (Rn00566938_m1), p22phox (Rn00577357_m1), HO-1 (Rn01536933_m1), and Cyclophilin peptidylprolyl isomerase A (PPIA) (Rn00690933_m1).

2.13.2 Real-time RT-PCR

Quantitative PCR for GPx, GST, Cu,Zn-SOD, p22phox, HO-1 and PPIA was performed using the TaqMan PCR Core Reagent Kit according to the manufacturer's protocol, being analyzed on a Real-Time 7300 PCR System, all from Applied Biosystems (Madrid, Spain). The thermal cycling comprised an initial step at 50 °C for 2 min, followed by a polymerase activation step at 95 °C for 10 min, and a cycling step with the following conditions: 40 cycles of denaturing at 95 °C for 15 s, annealing at 60 °C for 1 min. The relative levels of expression of the target gene were measured using PPIA mRNA as an internal control according to the $2^{-\Delta\Delta Ct}$ method.

2.13 Cytoplasm isolation

Cytosolic protein fraction was isolated from liver of experimental rats. Tissue was homogenized in sucrose buffer (250 mM sacarose, 50 mM Tris-HCl, 5mM MgSO4, 1 mM PMSF, 0.5 µg/ml aprotinin, and pH 7.4) to obtain the cytoplasm fraction. SODium deoxycholate and 0.1% SDS were added to the cytoplasm fraction. The protein content was determined by Bradford assay.

2.14 Western Blot analyses

Aliquots of the cytoplasmic (100 µg protein) were analyzed by Western blot. Briefly, the samples were placed in sample buffer (0.5 M Tris–HCl pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% (v/v) 2-β-mercaptoethanol, 0.05% bromophenol blue) and denatured by boiling at 95-100 °C for 5 min. Then the samples were separated by electrophoresis on 10% SDS polyacrylamide gels. The proteins were subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) using a transblot apparatus (Bio-Rad). The membranes were blocked for 1 h with 5% non-fat milk dissolved in TBS-T buffer (50 mM Tris, 1.5% NaCl, 0.2% Tween 20, pH 7.5). Afterward, the membranes were incubated overnight at 4 °C with primary monoclonal antibodies against HO-1 (ADI-OSA-150-F, Enzo Life Sciencies), Nrf2 (ADI-KAP-TF125-F, Enzo Life Sciencies) and α-Tubulin (T3526, Sigma). The blots were washed thoroughly in TBS-T buffer and incubated for 1 h with an anti-rabbit peroxidase-conjugated IgG antibody (Amersham Biosciences, GE Healthcare). Immunoreactive proteins were visualized using an enhanced chemiluminescence substrate kit (ECL plus; Amersham Biosciences, GE

Healthcare) according to the manufacturer's instructions. Digital images were taken with a GBOX Chemi XL 1.4 image system (Syngene). Band quantification was performed with Image J software (NIH, USA). The results were expressed as relative intensity (HO-1/α-Tubulin and Nrf2/α-Tubulin).

3. Results

3.1 Plasmatic AST and CRP levels

Plasma AST was assessed as a liver function maker. GSPE and GSPE+DHA-OR treatments significantly decreased the AST levels compared to the vehicle group, which only received lard oil. The DHA-OR treatment slightly decreased the liver function marker levels compared with control group (Figure 2A).

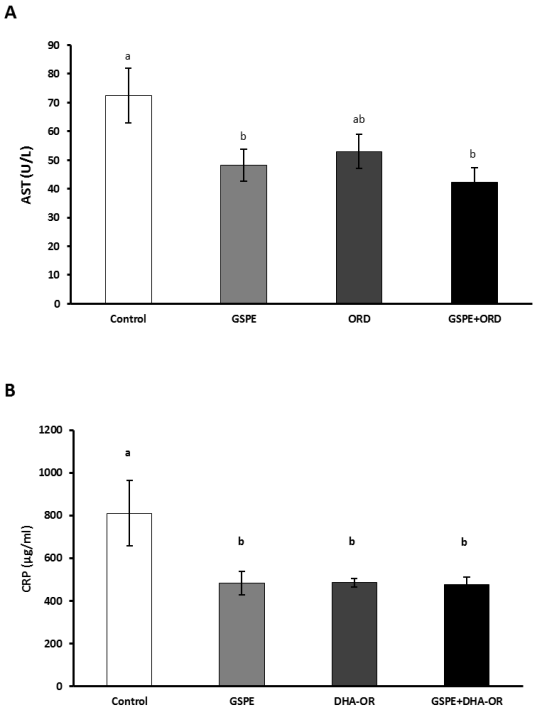

Figure 2. Plasmatic parameters. Plasmatic AST (A) and CRP (B) levels were assessed in experimental animals. The results are expressed as the mean±SEM for each group (n=6): control, GSPE (250 mg/kg), DHA-OR (250 mg/kg), and GSPE+DHA-OR. Different letters indicate statistically significant differences (p≤0.05) among different groups.

Moreover, plasma levels of CRP were assessed as a proinflammatory marker. As shown in Figure 2B, all treatments reduced the CRP plasma levels compared with animals that only received lard oil, revealing the anti-inflammatory properties of GSPE and DHA-OR; however, treatment with both compounds together did not show an additive effect.

3.2 Hepatic lipids

Total lipids, TGs and total cholesterol were assessed to study the effect of the treatments on hepatic lipids. Table 1 shows that hepatic TG was significantly decreased by the DHA-OR and GSPE+DHA-OR treatments after 5 h of administration. Liver cholesterol was slightly decreased by DHA-OR, although the difference was not significant (Table 1).

Hepatic lipid profile	Liver Postprandial state			
Mean±SEM	Control	GSPE	DHA-OR	GSPE+DHA-OR
Total Lipids (g/100 g liver)	4.76±0.39 a	5.32±0.24 a	4.66±0.48 a	4.09±0.46 a
Triglycerides (g/100 g liver)	1.87±0.18 ab	2.1±0.29 a	1.46±0.03 b	1.29±0.12 b
Total Cholesterol (g/100 g liver)	0.73±0.04 a	0.74±0.08 ab	0.48±0.04 b	0.64±0.09 ab

Table 1. Hepatic lipid profile. Total lipids, triglycerides and total cholesterol levels were assessed in in livers of rats fed lard oil with or without an acute dose of GSPE, DHA-OR and GSPE+DHA-OR. The results are expressed as the mean±SEM for each group (n=6). Different letters indicate statistically significant differences ($p \leq 0.05$) among different groups.

3.3 Hepatic lipid peroxidation

To evaluate the oxidative stress-related effects, liver lipid peroxidation was assessed. Lipid peroxidation is related to cellular ROS production; we did not find any significant change in liver MDA (Figure 3).

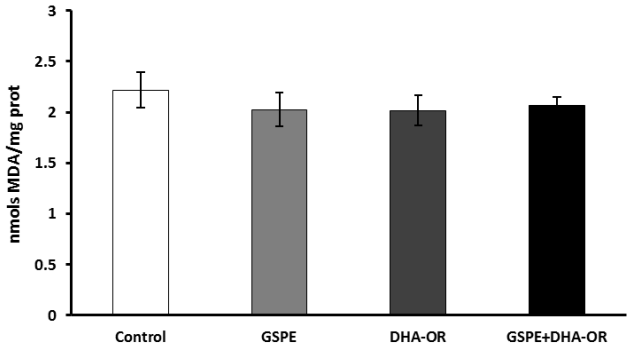

Figure 3. Hepatic lipid peroxidation. . Lipid peroxidation was determined by measuring MDA levels in liver homogenates. The results are expressed as the mean±SEM for each group (n=6): control, GSPE (250 mg/kg), DHA-OR (250 mg/kg), and GSPE+DHA-OR. Different letters indicate statistically significant differences ($p \leq 0.05$) among different groups.

3.4 Glutathione metabolism in blood and liver

Glutathione, which is present in high concentrations in the liver, plays an important role in detoxifying reactions. Therefore, we studied changes in the GSH/GSSG ratio in the blood and liver as a marker of non-enzymatic antioxidant defenses.

GSH levels in the blood were significantly increased by GSPE+DHA-OR treatment, but GSSG levels were not affected by different treatments. Accordingly, the GSH/GSSG ratio was significantly increased by GSPE+DHA-OR (Figure 4 A-B-C).

Interestingly, when glutathione levels were assessed in the liver, the results obtained were different from those found in blood. The GSH content was significantly decreased by DHA-OR and GSPE+DHA-OR. GSPE slightly decreased the GSSG levels and GSPE+DHA-OR slightly increased the hepatic GSSG levels. These changes resulted in a slight increase in the GSH/GSSG ratio after GSPE treatment and a significantly decreased GSH/GSSG ratio after GSPE+DHA-OR administration (Figure 4 D-E-F).

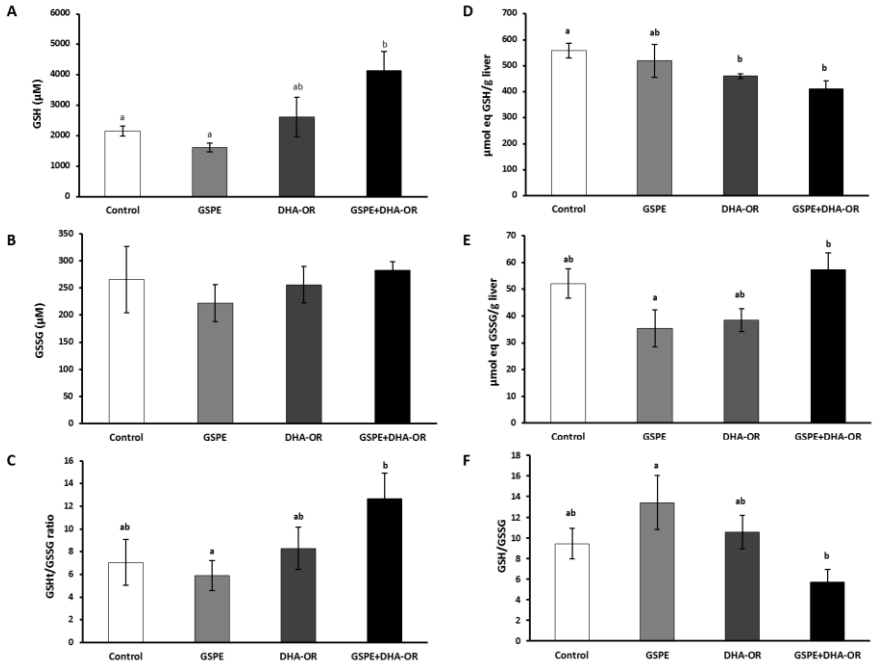

Figure 4. GSH metabolism. Total GSH/GSSG (C, F) was estimated by assessing total GSH (A, D) levels and GSSG content (B, E) in blood and liver homogenates. The results are expressed as the mean±SEM for each group (n=6): control, GSPE (250 mg/kg), DHA-OR (250 mg/kg), and GSPE+DHA-OR. Different letters indicate statistically significant differences (p≤0.05) among different groups.

3.5 Liver oxidative stress parameters

Due to the role that GPx plays in GSH metabolism, the activity and mRNA expression of the enzyme were assessed. Although the GPx mRNA expression was not altered by the different treatments, the GPx activity was significantly decreased by GSPE+DHA-OR treatment.

Moreover, due to the irreversible consumption of GSH by GST, the activity and mRNA expression of the GST enzyme were determined. GST mRNA expression was slightly decreased by the different treatments, whereas the enzyme activity was significantly increased by the DHA-OR supplementation.

We assessed the Cu/Zn-SOD mRNA levels as a marker of antioxidant enzymatic defenses. This enzyme removes the peroxide radical (O2-) by converting it to oxygen. The GSPE+DHA-OR treatment significantly upregulated the Cu/Zn-SOD mRNA levels.
mRNA levels of the NADPH-oxidase subunit p22phox were analyzed as a marker of oxidative stress. Our results showed that the GSPE, DHA-OR and GSPE+DHA-OR treatments downregulated the expression of the p22phox gene (p≤0.01).

All the results are shown in Table 2.

Liver oxidative stress parameters	Control	GSPE	DHA-OR	GSPE+DHA-OR	
Relative mRNA expression					ANOVA p-value
GPx	0.98±0.05	1.11±0.17	1.11±0.13	1.17±0.17	0.05
GST	1±0.18	0.75±0.04	0.73±0.04	0.82±0.08	0.05
Cu,Zn-SOD	1.01±0.06 a	1.12±0.08 ab	1.01±0.03 a	1.28±0.09 b	0.05
p22phox	1.25±0.13 a	0.99±0.02 b	0.93±0.08 b	0.86±0.08 b	0.1
Activity enzyme					
GPx (µmol/min*mg prot)	334.65±6.65 a	327.88±17.45 ab	316.09±10.77 ab	288.25±8.78 b	0.05
GST (nmol/min*mg prot)	434.31±5.55 a	450.99±25.66 ab	480.48±3.12 b	587.48±13.57 c	0.05

Table 2. Liver oxidative stress parameters. mRNA expression of GPx, GST, Cu,Zn-SOD and p22phox was analyzed by real-time RT-PCR. The relative levels of expression of the target gene were measured using PPIA mRNA as an internal control according to the $2^{-\Delta\Delta Ct}$ method. Liver GPx and GST activities were also assessed. The results are expressed as the mean±SEM for each group (n=6): control, GSPE (250 mg/kg), DHA-OR (250 mg/kg), and GSPE+DHA-OR. Different letters indicate statistically significant differences (p≤0.05) among different groups.

3.6 Hepatic HO-1 and Nrf2 protein expression

Western blot analyses showed that the hepatic HO-1 accumulation in the cytoplasmic fraction was significantly decreased by GSPE+DHA-OR treatment. The GSPE and DHA-OR treatments slightly decreased HO-1 protein expression (Figure 5A).
The GSPE+DHA-OR treatment inhibited Nrf2 nuclear translocation as a result of an increase in the Nrf2 accumulation in the cytoplasm of liver cells, which is cause by the stabilization of Nrf2 by the Keap1 protein. The cytoplasmic accumulation of Nrf2 was not changed by the GSPE treatment, but it was slightly increased by DHA-OR administration (Figure 5B).

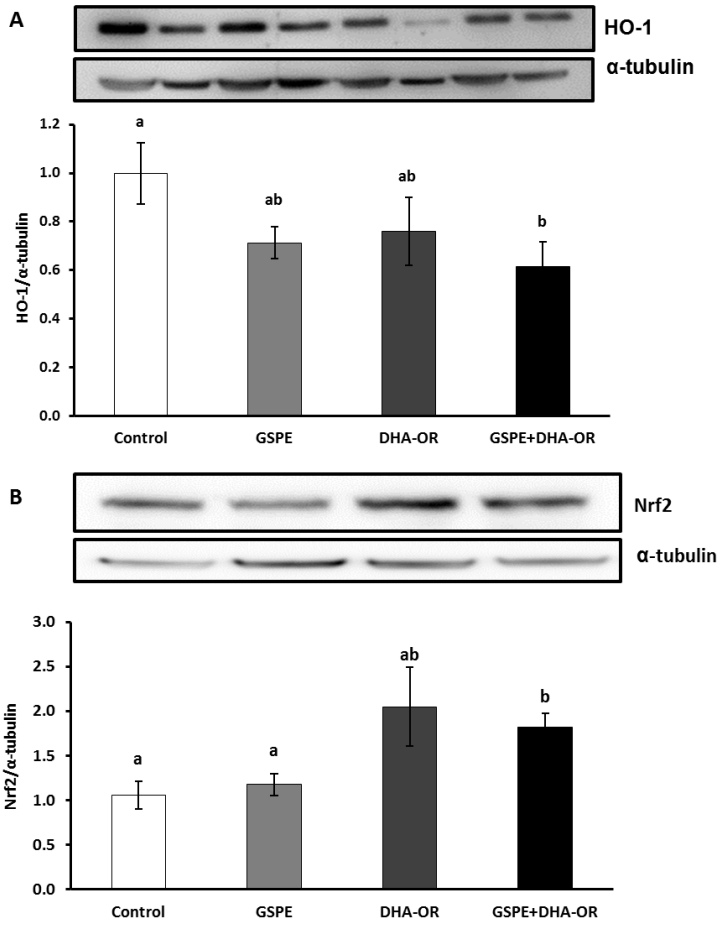

Figure 5. Western blot analyses. The cytoplasm fraction was isolated from liver of experimental rats and the HO-1 and Nrf2 protein content was analyzed by Western blot with a specific antibody. (A) HO-1 western blot image and HO-1 relative expression; (B) Nrf2 western blot image and Nrf2 relative expression. The results are expressed as the mean±SEM for each group (n=6): control, GSPE (250 mg/kg), DHA-OR (250 mg/kg), and GSPE+DHA-OR. Different letters indicate statistically significant differences ($p \leq 0.05$) among different groups.

4. Discussion

The plasma levels of hydroperoxides increase in the postprandial state and, in consequence, the oxidative stress also increases [26]. Some authors have suggested that dietary lipid hydroperoxides, which may be generated during digestion in the alkaline pH of the intestine, are the source of chylomicron remnant lipid hydroperoxides, which are elevated in the postprandial state [27]. Therefore, oxidative stress is hypothesized to be a significant mediator of the impairment of postprandial endothelial function as well as a stimulator of the inflammatory response following a high-fat meal [28]. In addition, it has been demonstrated that different biomarkers of oxidative stress, such as MDA, increase in response to the lipid meal [5]. Accordingly, it has been shown that acute administration of lard oil induces postprandial hyperlipidemia with the associated elevated levels of triglycerides [8], chylomicrons remnants, and free fatty acids that could result in oxidative stress and inflammation [29].

Natural compounds can mitigate postprandial oxidative stress after the ingestion of a high-fat meal [6; 13]. Furthermore, it has been reported that the oral intake of GSPE significantly blocks the increase in plasma TG levels induced by lard oil ingestion, reducing the risk of CVD [8]. On the other hand, n-3 PUFAs have favorable effects on postprandial triglyceride and triglyceride-rich lipoprotein metabolism [30], and a dose of n-3 PUFA reduces both fasting and postprandial TGs in patients with cardiovascular disease [31].

Therefore, the aim of this study was to investigate the effects of an acute dose of GSPE and DHA-OR and the possible additive effects of the combination of GSPE and DHA-OR on postprandial-associated oxidative stress.

The administration of 250 mg/kg bw of GSPE or DHA-OR did not show adverse effects, as was previously described for (a) GSPE in male rats [19] and (b) algal oil from *Schizochytrium* sp. in male and female rats [32; 20]. Furthermore, doses of 250 mg/kg bw of GSPE have been shown to improve the plasma lipid profile in healthy, chow-fed rats in a postprandial state by upregulating SHP expression in liver [12]. Moreover, it has been suggested that 250 mg/kg bw DHA supplementation may improve hepatocyte survival and protect against further liver damage [33].

The administration of mulberry water extracts, which contain polyphenols, alleviate the increased serum AST caused by LPS-induced acute inflammation in rats [34]. Furthermore, the increased level of serum AST after CCl_4-induced hepatic damage in mice was decreased after the administration of polyphenol-rich extract from Houttuynia cordata tea [35]. In addition, n-3 PUFA supplementation in humans is

associated with a positive effect on liver fat and a reduction of AST levels [36]. Although there was no induced hepatic damage in our study, the oral intake of GSPE significantly decreased plasma AST levels compared with control animals, which only were treated with lard oil. Moreover, the DHA-OR treatment slightly reduced the AST levels. Importantly, the combination of the two compounds significantly decreased the AST levels, suggesting a preventive effect given that the GSPE+DHA-OR treatment showed an AST level below the basal (control) state (Figure 2A).

The AST reduction by different treatments correlated with plasma CRP levels (Figure 2B), which were significantly decreased by GSPE, DHA-OR and GSPE+DHA-OR treatments. This result reinforces the preventive effect of such treatments on the postprandial hyperlipidemia effects, which was related to the improvement in the basal systemic CRP levels. Regarding these results, the inflammatory nature of postprandial hypertriglyceridemia has been correlated to immediate postprandial increases in CRP. However, a diet that includes whole plant foods and moderate levels of lean protein and beneficial fats, such as n-3 PUFAs, ameliorate postprandial oxidative stress and inflammation, reducing CRP by 20% to 40% after 3 weeks, even before any significant weight changes occur [29]. In addition, it has been demonstrated that a short corrective treatment of a high dose of GSPE is able to lower CRP plasma levels in obese rats [11; 37].

The liver is a key organ in controlling lipid metabolism [38], and the postprandial state promotes hyperlipidemia and oxidative stress. Therefore, we focused on the hepatic tissue to study the effects of natural compounds on antioxidant status in the lipidic postprandial state. The hepatic lipid profile (Table 1) was analyzed in the treated animals. It has been previously been shown that 3 hours after lard oil administration, the hepatic TG and cholesterol content increased by approximately 40% with respect to the basal condition [39]. In our study, DHA-OR treatment significantly reduced the total cholesterol in liver and, interestingly, the GSPE and DHA-OR combination treatment only slightly decreased cholesterol levels. These results are consistent with studies that have shown that long-chain PUFAs are able to reduce the total plasma cholesterol in rats [40] as well as in other animals [41].

A central role in the defense against oxidative stress has been attributed to the transcription factor Nrf2. Our results, presented in Figure 5B, showed that after the administration of lard oil with GSPE+DHA-OR, cytoplasmic Nrf2 protein levels were significantly increased compared with the control group. Therefore, we hypothesized that the cytoplasmic Nrf2 increase could be explained by the fact that Keap-1 protein levels increased after treatment, preventing the nuclear translocation of Nrf2 and preventing the expression of antioxidant genes [1]. This nuclear translocation reduction was corroborated by analyzing the HO-1 protein expression. As shown in Figure 5A,

the cytoplasmic HO-1 protein level decreased with the GSPE+DHA-OR treatment. In response to oxidative stress, cells attempt to fortify their antioxidant defenses. Although some studies have demonstrated Nrf2 induction by the polyphenol quercetin at both the mRNA and protein levels [(42)], it has also been reported that in the liver, most antioxidant genes are downregulated or not affected by treatment with phenolic apple juices concomitant with the absence of Nrf2 induction [(43)].
NADPH oxidases are likely to be the predominant source of ROS, and the activation of the NADPH oxidase enzyme complex requires the assembly of the cytosolic regulatory subunits (p47phox, p67phox, p40phox, and Rac) with the membrane-bound cytochrome b558 (gp91phox and p22phox). The postprandial state increases the expression of different NADPH-oxidase subunits (gp91phox, p22phox and p47phox), as shown by Perez-Herrera and coauthors [(44)]. In our study, the different treatments reduced the p22phox mRNA levels in the liver (Table 2), likely due to the preventive effects of GSPE and DHA-OR on the hepatic oxidative stress induced by the lipidic postprandial state.

It is well known that Cu/Zn-SOD and GPx are primary enzymes of the antioxidant defense system. The Cu/Zn-SOD decomposes superoxide radicals and produces H_2O_2 [(45)]. In our study, the Cu/Zn-SOD mRNA expression was upregulated by the GSPE+DHA-OR administration, although this was not reflected in the lipid peroxidation levels. Moreover, the results showed that GPx mRNA levels were not altered by any treatment. However, GPx activity was significantly reduced by the combined GSPE+DHA-OR treatment, compared to the control group.

GSH is involved in the detoxification of many xenobiotics through the formation of S-conjugates with toxic metabolites in the second phase of biotransformation [(46; 47)]. GSH also forms S-conjugates with the products of lipid peroxidation [(48; 49)]. The S-conjugation reaction can be significantly accelerated by glutathione S-transferase (GST) [(46)].

In our study, GST mRNA levels were not significantly modified by GSPE+DHA-OR administration, although the enzymatic activity was significantly increased.

Changes in the activity of GPx and GST can cause disturbances in the concentration of GSH. Interestingly, an altered glutathione status has repeatedly been reported under conditions of chronic or acute oxidative challenge related to both normal and pathophysiological states [(50)]. In particular, DHA-OR and GSPE+DHA-OR significantly reduced hepatic GSH levels compared to the control group in our study. Moreover, GSPE+DHA-OR treatment slightly increased the hepatic GSSG content. Consequently, the GSH/GSSG ratio was decreased approximately 40% compared to the control group (Figure 4 D, E, F).

The decrease of the hepatic GSH content correlated with the increase in GST activity, particularly in the GSPE+DHA-OR group, suggesting that the fate of GSH is S-conjugation with lipid hydroperoxides. Interestingly, it has been shown that GST not only reduces lipid hydroperoxides through their S-independent glutathione peroxidase activity but also detoxifies the lipid peroxidation end product [51]. Accordingly, GPx activity is reduced due to the hypothetical depletion GSH toward GST activity.

In a previous study, a decrease in plasma GSH was detected in the postprandial plasma after a test meal in healthy men [6]. Our results showed that GSPE+DHA-OR significantly increased blood GSH compared with the vehicle and GSPE groups, suggesting that GSPE and DHA-OR together have the ability to increase and maintain cellular glutathione levels, allowing the cells to combat the oxidative stress that occurs as a result of a lipidic postprandial state (Figure 4 A, B, C).

Lipid peroxidation was not reduced by the GSPE and/or DHA-OR treatments, as indicated by the MDA levels shown in Figure 3. This may be because the origin of the insult influences this effect. Because lard oil is mainly composed of cholesterol and monounsaturated fats, it is feasible that it could be rapidly incorporated into membrane phospholipids, where it could be more available to lipid oxidation, and GSPE and DHA-OR are not potent enough to revert this harmful effect [52].

Finally, this study provides evidence that the combination of grape seed proanthocyanidin extract and oil rich in docosahexaenoic acid modifies the hepatic antioxidant status in the context of a single high-fat meal, promoting a beneficial effect through genomic (Nrf2 translocation inhibition) and non-genomic (antioxidant enzymatic activities) mechanisms. This treatment could represent an interesting option for the prevention of the transient imbalance between the lipid hydroperoxide level and antioxidant status related to a lipidic postprandial state, which represents a harmful situation in the western diet.

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The authors declare no conflicts of interest.

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3.4 Effect of grape seed proanthocyanidin extract and omega-3 oil rich on oxidative stress in diet-induced obesity

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Keywords: Cafeteria diet / Heme oxygenase-1 / Nrf2 / Omega-3 PUFAs / Oxidative Stress / Proanthocyanidin

Abbreviations: 4-HAE, 4-hydroxyalkenals; **AST,** Aspartate aminotransferase; **CRP,** C-reactive protein; **DCFH-DA,** 2',7'-Dichlorofluorescin diacetate; **DHA-OR;** Oil rich in DHA; **GSH,** Reduced glutathione; **GSPE;** Grape seed proanthocyanidin extract; **GSSG,** Oxidized glutathione; **HFD,** High-fat diet; **HO-1,** Heme oxygenase-1; **MDA,** Malondialdehyde; **NAFLD,** Non-alcoholic fatty liver disease; **NASH,** Non-alcoholic steatohepatitis; **Nrf2,** Nuclear factor erythroid 2; **ROS,** Reactive oxygen species; **TBST-T,** Tris-Buffered saline Tween; **TG,** Triglycerides.

Abstract

Many studies suggest that obesity and associated hepatic fat accumulation induced by HFD can cause oxidative stress. In this study we evaluated the beneficial effects of GSPE and/or DHA oil rich supplementation against: (a) oxidative stress induced by HFD ingestion and, (b) associated hepatic steatosis.

The results showed that GSPE supplemented with cafeteria diet resulted in oxidative stress amelioration. In this sense, GSPE decreased the GSSG content resulting in total antioxidant capacity increase and inflammation amelioration. Moreover, DHA-OR was able to significantly increase the GSH/GSSG ratio that was reduced by the cafeteria diet ingestion. The combination of GSPE and DHA-OR was not able to counteract the oxidative stress induced by cafeteria diet, evidenced by the GSH/GSSG ratio results, although GSPE+DHA-OR supplementation led to increase the HO-1 protein expression and to decrease the accumulation of the Nrf2 in cytoplasm. Surprisingly, this slight Nrf2 pathway activation did not influence on the oxidative stress state, although it has been describes that Nrf2 nuclear translocation could be a potential target for the treatment on the most common liver diseases including NAFLD.

1 Introduction

Obesity is associated with a high incidence of many metabolic disorders [1]. The metabolic syndrome (MetS) is characterized by a group of metabolic risk factors in an individual, including abdominal obesity, atherogenic dyslipidemia, elevated blood pressure, and insulin resistance or glucose intolerance [2]. In a pathological state such as the MetS, an increased oxidant capacity coupled with decreased antioxidant activity creates an unbalanced environment that results in oxidative stress [3].

Cafeteria diet (CD) feeding is recognized model of obesity induced by high-fat diet (HFD) that shares common Western diet features and drives to MetS conditions [4, 5]. Over nutrition supply or HFD consumption leads to mitochondrial oxidative phosphorylation increase and to reactive oxygen species (ROS) over-production [6]. In this context, it has been described that adiposity and hyperinsulinemia may enhance liver oxidative stress in CD fed rats in comparison to standard diet fed rats [7]. Moreover, in this situation, the excessive accumulation of fat in the liver is the most common type of liver disease which is primarily characterized by triglycerides (TG) accumulation in the liver or non-alcoholic fatty liver disease (NAFLD). There is considerable evidence that the accumulation of lipids in the liver and the presence of reactive oxygen species (ROS) promotes lipid peroxidation, and in consequence produces oxidative stress [8].

Mammals, however, have developed an endogenous anti-oxidative system to prevent oxidative stress [1]. These include a battery of cytoprotective proteins that protect cells against oxidative stress and promote cell survival. Among these cytoprotective proteins there are the phase II defenses such as heme oxygenase-1 (HO-1). HO-1 is known for its cytoprotective effects against oxidative stress. Coordinated induction of cytoprotective gene transcription through the antioxidant response element (ARE) is essential for cellular protection against oxidative stress and related disorders [9]. Such induction is controlled by the nuclear factor erythroid 2-related factor 2 (Nrf2). This transcription factor is found ubiquitous in all the tissues, but is activated in response to a wide range of oxidative and electrophilic stimulation, including ROS and some chemicals agents. Under normal physiological conditions and a low oxidative stress environment, Nrf2 is confined to the cytoplasm associated with the suppressor protein Keap1, and is degraded by the ubiquitin proteasome pathway. Oxidative and electrophilic stress factors stimulate dissociation of the Nrf2-Keap1 complex, thereby promoting the release and translocation of Nrf2 into the nucleus, where it binds to the ARE, increasing in consequence gene expression of antioxidant/detoxifying enzymes [10].

Several studies have indicated that HFD resulted in more severe NAFLD/ non-alcoholic steatohepatitis (NASH) in Nrf2-null mice than wild-type mice. This indicates that Nrf2 plays a pivotal role in protection against the development of NAFLD/NASH and could be a useful therapeutic target to inhibit this development [5, 11].

Interestingly, several studies have proposed biological effects of polyphenols including antioxidant activity, amelioration of cardiovascular diseases, prevention of several degenerative age-related diseases and prevention of several kinds of cancer [12]. Proanthocyanidins are the oligomeric and polymeric forms of flavanols, and are present in fruits, beans, nuts, cocoa, tea and wine [13]. It has been reported that grape seed proanthocyanidin extract (GSPE) improves dyslipidemia induced by HFD in rats [14], enhances thermogenic capacity and improves mitochondrial function in brown adipose tissue [15], decreases oxidized LDL in hipercholesterolemic humans and improves endothelial function by modifying NO production. Besides their antioxidant activity and their effects on the vascular endothelium, the antiatherogenic properties of proanthocyanidins are also related to an improved serum lipid profile [14]. Furthermore, it has been demonstrated that polyphenols ameliorated hepatic steatosis in genetically obese mice [16], and that green tea polyphenols reduced the severity of liver injury in an experimental model of NAFLD [17].

Additionally, polyunsaturated fatty acids (PUFAs) are considered important bioactive nutrients. Diets rich in PUFAs, particularly omega-3 PUFAs, are considered effective in the prevention of many chronic diseases [18]. Moreover, some studies have suggested that liver TG or cholesterol content in rats fed with a HFD is decreased by PUFAs omega-3 administration [19]. Eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid are long-chain PUFAs present mainly in oily cold-water fish [20] and in plant food and vegetables oils [21]. Despite the benefits associated with increased EPA and DHA consumption, it has been also reported an increase of oxidative stress in both animals and humans due to the oxidation of these unsaturated fatty acids [18]. It has been showed that fatty acid composition of the liver can influence the degree of liver injury and disease progression. NAFLD associated with obesity was linked to the depletion of n-3 PUFAs, suggesting that a decrease in n-3 PUFAs contributes to the progression of NASH via the associated oxidative stress-induced damage and inflammation [22, 23]. Additionally, it has been demonstrated that DHA attenuated oxidative stress and hepatic damage in male mice, concluding that DHA dietary supplementation could be useful for the prevention and treatment of NASH in obese humans [24].

With these premises, the aim of this study was to evaluate the beneficial effects of GSPE and/or DHA oil rich (DHA-OR) supplementation against the oxidative stress induced by CD ingestion and the associated hepatic steatosis.

2 Materials and methods

2.1 Grape Seed Proanthocyanidin extract

GSPE was provided by *Les Dérives Résiniques et Terpéniques* (Dax, France). According to the previous analyses [25], the GSPE contained catechin (58 µmol/g), epicatechin (52 µmol/g), epigallocatechin (5.50 µmol/g), epicatechin gallate (89 µmol/g), epigallocatechin gallate (1.40 µmol/g), dimeric procyanidins (250 µmol/g), trimeric procyanidins (15.68 µmol/g), tetrameric procyanidins (8.8 µmol/g), pentameric procyanidins (0.73 µmol/g), and hexameric procyanidins (0.38 µmol/g).

2.2 DHA Oil Rich

DHA-OR (Martek DHA^{TM}-S) was nutritional oil derived from marine algae *Schizochytrium sp.* that contained 38.8% of DHA, 1.1% of EPA and 60.1% of other fatty acids. In addition the PUFAs omega-6/omega-3 ratio was 0.45.

2.3 Animals and experimental design

Males Wistar rats weighing 150 g (Charles River Laboratories, Barcelona, Spain) were housed in cages individually at 22ºC with 12 h light/dark cycle and were fed *ad libitum* with standard diet (Panlab A-04, Barcelona, Spain) and water. After a week of adaptation, the animals were divided in 2 experimental groups: SD group (n=7) and CD group (n=28) fed with bacon, biscuits with pâté, biscuits with cheese, muffins, carrots and milk with sugar providing 11.5% protein, 34.5% carbohydrates and 37.8% fat, in addition to standard diet. Animals were fed ad libitum from 7 pm to 9 am every day for 13 weeks. The last 3 weeks the CD group were divided into 4 subgroups (n=7): vehicle (Arabic gum 5% in water) (G9752, Sigma-Aldrich), GSPE (vehicle + 25 mg/kg*body weight (bw)), DHA-OR (vehicle + 500 mg DHA/kg*bw) and GSPE + DHA-OR (vehicle + 25 mg GSPE/kg*bw + 500 mg DHA/kg*bw) (Figure 1). After 21 days of treatments, animals were fasted for 3 h and they were sacrificed by exsanguination from abdominal aorta. Plasma was obtained by centrifugation, and liver and adipose tissue (mesenteric, perirenal and epididymal) were excised, weighed and frozen immediately in liquid nitrogen and stored at -80 °C until analysis.

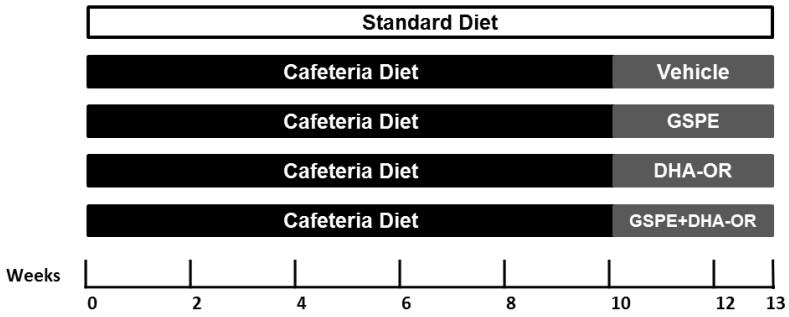

Figure 1. Schematic experimental design. Rats were fed with standard diet (SD) or SD plus cafeteria diet (CD). After 10 weeks, rats fed with SD plus CD were orally treated with GSPE (25 mg/kg bw), DHA-OR (500 mg/kg bw) or GSPE+DHA-OR (25 mg/kg bw + 500 mg/kg bw) for 3 weeks.

The Animal Ethics Committee of our University approved all procedures, with the permission number 4249 of the Catalonia Government (Generalitat de Catalunya. Departament de Medi Ambient i Habitatge).

2.4 Body weight gain and adiposity measurement

Body weight was monitored weekly during the whole experiment including the last 3 weeks. Tissue weight was represented as % of body weight, and adiposity index was calculated as total adipose tissue versus total body weight.

2.5 Assessment of liver function (AST)

Serum aspartate aminotransferase (AST) activity was measured according to the manufacturer's instructions of GOT/AST UV kit (QCA, Tarragona, Spain). Briefly, the AST activity is based in the reaction of aspartate and α-ketoglutarate to oxaloacetate and glutamate. The AST activity was expressed as U/L.

2.6 Plasma CRP levels

CRP is the most extensively studied marker of systemic inflammation. Hence, plasma CRP levels were quantified using a specific enzyme immunoassay according to the manufacturer's instructions (Immunology Consultants Laboratory). The assay consists of a double polyclonal antibody sandwich enzyme immunoassay. The results were expressed as μg CRP/mL.

2.7 Hepatic GSH/GSSG ratio measurement

The presence of sulfhydryl group in GSH makes is the responsible of its antioxidant function, protecting against free radicals and other oxidants. The reduced GSH form is oxidized to GSGG in an oxidative stress environment. Therefore, he GSH/GSSG ratio and the quantification of GSSG are useful non-enzymatic indicators of oxidative stress in cells and tissues [26]. For this assay, the GSH and GSSG present in liver homogenate were determined by an enzymatic method reported by Griffith, et al. [27] . It is based in the reaction of the GSH with DTNB (5,5'-Dithiobis(2-nitrobenzoic acid) (D8130, Sigma-Aldrich)) to generate 2-nitro-5-thiobenzoic acid, a yellow compound that absorbs at λ=412 nm, and GSSG. GSH is regenerated from GSSG due to the presence of glutathione reductase in excess. Under these conditions, total GSH in the sample was determined. The determination of "GSSG only" was measured due to the reaction of GSH and 2-vinylpyridine, a thiol-scavenging reagent to form a pyridinium salt. For this assay, liver was homogenized and deproteinized with metaphosphoric acid 6% and centrifuged at 5000 g for 15 min at 4 °C. The supernatants were used for the analysis. GSH and GSSG content was expressed as µmol equivalents per g of liver (µmol eq/g liver). The GSH/GSSG ratio was calculated by dividing the difference between the GSH and twice GSSG concentrations by the concentration of GSSG (ratio= (GSH-2GSSG)/GSSG).

2.8 Hepatic oxidative stress markers (lipid peroxidation, ROS)

The content of lipid peroxides in liver homogenates was determined by the classical method of measuring TBARS [28]. Concretely, malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) levels were determined by colorimetric assay based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA and HAE to yield a stable chromophore with maximal absorbance at λ=586 nm. Briefly, liver was homogenized in Tris Buffer, pH=7.4 and 10 µl of 0.5M butylated hydroxytoluene (BHT) stock solution per 1 ml of homogenate was added to prevent sample oxidation. The homogenates were centrifuged at 3000 g for 10 min at 4 °C. Subsequently, MDA+HAE levels were assessed using Lipid Peroxidation Microplate Assay Kit FR-22 according to the manufacturer's instructions (Oxford Biomedical Research, Barcelona, Spain). The results were expressed as mM MDA+HAE/mg protein.

Additionally, the intracellular formation of ROS was detected using the fluorescent probe 2',7'-dichlorfluorescein-diacetate (DCFH-DA) [29]. The nonpolar DCFH-DA crosses cell membranes and is hydrolyzed enzymatically by intracellular esterases to nonfluorescent DCFH. In the presence of ROS, DCFH is oxidized to highly fluorescent dichlorofluorescein (DCF) [30]. For this assay liver was homogenized on ice with 1 mM EDTA–50 mM sodium phosphate buffer (pH 7.4). 25 µM DCFH-DA was added to homogenates. Afterwards, it was incubated for 1 h at 37 °C, and fluorescence values

were determined at a λ_{ex}=485 nm and a λ_{em}=530 nm using the FLx800 Multi-Detection Microplate Reader (Biotek, Winooski, USA). Cellular ROS levels were expressed as fluorescence units (RFU) per mg protein.

The protein content was determined by a colorimetric assay (Bradford, Sigma Aldrich).

2.9 Oxygen Radical Absorbance Capacity (ORAC) assay

2.9.1 Preparation of plasma samples

Plasma samples were prepared as described by Leite et al. [31]. Briefly, plasma was mixed with ethanol and distilled water. After the solution was vortexed, metaphosphoric acid was added and the mixture was vortexed once again. The solution was centrifuged at 2000 g for 5 min at 10 °C. For the ORAC assay 80 µL of the supernatant was diluted with 420 µL of phosphate buffer.

2.9.2 ORAC assay

The ORAC assay was carried out using the method described by Huang et al. [16]. Concisely, 25 µL of plasma solution and 150 µL of 59.8 nM fluorescein (FL) (Sigma-Aldrich, Madrid, Spain) were added into the 96-well microplate. The fluorescence was measured at λ_{ex}=485 nm and λ_{em}=520 nm every 1 min, for 90 min in the FLx800 Multi-Detection Microplate Reader (Biotek, Winooski, USA) after the injector addition of 25 µL of 73 mM of the radical generator 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH, Acros Organics, Belgium). Different concentrations (0, 3.125, 6.25, 12.5, 25, 50, 100 µM) of Trolox solution (Sigma-Aldrich, Madrid, Spain) was used as standard. The final ORAC values were calculated by a regression equation between the Trolox concentration and the net area under the FL decay curve and were expressed as µmol Trolox equivalents per liter.

2.10 Nrf2 and HO-1 expression

2.10.1 Cytoplasm isolation

Cytoplasm protein extract was isolated from liver of experimental rats. Tissue was homogenized in isotonic buffer at pH 7.4 (250 mM sucrose, 50 mM Tris-HCl, 5 mM $MgSO_4$, 1 mM PMSF and 0.5 µg/ml aprotinin) and centrifuged at 800 g for 10 min. The resulting supernatant was collected and stored at -80 °C. The protein content was determined by a colorimetric assay. (Bradford, Sigma Aldrich).

2.10.2 Western Blot analysis

Equal amounts of cytoplasmic protein (100 µg) were analyzed by western blot. Briefly, samples were placed in sample buffer (0.5M Tris–HCl pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% (v/v) 2-β-mercaptoethanol, 0.05% bromophenol blue), and denatured by boiling at 95-100 °C for 5 min. Samples were then separated by electrophoresis on 10% SDS polyacrylamide gels. Proteins were subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, GE Healthcare) using a transblot apparatus (BioRad). The membranes were blocked for 1 h with 5% non-fat milk dissolved in TBS-T buffer (50mM Tris, 1.5% NaCl, 0.2% Tween 20, pH 7.5). They were then incubated overnight at 4 °C with primary monoclonal antibodies against HO-1 (ADI-OSA-150-F, Enzo Life Sciencies), Nrf2 (ADI-KAP-TF125-F, Enzo Life Sciencies), and α-tubulin (T3526, Sigma). The blots were washed thoroughly in TBS-T buffer and incubated for 1 h with a peroxidase-conjugated IgG antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence substrate kit (ECL plus; Amersham Biosciences, GE Healthcare) according to the manufacturer's instructions. Digital images were taken with a GBOX Chemi XL 1.4 system (Syngene), which permits semi-quantification of the band intensity. The protein load was periodically monitored via the immuno-detection of tubulin.

2.11 HO-1 and p22phox mRNA expression

HO-1 and p22phox mRNA expression were quantified in liver. Total RNA was obtained from liver using RNeasy Mini Kits (Qiagen, Valencia, CA) according to the manufacturer's protocol. DNase I, RNAase-free kits (High Capacity complementary DNA Reverse transcription kits, Applied Biosystems, Madrid, Spain) were used to remove the genomic DNA from RNA preparations. The cDNA was subsequently amplified by PCR using specific TaqMan Assay-on-Demand Probe for HO-1 (Rn01536933_m1), p22phox (Rn00577357_m1), and Cyclophilin peptidylprolyl isomerase A (PPIA) (Rn00690933_m1).

Quantitative PCR for different genes was performed using TaqMan PCR Core Reagent Kits according to the manufacturer's protocol and analyses on a Real-Time 7300 PCR System, all from Applied Biosystems. The relative levels of expression of the target gene were measured using PPIA mRNA as an internal control according to the $2^{-\Delta\Delta Ct}$ method.

3 RESULTS

3.1 Obesity and metabolic syndrome markers

Comparing with SD group body weight was increased around 20% due to CD, although the body weight difference was already significant after 2 weeks of diet (Figure 2a), which indicates that the CD was a good experimental model to induce obesity. After 3 weeks of administration, GSPE treatment significantly decreased the relative body weight gain compared with the CD group. In contrast, rats treated with GSPE+DHA-OR significantly increased body weight gain compared with GSPE group, although DHA-OR slightly decreased the body weight gain associated with CD (Figure 2b).

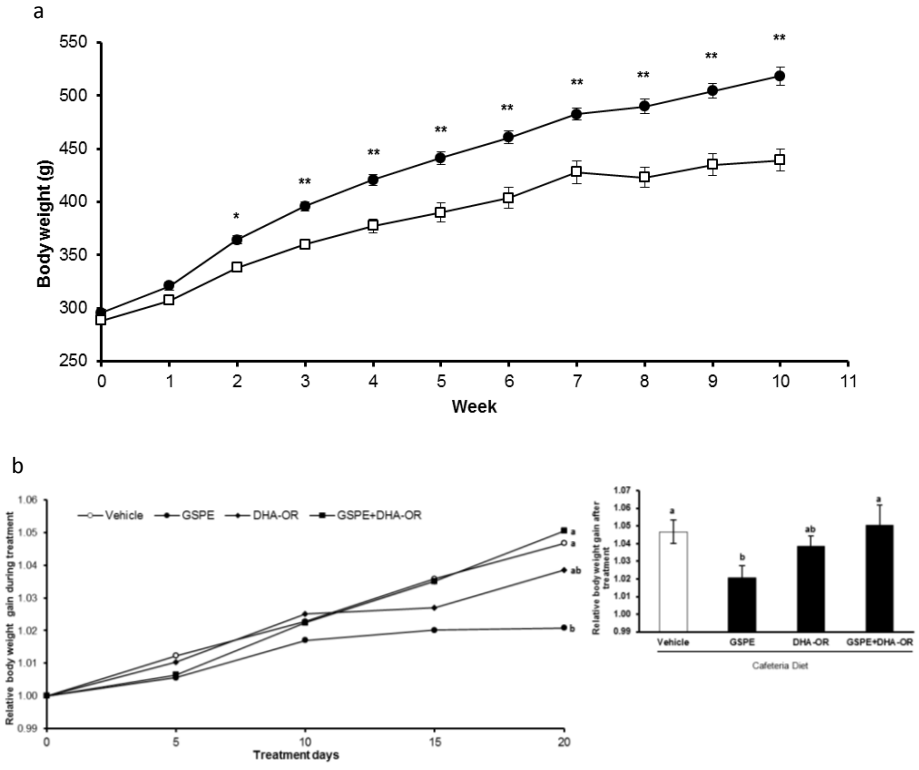

Figure 2. Evolution of body weight. Rats were fed with standard diet (□) or cafeteria diet (●)(a). for 10 weeks. After, rats were fed for 3 weeks with cafeteria diet (vehicle) (○), and CD with GSPE (25 mg/kg) (GSPE) (●), CD with DHA-OR (500 mg/kg) (DHA-OR) (, and CD with GSPE+DHA-OR (GSPE+DHA-OR) (▪) (b). The results are expressed as the mean±SEM for each group. Different letters indicate statistically significant differences (p≤0.05) among different groups.

On the other hand, CD increased mesenteric, epididymal and perirenal fat weight resulting in an adiposity index significantly increased. However, all treatment were able to revert these fat weight gain related to CD ingestion. Despite body weight gain due to CD feeding, liver weight was not incremented, and any of the assayed treatments (GSPE, DHA-OR and GSPE+DHA-OR) had no effect on liver weight (Table 1).

		Cafeteria Diet			
% of body weight	SD	Vehicle	GSPE	DHA-OR	GSPE+DHA-OR
Liver	3.0±0.01 a	3.06±0.07 ab	2.94±0.06 a	3.19±0.01 b	3.19±0.04 b
Mesenteric fat	1.41±0.14 a	2.61±0.22 b	2.533±0.13 b	2.548±0.25 b	3.3±0.43 b
Epididimal fat	2.29±0.14 a	4.02±0.36 b	3.55±0.19 ab	4.78±0.47 b	4.54±0.34 b
Perirenal fat	2.12±0.13 a	3.78±0.32 bc	3.81±0.19 b	3.86±0.24 bc	4.46±0.34 c
Adiposity index	5.8±0.3 a	10.4±0.8 b	9.9±0.2 b	10.8±1.0 b	12.3±1.1 b

Table 1. Tissues weight of experimental rats. The results are expressed as the mean±SEM for each group (n=7): standard diet group (SD), vehicle group (CD), CD rats treated with GSPE (25 mg/kg) (GSPE), CD rats treated with DHA-OR (500 mg/kg) (DHA-OR), and CD rats treated with GSPE+DHA-OR (GSPE+DHA-OR). Different letters indicate statistically significant differences ($p \leq 0.05$) among different groups.

3.2 Plasmatic measurements

3.2.1 Liver function marker: AST

Plasma AST levels were not modified in CD group. GSPE slightly decreased AST activity (Figure 3).

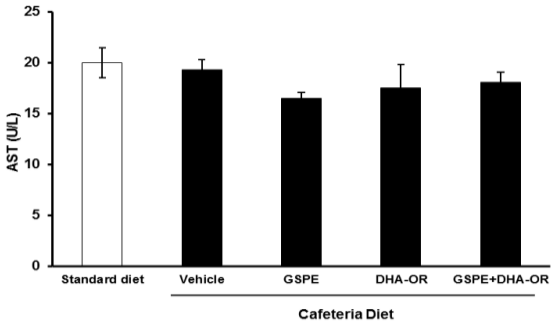

Figure 3. Liver function. Plasmatic AST activity was assessed in experimental animals. The results are expressed as the mean±SEM for each group (n=7): standard diet group, vehicle group (CD), CD rats treated with GSPE (25 mg/kg) (GSPE), CD rats treated with DHA-OR (500 mg/kg) (DHA-OR), and CD rats treated with GSPE+DHA-OR (GSPE+DHA-OR).

3.2.2 Pro-inflammatory marker: CRP

The plasma analysis of CRP levels revealed that CD significantly increased this inflammatory parameter. Additionally, GSPE treatment was able to revert the increased CRP levels related to cafeteria diet ingestion. The DHA-OR treatment slightly decreased the CRP levels induced by cafeteria diet, and the two compounds together significantly decreased the inflammatory marker content compared with CD group (Figure 4).

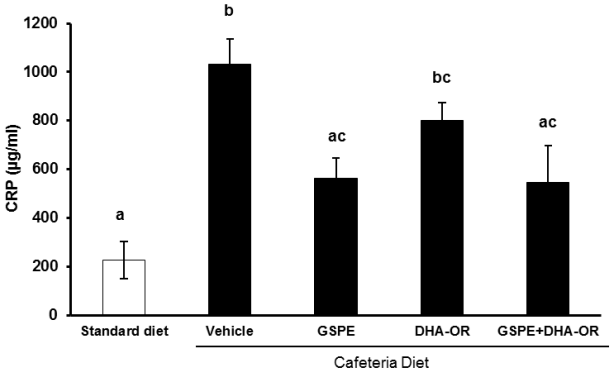

Figure 4. Plasmatic CRP content was assessed in experimental animals. The results are expressed as the mean±SEM for each group (n=7): standard diet group, vehicle group (CD), CD rats treated with GSPE (25 mg/kg) (GSPE), CD rats treated with DHA-OR (500 mg/kg) (DHA-OR), and CD rats treated with GSPE+DHA-OR (GSPE+DHA-OR). Different letters indicate statistically significant differences ($p \leq 0.05$) among different groups.

3.2.3 Antioxidant capacity: ORAC

Plasma total antioxidant capacity was significantly increased by the GSPE administration despite CD ingestion. In contrast the GSPE+DHA-OR treatment reduced but not significantly the plasma antioxidant capacity (Figure 5).

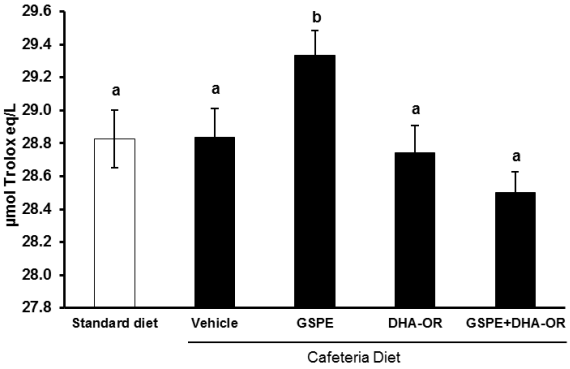

Figure 5. Total antioxidant capacity (ORAC). Plasmatic total antioxidant capacity was assessed using the ORAC method. The results are expressed as the mean±SEM for each group group (n=7): standard diet group, vehicle group (CD), CD rats treated with GSPE (25 mg/kg) (GSPE), CD rats treated with DHA-OR (500 mg/kg) (DHA-OR), and CD rats treated with GSPE+DHA-OR (GSPE+DHA-OR). Different letters indicate statistically significant differences ($p \leq 0.05$) among different groups.

3.3 Oxidative status of the liver

3.3.1 GSH metabolism

We investigated hepatic total GSH/GSSG ratio as indicator of non-enzymatic antioxidant defense. The results showed that GSH levels decreased by GSPE+DHA-OR treatment compared with SD group (715.7 ± 34.5 for SD vs 545.5 ± 39.9 for GSPE+DHA-OR; $p \leq 0.05$). On the contrary, the GSSG levels were significantly increased by CD group (52.3 ± 8 for SD vs 115.6 ± 21.6 for CD; $p \leq 0.05$). However, GSPE and DHA-OR significantly reduced the GSSG content until SD levels (115.6 ± 21.6 for CD vs 68.4 ± 8.5 for GSPE, and 69.1 ± 10.3 for DHA-OR; $p \leq 0.05$). Consequently, CD significantly decreased the total GSH/GSSG ratio compared with SD group (12.3 ± 1.4 for SD vs 4.6 ± 1.5 for CD; $p \leq 0.05$). Nevertheless, the GSH/GSSG ratio significantly increased in DHA-OR group. Surprisingly, when cafeteria diet is combined with two compounds together, the GSH/GSSG ratio was significantly decreased (12.3 ± 1.4 for SD vs 4.9 ± 1.2 for GSPE+DHA-OR; $p \leq 0.05$) due to the GSH content diminished (Figure 6).

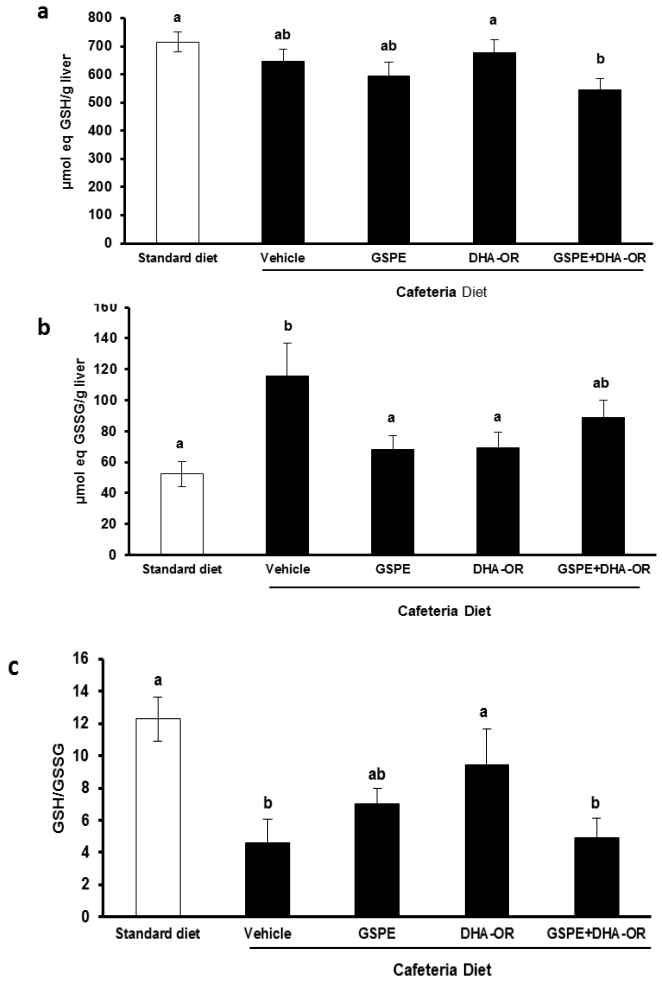

Figure 6. GSH metabolism. Total GSH/GSSG (c) was estimated by assessing total GSH (a) levels and GSSG content (c) in liver homogenates. The results are expressed as the mean±SEM for each group (n=7): standard diet group, vehicle group (CD), CD rats treated with GSPE (25 mg/kg) (GSPE), CD rats treated with DHA-OR (500 mg/kg) (DHA-OR), and CD rats treated with GSPE+DHA-OR (GSPE+DHA-OR). Different letters indicate statistically significant differences ($p \leq 0.05$) among different groups.

3.3.2 Hepatic oxidative stress markers (MDA, ROS and p22phox)

Lipid peroxidation is a well-established mechanism of cellular injury, and is used as an indicator of oxidative stress in cells and tissues. The measurement of MDA and HAE was used as indicator of lipid peroxidation. CD did not present any effect on lipid peroxidation in liver. Moreover, any of the treatments influenced cell lipid peroxidation (Figure 7a).

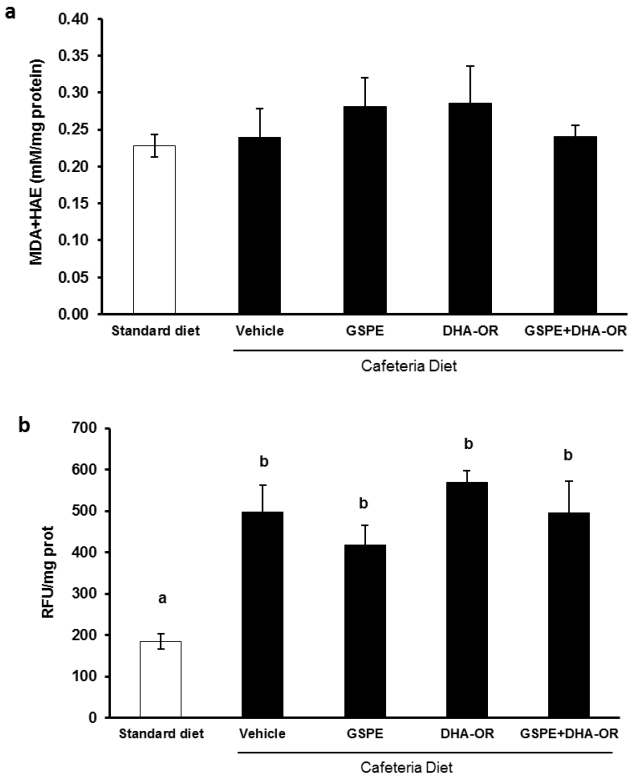

Figure 7. Hepatic lipid peroxidation and ROS content. Lipid peroxidation was determined by measuring MDA+4-HAE levels in liver homogenates (a). Liver ROS production was assessed by the DCFH method (b). The results are expressed as the mean±SEM for each group (n=7): standard diet group, vehicle group (CD), CD rats treated with GSPE (25 mg/kg) (GSPE), CD rats treated with DHA-OR (500 mg/kg) (DHA-OR), and CD rats treated with GSPE+DHA-OR (GSPE+DHA-OR). Different letters indicate statistically significant differences ($p \leq 0.05$) among different groups.

The DCFH-DA method was used to determine ROS production in liver (Figure 7b). A significantly increase in ROS production was observed CD group compared with SD group (185.2 ± 18.1 for SD vs 498.3 ± 64.6 for CD; p≤0.05). On the other hand, the different treatments did not affect ROS production in liver, and only the GSPE treatment slightly decreased ROS levels.

Moreover, p22phox mRNA expression was analyzed as NADPH oxidase subunit. NADPH oxidases are likely to be the predominant source of ROS, and the increase of gene expression of different NADPH-oxidase subunits is associated with an oxidative stress state. Surprisingly, although CD significantly increased ROS production, the p22phox mRNA expression was not increased by CD ingestion. On the contrary, DHA-OR and GSPE+DHA-OR significantly decreased the p22phox expression compared with CD group (Figure 8).

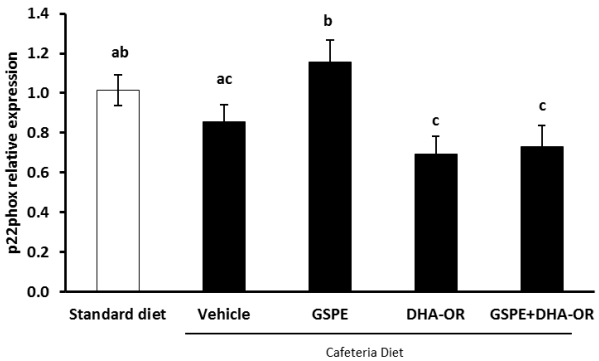

Figure 8. The mRNA expression of prooxidant enzyme. The mRNA expression of liver p22phox was analyzed by real-time RT-PCR. The relative levels of expression of the target gene were measured using PPIA mRNA as an internal control according to the $2^{-\Delta\Delta Ct}$ method. The results are expressed as the mean±SEM for each group (n=7): standard diet group, vehicle group (CD), CD rats treated with GSPE (25 mg/kg) (GSPE), CD rats treated with DHA-OR (500 mg/kg) (DHA-OR), and CD rats treated with GSPE+DHA-OR (GSPE+DHA-OR). Different letters indicate statistically significant differences (p≤0.05) among different groups.

3.3.3 Nrf2 signaling pathway: HO-1 expression

Cytoplasmic western blot analyses revealed that HO-1 protein expression slightly increased in animals fed with CD. When rats were also administered with DHA-OR and GSPE+DHA-OR in addition to cafeteria diet, the HO-1 protein content significantly

increased compared with SD group. On the other hand, the cytoplasmic Nrf2 accumulation did not present any change between different groups, although DHA-OR and GSPE+DHA-OR slightly decreased the cytoplasmic Nrf2 content and GSPE treatment led to increase the cytoplasmic Nrf2 expression (Figure 9).

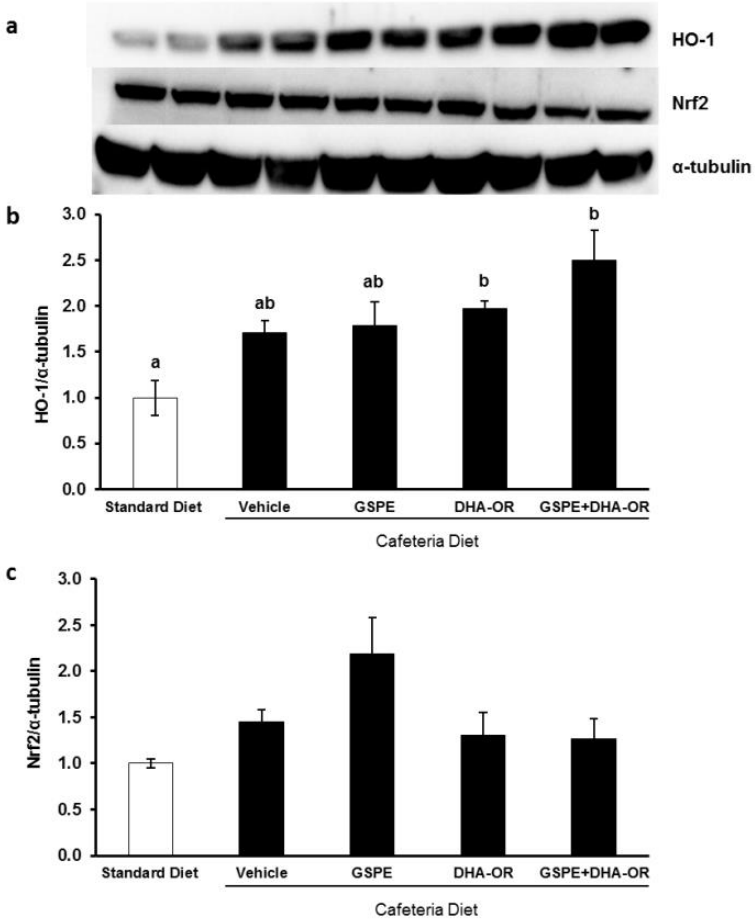

Figure 9. Western blot analyses. The cytoplasm fraction was isolated from liver of experimental rats, and the HO-1 and Nrf2 protein content was analyzed by Western blot with a specific antibody: western blot image (a), HO-1 relative expression (b), and Nrf2 relative expression (c). The results are expressed as the mean±SEM for each group (n=7): standard diet group, vehicle group (CD), CD rats treated with GSPE (25 mg/kg) (GSPE), CD rats treated with DHA-OR (500 mg/kg) (DHA-OR), and CD rats treated with GSPE+DHA-OR (GSPE+DHA-OR). Different letters indicate statistically significant differences ($p \leq 0.05$) among different groups.

4 Discussion

Many studies suggest that obesity and associated hepatic fat accumulation induced by HFD can cause oxidative stress via variety of mechanisms including induction of mitochondrial dysfunction, depletion of GSH and decrease of antioxidant enzymes activities [17, 32]. The CD was a good model to induce obesity in rats and related disorders, including inflammation and GSH metabolism deregulation in liver [33].

In this study, the CD increased several obesity markers (the body weight, the proinflammatory marker CRP). Moreover, previous studies showed the plasmatic TG and cholesterol increase [34], and the insulin resistance development (doi:10.1155/2013/875314). Therefore, the CD is defined as a robust model of human MetS with liver inflammation [33].

Liver steatosis is defined as TG accumulation in liver [8]. The CD is able to accumulate lipids in the liver and increase liver weight and TG levels [32]. In our study it has been previously shown that CD increased liver TG content compared with SD group as well as total liver lipids (published data) [34], although in agreement with Vial et al [35] liver weight was unchanged.

Therefore, we could assume that the experimental rats in this study developed fatty liver and, in consequence, presented hepatic steatosis. It has been reported that feeding obese rats a HFD was sufficient to cause oxidative stress associated with hepatic steatosis [36]. Consistent with a role for oxidative stress, in the present study, we found that rats fed with CD had a greater oxidative stress, as evidenced by a low GSH/GSSG ratio. This oxidative stress could be linked to an excessive production of ROS. In consequence, the plasma antioxidant defense mechanisms, including GSH metabolism, could be compromised. In this sense, it has been reported the correlation between hepatic ROS production increase and NAFLD development [37].

Moreover, the tendency of higher HO-1 protein expression in cytoplasm by CD could represent a cell response in order to fortify the antioxidant defense for the prevention of the NAFLD. Therefore, the ROS overproduction induced by CD could be the HO-1 increase inductor. Hence, the obesity development was associated not only with the energy intake but also with oxidative stress changes.

In this study, the GSPE supplemented with CD resulted in oxidative stress amelioration. It has been reported that grape seed extract has beneficial effects on preventing diet-induced obesity by improving oxidative stress markers, including the down-regulation of antioxidant enzymes as a consequence of the sparing effect of dietary antioxidants that are able to scavenge the oxygen radicals, and in

consequence, reducing the requirement for enzymatic endogenous antioxidants [38]. In this sense, GSPE decreased the GSSG content resulting in a total antioxidant capacity increase and in inflammation amelioration. The plasmatic CRP reduction was in accordance with Terra et al. [39] who showed that ingestion of procyanidins diminished CRP levels reducing the diet-induced low-grade inflammation.

On the other hand, DHA-OR was able to significantly increase the GSH/GSSG ratio that was reduced by the CD ingestion. This increase was due to the significant GSSG content decrease. However, DHA-OR treatment was not able to: (a) increase the total antioxidant capacity compared with CD, and (b) did not affect in other oxidative stress parameters. Moreover, the DHA-OR supplementation had no influence on the body weight gain. In this regard, it has been obtained conflicting results about the effects of omega-3 PUFAs on body weight and composition. Couet et al. [40] observed in healthy adults that replacement of 6 g of visible fat in diet with 6 g of fish oil did not affect body weight loss. However, Vasickova et al. [41] suggest a possible beneficial effect of DHA intake on body weight reduction in obese children.

It has been reported that the anti-inflammatory and the antioxidant activities of curcumin, a phenolic compound, can be enhanced by combining with omega-3 PUFAs [42]. In this sense, we evaluated the possible antioxidant effect of a simultaneous administration of GSPE and DHA-OR in combination with CD ingestion. Our results showed that when DHA-OR is administered in combination with GSPE, was not able to counteract the oxidative stress induced by CD, evidenced by the GSH/GSSG ratio results. Moreover, GSPE+DHA-OR slightly decreased the GSH content. However, the two compounds combination significantly reduced de pro-inflammatory marker CRP. This inflammation amelioration could due in part of the HO-1 anti-inflammatory effect [43]. Our results showed that GSPE+DHA-OR led to increase the HO-1 protein expression and to decrease the accumulation of the Nrf2 in cytoplasm. Surprisingly, this slightly induction did not influence on the oxidative stress state, although the activation of Nrf2 pathway it has been describes as a potential target for the treatment on the most common liver diseases including NAFLD [28].

In this study, we demonstrated that rats fed with CD and treated simultaneously with GSPE presented: (a) increase on total antioxidant capacity, (b) an improved hepatic GSH metabolism, and (c) a decreased the proinflammatory marker CRP. Moreover, GSPE reduced body weight gain after 3 weeks of treatment. Furthermore, we demonstrated that even though GSH levels were reduced by the combination of GSPE with DHA-OR, the CRP levels were decreased. Despite this results, GSPE+DHA-OR was able to increase the HO-1 which could subsequently act against oxidative stress.

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4 SUMMARY

Obesity is considered a metabolic disorder, whose prevalence is increasing dramatically in most developed countries over the last 20 years. Obesity is associated with an increased risk of developing chronic morbidities (hypertension, insulin resistance, dyslipidemia), which constitute the major components of the MetS [1]. Emerging evidence suggests that oxidative stress play an important role in the pathophysiology of MetS-related manifestations. In this sense, hepatic steatosis has been associated with obesity, and in consequence, the hepatic lipid accumulation and the presence of ROS promote oxidative stress [2].

Importantly, several studies have showed the amelioration of obesity-related manifestations using nutritional supplements such as polyphenols [3, 4] and n-3 PUFAs [5, 6]. In accordance, the hypothesis of this research was that polyphenols and n-3 PUFAs could have additive antioxidant effects in different oxidative stress conditions both *in vitro* and *in vivo*.

The research of our group is focused on the beneficial effects of polyphenols. For this reason, our first objective was to assess the GSPE effect on oxidative stress conditions associated to a genetic obesity model rats (Zucker rats). The animals were administered daily with 35 mg GSPE/kg bw for 10 weeks, and we studied the GSPE effects on obesity-induced oxidative stress as well as on hepatic steatosis. Our results show that GSPE improved hepatic GSH metabolism evidenced by higher total GSH/GSSG ratio and by lower GSSG content, and also increased total antioxidant capacity of the cell compared with obese rats. In this sense, the cellular antioxidant defense (GPx, GR and GST enzymes) was not activated.

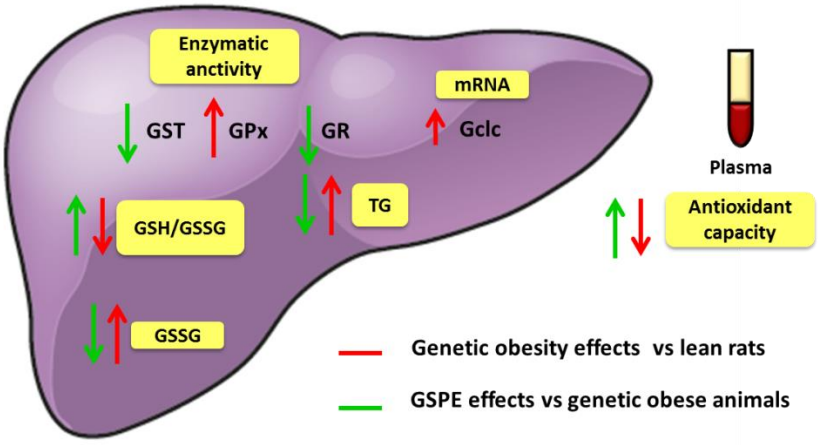

Figure 22. GSPE effect on oxidative stress in genetic obesity rats.

In agreement, it had been reported that the animals treated with antioxidant compounds showed lower antioxidant enzyme activities, which might be due to the oxidative stress-reducing capacity and inhibition of the generation of superoxide and hydroxyl free radicals of such compounds [7] (Figure 22).

Once the GSPE antioxidant effects in an obesity-induced oxidative stress condition were assessed, we wanted to study whether n-3 PUFAs were able to mitigate oxidative stress in combination with GSPE.

Firstly, we evaluated two pure compounds *in vitro* rat hepatocyte cells. Despite the benefits related with n-3 PUFAs consumption, it had been reported that these fatty acid could increase lipid peroxidation due to the susceptibility to free radical oxidation, increasing the oxidative stress [8]. Therefore, we explored if DHA supplementation could increase the sensitivity of hepatocytes to *tert*-BHT induced oxidative stress, and, on the contrary, if the tea green polyphenol EGCG was able to protect such detrimental effect. We observed that DHA supplementation exacerbated the *tert*-BHP-induced oxidative damage, resulting in ROS and lipid peroxidation production, and GSH/GSSG decrease. In consequence, the Nrf2 nuclear translocation, considered a master regulator of antioxidant defense mechanisms, was activated with the subsequent increase of the antioxidant HO-1 expression. In this sense, it is well known that DHA is rapidly incorporated into membrane phospholipids and it enhances the liver susceptibility to lipid peroxidation [9]. Interestingly, when hepatocytes were treated with the combination of DHA and EGCG, the polyphenol was able to protect the cell of oxidative damage induced by *tert*-BHP and DHA. Our results show that the polyphenol inhibited the Nrf2 nuclear translocation and decreased HO-1 protein content as an indicator of redox balance improvement, evidenced by ROS and lipid peroxidation decrease and by cell viability and GSH/GSSG increase. Despite further studies are needed, or *in vitro* results suggest that it would also be important to consider global oxidative status when PUFAs will be administered.

Afterwards, we studied polyphenols and n-3 PUFAs effects under oxidative stress conditions *in vitro*, we decided to carry out *in vivo* experiments. In this sense, firstly we evaluated the GSPE and DHA-OR in a lipidic postprandial state in male Wistar rats. Postprandial oxidative stress is characterized by an increased susceptibility of the organism towards oxidative damage after consumption of a meal rich in lipids and/or carbohydrates [10]. In this study postprandial state was induced by saturated fat administration [11], and we investigated the GSPE and DHA-OR effects on postprandial oxidative stress manifestations [12], including glutathione metabolism and the Nrf2 pathway. The results showed that after 5 h of GSPE administration it occurred: (a) a reduction on the AST levels; (b) an amelioration of inflammation evidenced by CRP levels; and (c) a reduction on the p22phox mRNA expression. Moreover, DHA-OR

treatment reduced hepatic TG and cholesterol content as well as reduced CRP levels and p22phox mRNA expression. Importantly, in this study we demonstrated that DHA-OR and GSPE+DHA-OR reduced hepatic GSH levels. The decrease on the hepatic GSH content correlates with the increase of GST activity particularly on the GSPE+DHA-OR group, suggesting that the GSH fate is mainly the S-conjugation with lipid hydroperoxides.

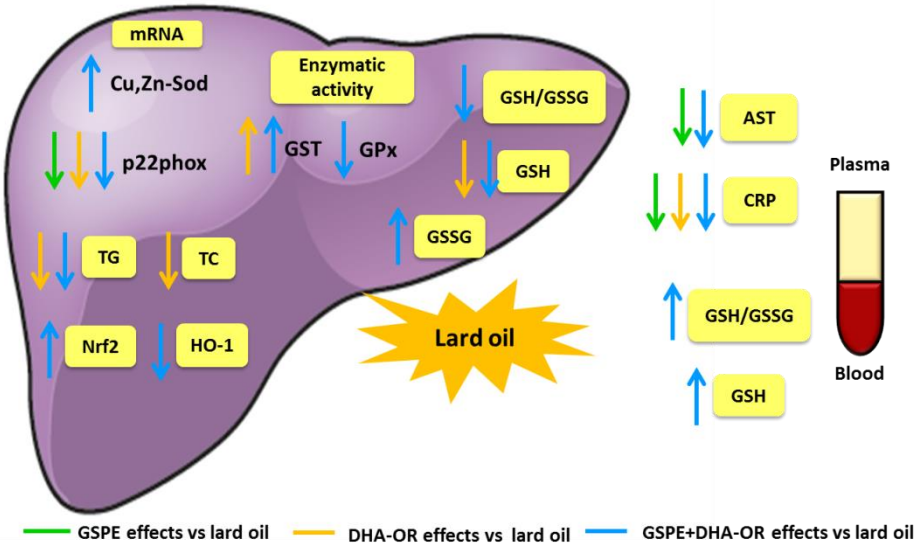

Figure 23. GSPE and/or DHA-OR effects on antioxidant status in lipidic postpandrial state

However, GSPE and DHA-OR combination increased plasmatic GSH levels and consequently increased the GSH/GSSG ratio, suggesting that GSPE and DHA-OR together have the ability to increase and maintain the cellular glutathione levels, allowing the cells to combat oxidative stress that occurs as a result of a lipidic postprandial state. Finally, this study suggest that GSPE and DHA-OR have additive antioxidant effects in a lipidic postprandial state (Figure 23).

Another useful animal model to study obesity is a diet-induced obesity rat model. In this sense cafeteria diet was a good model to induce obesity and related disorders in rats [13]. Some studies suggest that obesity and associated hepatic fat accumulation (hepatic steatosis) induced by HFD can cause oxidative stress via variety of mechanisms including induction of mitochondrial dysfunction, depletion of GSH and decrease of antioxidant enzymes activities [14]. In this sense, it has been described that polyphenols and n-3 PUFAs could ameliorate NAFLD development. In this regard, male

Wistar rats were fed with cafeteria diet. Once we confirmed the obesity in rats, they were treated with GSPE, DHA-OR and GSPE+DHA-OR to evaluate the effects of these natural compounds individually and combined in the oxidative stress induced by cafeteria diet ingestion.

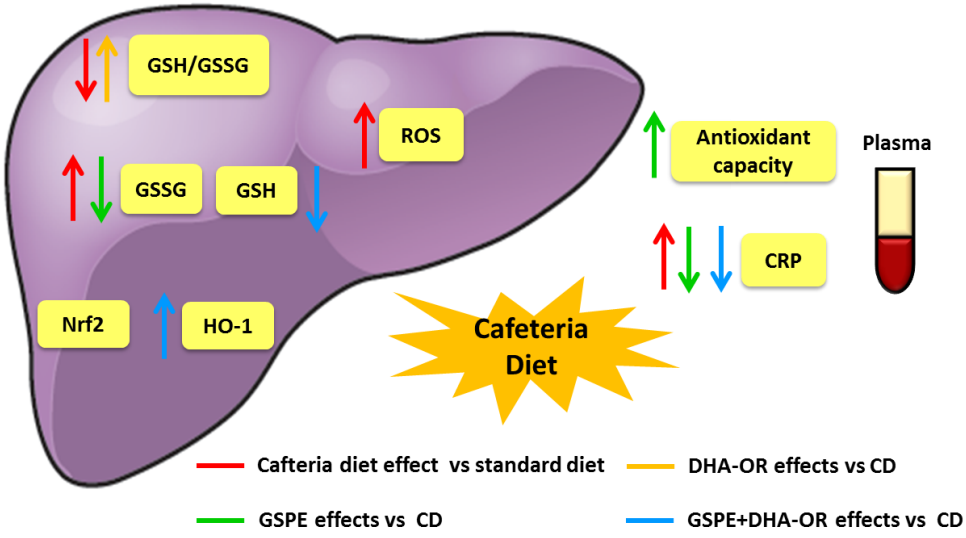

Figure 24. GSPE and/or DHA-OR effects on oxidative stress in diet-induced obesity

It has been described that polyphenols prevent diet-induced obesity by improving oxidative stress [15]. In this sense, GSPE decreased the GSSG content and consequently improved total antioxidant capacity of the cell, and also decreased the pro-inflammatory marker. Moreover, GSPE treatment was able to significantly reduce the body weight gain after 3 weeks of treatment.

On the other hand, DHA-OR increased the GSH/GSSG ratio but neither total antioxidant capacity nor body weight were influenced by this treatment. In this regard, there are conflicting results about the n-3 PUFAs effects. Some authors reported beneficial DHA effects on body weight [16], however, other authors showed that fish oil ingestion was not able to reduced body weight [17].

Finally, GSPE and DHA-OR although reduce the CRP levels, were not able to improve oxidative stress markers as evidenced by the decrease on the GSH/GSSG ratio. In this sense, the two compounds administered together activated the antioxidant HO-1 protein to counteract oxidative stress. In this case, GSPE+DHA-OR could ameliorate

oxidative stress via the Nrf2 activation and not interacting directly with GSH (Figure 24).

Globally, the results obtained in the *in vivo* experiments suggest that flavonoids, under oxidative stress environment such as genetic obesity, lipidic postprandial state and diet-induced obesity, are able to modulate the GSH metabolism. Consequently, proanthocyanidins increase the total antioxidant capacity of the cell. When animals were treated with n-3 PUFAs in a postprandial state condition or in diet-induced obesity model, the results also showed an oxidative stress improvement evidenced by: (a) GSH metabolism modulation, and (b) homeostatic redox balance.

Therefore, these *in vivo* results suggest that the combination of GSPE and DHA-OR modifies hepatic antioxidant status in lipidic postprandial state and could be an interesting option for the prevention of the transient redox unbalance related to a lipidic postprandial state. On the other hand, GSPE and DHA-OR shows different effects on oxidative stress in diet-induced obesity, although GSPE+DHA-OR combination increase the HO-1 protein expression to counteract the oxidative stress related with the obesity.

Otherwise, the *in vitro* experiment suggest that DHA is not always beneficial for cells and can be considered a double-edged sword in terms of benefits and risks, especially for situations of sustained oxidative stress conditions. However, the combination with EGCG could be an appropriate strategy to reduce the risks related with DHA supplementation.

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5 CONCLUSIONS

The principal conclusions of this research are:

A) *In vitro* experiment

1) DHA increase oxidative stress in a ROS environment.

In n-3 PUFAs pretreated hepatocyte cells, DHA is oxidized by *tert*-BHP-produced ROS, and is able to produce more oxidative damage. These oxidative inputs (ROS and MDA increase and, GSH/GSSG decrease) strongly induce Nrf2 nuclear translocation, which activates the antioxidant cell defense (HO-1 and CAT).

2) EGCG protects cells from "DHA supplementation damage" in an oxidative stress environment.

When cells are pretreated with the combination of EGCG and DHA, the n-3 PUFA is preserved due to the polyphenol effect, and less oxidative stress is generated. In consequence, Nrf2 nuclear translocation and HO-1 expression is repressed. Moreover, EGCG+DHA decrease lipid peroxidation and restore the GSH/GSSG ratio.

3) DHA is not always beneficial for cells and can be considered a double-edged sword in terms of benefits and risks, especially for situations of sustained oxidative stress conditions.

B) *In vivo* experiments

- **Genetic obesity rat model (Zucker)**

1) GSPE mitigate the glutathione alteration in the genetic obesity-induced oxidative stress.

GSPE administration to a genetic obesity rat model (Zucker), decrease the GSSG content and, consequently, increase the GSH/GSSG ratio suggesting an oxidative stress improvement. This oxidative stress amelioration leads to decrease the activation antioxidant enzymes (GPx, GR and GST) and to an increase in the total antioxidant capacity of the cell.

2) GSPE not change gene expression of antioxidant enzymes, although it is able to modify several antioxidant enzymes activities.

- **Lipidic postprandial state model**

1) The combination of GSPE and DHA-OR inhibit the Nrf2 nuclear translocation and the HO-1 expression in lipidic postprandial state.

The decrease of Nrf2 nuclear translocation could be an indicator of oxidative stress amelioration by the proanthocyanidins and n-3 PUFAs combination.

2) The combination of GSPE and DHA-OR modifies antioxidant status in lipidic postprandial state.

GSPE and DHA-OR combination decrease hepatic GSH content and increase GST activity, suggesting that the GSH fate is the S-conjugation with lipid hydroperoxides.

3) The GSPE and DHA-OR combination could represent an interesting option for the prevention of the transient unbalance between lipid hydroperoxide level and antioxidant status related to a lipidic postprandial state.

- **Cafeteria diet model**

1) Cafeteria diet is a good model to induce oxidative stress associated with obesity.

Cafeteria diet ingestion increase hepatic ROS production and, consequently, increase hepatic GSSG content and decrease GSH/GSSG ratio.

2) GSPE reduce hepatic GSSG content and increase plasmatic total antioxidant capacity in diet-induced obesity.

3) The combination of GSPE and DHA-OR ameliorate oxidative stress via the Nrf2 activation and not interacting directly with GSH.

Although GSPE+DHA-OR decrease hepatic GSH levels, the combination of two compounds increase the HO-1 protein expression to combat the oxidative stress.

6 ANNEXES

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Original Contribution

Grape seed procyanidin extract reduces the endotoxic effects induced by lipopolysaccharide in rats

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ABSTRACT

Acute inflammation is a response to injury, infection, tissue damage, or shock. Bacterial lipopolysaccharide (LPS) is an endotoxin implicated in triggering sepsis and septic shock, and LPS promotes the inflammatory response, resulting in the secretion of proinflammatory and anti-inflammatory cytokines such as the interleukins (IL-6, IL-1β, and IL-10) and tumor necrosis factor-α by the immune cells. Furthermore, nitric oxide and reactive oxygen species levels increase rapidly, which is partially due to the activation of inducible nitric oxide synthase in several tissues in response to inflammatory stimuli. Previous studies have shown that procyanidins, polyphenols present in foods such as apples, grapes, cocoa, and berries, have several beneficial properties against inflammation and oxidative stress using several *in vitro* and *in vivo* models. In this study, the anti-inflammatory and antioxidant effects of two physiological doses and two pharmaceutical doses of grape seed procyanidin extract (GSPE) were analyzed using a rat model of septic shock by the intraperitoneal injection of LPS derived from *Escherichia coli*. The high nutritional (75 mg/kg/day) and the high pharmacological doses (200 mg/kg/day) of GSPE showed anti-inflammatory effects by decreasing the proinflammatory marker NOx in the plasma, red blood cells, spleen, and liver. Moreover, the high pharmacological dose also downregulated the genes *Il-6* and *iNos;* and the high nutritional dose decreased the glutathione ratio (GSSG/total glutathione), further illustrating the antioxidant capability of GSPE. In conclusion, several doses of GSPE can alleviate acute inflammation triggered by LPS in rats at the systemic and local levels when administered for as few as 15 days before the injection of endotoxin.

Acute inflammation is a response to mechanical injury, infection, chemical injury, burns, radiation tissue injury, or shock [1]. Bacterial lipopolysaccharide (LPS)[1] is a glycolipid component of the cell wall of gram-negative bacteria and is an endotoxin implicated in triggering sepsis and septic shock [2]. LPS causes an acute inflammatory response, which evokes a state characterized by fever, hypotension, and multiorgan system failure [3]. The inflammation associated with endotoxic shock promotes an early response that involves the secretion of cytokines, chemokines, and other mediators, which triggers a sequence of events such as blood vessel dilation, increased blood flow, leukocyte infiltration, release of proteases, and the formation of oxygen free radicals [1].

In response to endotoxin, proinflammatory cytokines such as the interleukins (IL-6 and IL-1β) and tumor necrosis factor-α (TNF-α) and anti-inflammatory cytokines such as IL-10 are produced by inflammatory cells [4]. Furthermore, nitric oxide (NO) levels increase rapidly within minutes to hours in response to inflammatory stimuli [5]. In fact, inducible nitric oxide synthase (iNOS), which is induced upon exposure to endotoxin (LPS) as well as by cytokines such as TNF-α and IL-1β, produces NO in large amounts. iNOS is expressed by a variety of cells including macrophages, Kupffer cells, hepatocytes, and glial cells [4,6,7]. Thus, NO is a crucial factor during acute inflammation and sepsis and has been used as a marker of inflammation in many studies [4,6]. Moreover, TNF-α, IL-1β, and NO levels are altered at the systemic level in blood cells such as the erythrocytes [6] as well as in peripheral tissues such as the liver, spleen, brain, and adipose tissue. Therefore, many studies have focused on assessing how endotoxic shock increases the production of these proteins and molecules in these tissues as well as their relevance to the pathogenesis of endotoxemia [4,6,8].

Previous studies have shown that molecules derived from food have anti-inflammatory effects in endotoxin-treated rats [9–12].

Abbreviations: BSA, body surface area; CRP, C-reactive protein; DIAS, diastolic pressure; GSH, reduced glutathione; GSPE, grape seed procyanidin extract; GSSG, oxidized glutathione; ip, intraperitoneally; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NOx, nitrate and nitrite; PA, proanthocyanidin; PBS, phosphate-buffered saline; RBC, red blood cells; SYS, systolic pressure; TNF-α, tumor necrosis factor-α

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Several of these studies have shown that procyanidins, a class of polyphenols that belongs to the flavanol group and is present in food such as apples, grapes, cocoa, and berries and in beverages such as wine or tea, have several beneficial properties against inflammation and oxidative stress in several *in vitro* [13–16] and *in vivo* [17–19] models.

In this study, the anti-inflammatory and antioxidant properties of grape seed procyanidin extract (GSPE) were analyzed using a rat model of septic shock by the intraperitoneal injection of LPS derived from *Escherichia coli.* Treatment with a moderate–high and a high nutritional doses (50 and 75 mg/kg/day GSPE, respectively) and two high pharmaceutical doses (100 and 200 mg/kg/day GSPE) were tested to assess the preventive action of GSPE against an acute inflammatory response *in vivo*.

Materials and methods

Reagents

GSPE was provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, the GSPE contained monomeric (16.6%), dimeric (18.8%), trimeric (16.0%), tetrameric (9.3%), and oligomeric (5–13 U; 35.7%) procyanidins and phenolic acids (4.2%). The vehicle of GSPE was sweetened condensed milk diluted in water in a 1:6 ratio. The milk composition per 100 g was as follows: 8.9 g protein, 0.4 g fat, 60.5 g carbohydrates, and 281 kcal.

LPS from *E. coli* 0111:B4 (Ref. L2630, Lot 030M4114) was purchased from Sigma–Aldrich (St. Louis, MO, USA). LPS was diluted in salt serum and was administered to the animals intraperitoneally (ip).

Animals and experimental protocol

Wistar female rats (Charles River Laboratories, L'Arbresle Cedex, France) weighing between 200 and 225 g at 11 weeks of age were housed in cages in pairs or trios at a constant temperature (22 °C). The rats were subjected to a standard 12-h light/dark cycle and were provided with water and feed *ad libitum*. The rats were allowed to adapt to their environment for 1 week before the beginning of the experiments.

Rats were randomly distributed in groups according to the treatment they received: negative control (C−; $n=3$) and positive control groups (C+; $n=5$) were treated with vehicle. Four groups were treated with various doses of GSPE: 50 mg/kg/day GSPE (50PE, $n=5$), 75 mg/kg/day GSPE (75PE, $n=5$), 100 mg/kg/day GSPE (100PE, $n=5$), and 200 mg/kg/day GSPE (200PE, $n=5$; Fig. 1). The treatment was given before LPS administration.

All groups received treatment at 7:00 PM for 15 days by controlled oral intake using a syringe until a day before the animals were sacrificed. During the experimental period, the rats were provided with free access to water and feed immediately after the treatment until the morning to improve the absorption of GSPE with the diet. In the morning, the feed was removed from the animal cages and was replenished after GSPE treatment. The food intake and body weight were measured weekly. LPS was injected (ip) after 15 days of treatment at 7 mg/kg into the C+, 50PE, 75PE, 100PE, and 200PE groups on the day the animals were sacrificed (Fig. 1). After LPS injection, the rats were fasted until euthanasia, and during the fasting period, blood samples were taken from the tail at 0 and 4 h. Seven and a half hours after the LPS injection, the rats were anesthetized with 75 mg/kg sodium pentobarbital (ip) and were sacrificed by exsanguination to obtain blood from the aorta, collected in prerinsed tubes with EDTA. The liver and spleen were surgically excised. Plasma was obtained by centrifuging the blood samples at 2000*g* for 10 min. All of the samples were stored at −80 °C.

Nitrate and nitrite measurements in the plasma, red blood cells, liver, and spleen

For the quantification of nitrate and nitrite (NOx) levels, a colorimetric assay kit (Ref. 780001) purchased from Cayman Chemical (Madrid, Spain) was used. The preparation of the samples was performed as follows: Red blood cell (RBC) samples were obtained from the blood after separating the plasma. RBC lysis buffer (0.154 M NH_4Cl, 9.988 mM $KHCO_3$, 0.126 mM EDTA; autoclaved) was added to the blood, and then the samples were vortexed and centrifuged at 2000*g* for 5 min. The supernatants were collected and were stored at −80 °C. Liver and spleen samples were homogenized in autoclaved PBS, centrifuged at 10,000*g* for 20 min, and stored on ice until the deproteinizing treatment. Plasma, RBC, liver, and spleen samples were deproteinized using prerinsed Amicon Ultra-0.5 centrifugal filter devices (Ref. UFC501096; 10 K) purchased from Millipore (Billerica, MA, USA). Then, the NOx levels were quantified using a colorimetric kit according to the manufacturer's instructions. Before deproteinization, protein concentrations in all samples were determined by using the Bradford assay [20] to normalize the results.

TNF-α and IL-10 measurements in plasma

For the TNF-α and the IL-10 assays, two ELISA kits were used (Refs. KRC3011 and KRC0101, respectively). These kits were purchased from Invitrogen (Camarillo, CA, USA). TNF-α and IL-10 levels were quantified according to the manufacturer's instructions.

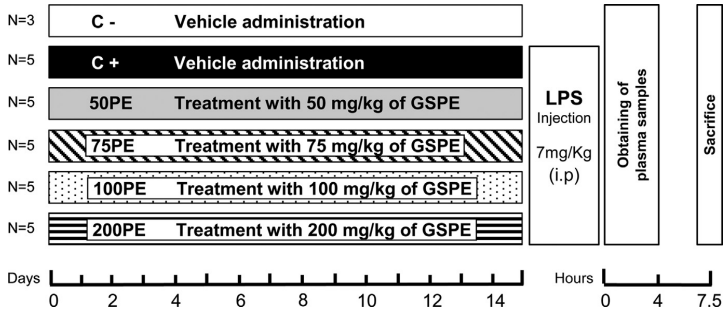

Fig. 1. Time line for the groups and treatments applied in the experiment. GSPE, grape seed procyanidin extract. LPS, lipopolysaccharide.

Semiquantitative real-time PCR of proinflammatory and anti-inflammatory genes from the liver

RNA from liver tissue was isolated with the RNeasy Mini Kit from Qiagen (Ref. 74104; Barcelona, Spain). cDNA was synthesized from 2 μg of total RNA using the High Capacity cDNA reverse transcription kit (Ref. 4368814; Invitrogen). cDNA (20 ng) was subjected to quantitative RT-PCR amplification using SYBR Green PCR Master Mix from Applied Biosystems (Ref. 4309155; Madrid, Spain). The forward and reverse primers for the rat genes analyzed are shown in Table 1. Reactions were run on a quantitative real-time PCR system (Applied Biosystems); the thermal profile settings were 50 °C for 2 min and 95 °C for 2 min and then 40 cycles at 95 °C for 15 s and 60 °C for 2 min. Finally, statistical data were converted and normalized to the linear form by the 2^{-CT} ($\Delta\Delta C_T$) calculation [21]. The relative expression levels of the proinflammatory genes *Crp, Il-6, iNos,* and *Tnf-α* and the anti-inflammatory gene *Il-10* were assessed in the liver and were normalized to cyclophilin mRNA levels.

Table 1
Rat-specific primer sequences.

Gene	Primer sequence
Crp	Fw: 5′-GGCTTTTGGTCATGAAGACATG-3′ Rv: 5′-TCTTGGTAGCGTAAGAGAAGA-3′
Tnf-α	Fw: 5′-CCTCACACTCAGATCATCTTCTC-3′ Rv: 5′-TTGGTGGTTTGCTACGACGTG-3′
Il-6	Fw: 5′-CTCTCCGCAAGAGACTTCC-3′ Rv: 5′-GCCATTGCACAACTCTTTTCTC-3′
Il-10	Fw: 5′-GCAGGACTTTAAGGGTTACTTGG-3′ Rv: 5′-GGAGAAATCGATGACAGCGT-3′
iNos	Fw: 5′-CACCCGAGATGGTCAGGG-3′ Rv: 5′-CCACTGACACTCCGCACAA-3′
Ppia	Fw: 5′-CTTCGAGCTGTTTGCAGACAA-3′ Rv: 5′-AAGTCACCACCCTGGCACATG-3′

Crp, C-reactive protein; *Tnf-α,* tumor necrosis factor-α; *Il-6,* interleukin-6; *Il-10,* interleukin-10; *iNos,* inducible nitric oxide synthase; *Ppia,* cyclophilin A. Fw, forward primer sequence; Rv, reverse primer sequence.

Oxidative stress analysis by measuring the hepatic glutathione ratio (oxidized glutathione (GSSG)/total glutathione)

For the reduced/oxidized glutathione analyses, the enzymatic method reported by Griffith was used [22]. Briefly, this method is based on the reaction of the glutathione present in the sample with 5,5′-dithiobis(2-nitrobenzoic acid) (D8130; Sigma–Aldrich) to generate 2-nitro-5-thiobenzoic acid, a yellow compound that absorbs at 412 nm, and GSSG. To determine total glutathione in the sample, the reduced form is regenerated from the oxidized form by glutathione reductase present in excess. On the other hand, the determination of GSSG exclusively could be measured from the reduced glutathione (GSH) scavenged by 2-vinylpyridine, a thiol-scavenging reagent, to form a pyridinium salt. For this assay, livers were homogenized and deproteinized with metaphosphoric acid (6%) and were centrifuged at 5000*g* for 15 min at 4 °C. The supernatants were used for the GSSG and the total glutathione analysis.

Statistical analysis

The results are presented as the mean with the associated standard error (SE). The data were analyzed using a one-way ANOVA to determine the significant difference using IBM SPSS statistical software (version 19.0 for Windows; SPSS, Inc.). p values <0.05 were considered statistically significant.

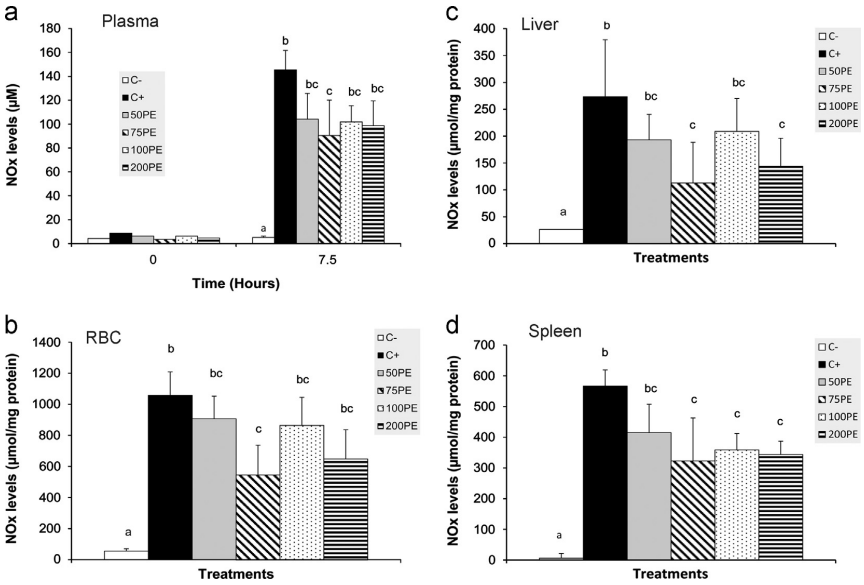

Fig. 2. NOx (nitrate+nitrite) levels (μmol/mg protein) in (a) plasma, (b) red blood cells, (c) liver, and (d) spleen. The results are presented as the mean ± SE for each group ($n=5$; C−, $n=3$): negative control group (C−), positive control group (C+), and groups treated with 50, 75, 100, and 200 mg/kg grape seed procyanidin extract (50PE, 75PE, 100PE, and 200PE, respectively). Different letters (a, b, c) indicate significant differences between groups. Statistical significance was determined using a one-way ANOVA, and p values <0.05 were considered statistically significant.

Results

NOx levels are reduced in blood and tissues in GSPE groups

NOx levels were analyzed in the liver, spleen, plasma, and RBCs. The NOx levels of the C+ group were increased relative to those of the C− group in all tissues analyzed (Figs. 2a–d). In the plasma (Fig. 2a) and RBCs (Fig. 2b), rats treated with 75 mg/kg GSPE (75PE) showed significantly decreased levels of NOx compared with the C+ group. Moreover, the hepatic NOx levels of the 75PE and 200PE groups decreased significantly compared with the NOx levels of the C+ group (Fig. 2c), whereas the splenic levels of NOx in the 75PE, 100PE, and 200PE groups were also decreased relative to the C+ group (Fig. 2d). Furthermore, all groups treated with GSPE showed decreased levels of NOx compared to the C+ group; however, only the 75PE group reached statistical significance in all the locations assessed.

Plasma levels of IL-10 decrease upon GSPE treatment

The plasma levels of the proinflammatory molecule TNF-α and the anti-inflammatory molecule IL-10 were assessed after the injection of LPS. On one hand, TNF-α levels were increased in all of the groups 4 h after LPS injection relative to the basal levels of TNF-α (Fig. 3a). On the other hand, IL-10 levels were elevated at 4 and 7.5 h after LPS injection (Fig. 3b). Importantly, the IL-10 levels of the 50PE and 200PE groups were significantly decreased compared with the C+ group 7.5 h after LPS injection (Fig. 3b).

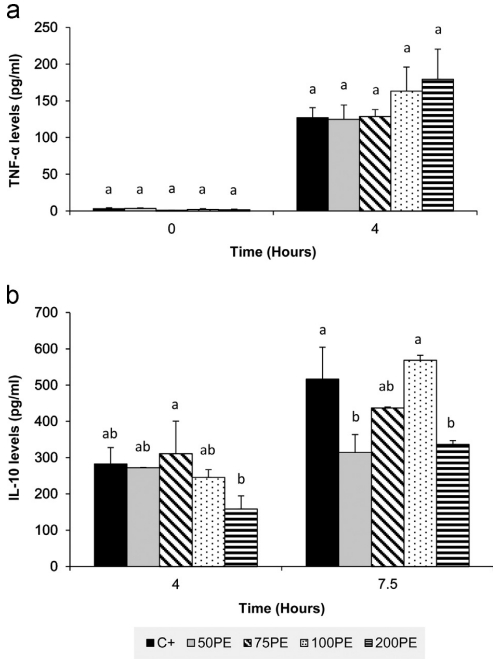

Fig. 3. (a) Tumor necrosis factor-α (TNF-α; pg/ml) and (b) interleukin-10 (IL-10; pg/ml) levels in the plasma. The results are presented as the mean ± SE for each group ($n=5$): positive control group (C+) and groups treated with 50, 75, 100, and 200 mg/kg grape seed procyanidin extract (50PE, 75PE, 100PE, and 200PE, respectively). Different letters (a, b) indicate significant differences between groups. Statistical significance was determined using the one-way ANOVA, and p values < 0.05 were considered statistically significant.

Downregulation of proinflammatory genes in liver by GSPE treatments

The relative hepatic mRNA expression levels of the proinflammatory genes *Tnf-α, iNos, Il-6,* and *Crp* and the anti-inflammatory gene *Il-10* were analyzed. Expression of *Tnf-α, iNos, Il-6,* and *Il-10* was upregulated upon injection with LPS (C+) in contrast to the non-LPS group (C−) (Figs. 4a–d), whereas *Crp* gene expression was downregulated (Fig. 4e). Expression of *iNos* was downregulated by GSPE pretreatment in the 50PE, 100PE, and 200PE groups (Fig. 4b), whereas *Il-6* gene expression was downregulated in the 200PE group relative to the C+ group (Fig. 4d). In contrast, *Il-10* and *Crp* gene expression was not transcriptionally regulated by any of the GSPE dosages relative to the C+ group (Figs. 4c and e).

GSPE reverses hypotension upon injection of LPS

Treatment with GSPE for 15 days had no effect on body weight (Table 2) or the amount of food ingested (data not shown). However, pretreatment with GSPE reversed the drop in systolic pressure (SYS), diastolic pressure (DIAS), and the SYS/DIAS ratio at the 7.5-h time point after LPS injection (Table 2). In particular, the 200PE group reversed the drop in the DIAS and the SYS/DIAS ratio compared with the C+ group, whereas the 75PE, 100PE, and 200PE groups reversed the drop in the SYS, DIAS, and SYS/DIAS ratio (Table 2).

A high nutritional dose of GSPE decreases the GSSG/total glutathione ratio in liver

When cells are exposed to increased levels of oxidative stress, the oxidized form of glutathione (GSSG) accumulates, and the GSSG/total glutathione ratio increases. The GSSG, total glutathione, and GSSG/total glutathione ratio were measured as useful indicators of oxidative stress in the liver. The 75PE group showed significantly increased levels of total glutathione and decreased GSSG/total glutathione ratio at 7.5 h after LPS injection (Figs. 5a and b).

Discussion

In recent years, the study of the anti-inflammatory effects of bioactive molecules present in food has become more popular. How bioactive molecules influence the proinflammatory and anti-inflammatory effects triggered during acute inflammation remains to be fully understood.

In this work, a grape seed extract with large amounts of procyanidins, a type of proanthocyanidin (PA), was used to assess their anti-inflammatory effects in a model of acute inflammation *in vivo*. Several doses of GSPE were used to ascertain if their effects could be dose-dependent and to assess which can be the most effective. Considering that the maximum intake of PAs in various human populations can reach 400–450 mg/day [23,24], the dose of 50 mg/kg used in rats could be considered a moderate–high dose. This extrapolation between human and animal doses has been normalized using the body surface area (BSA)-based dose calculation [25], which is considered more appropriate than the simple conversion based on body weight. A human diet with foods rich in procyanidins, such as red wine, baking chocolate, apples, and dried fruits, could reach dosages up to 700–800 mg/day of procyanidins (using tables of foods containing PAs) [26,27], which would be approx 70–80 mg/kg in rats

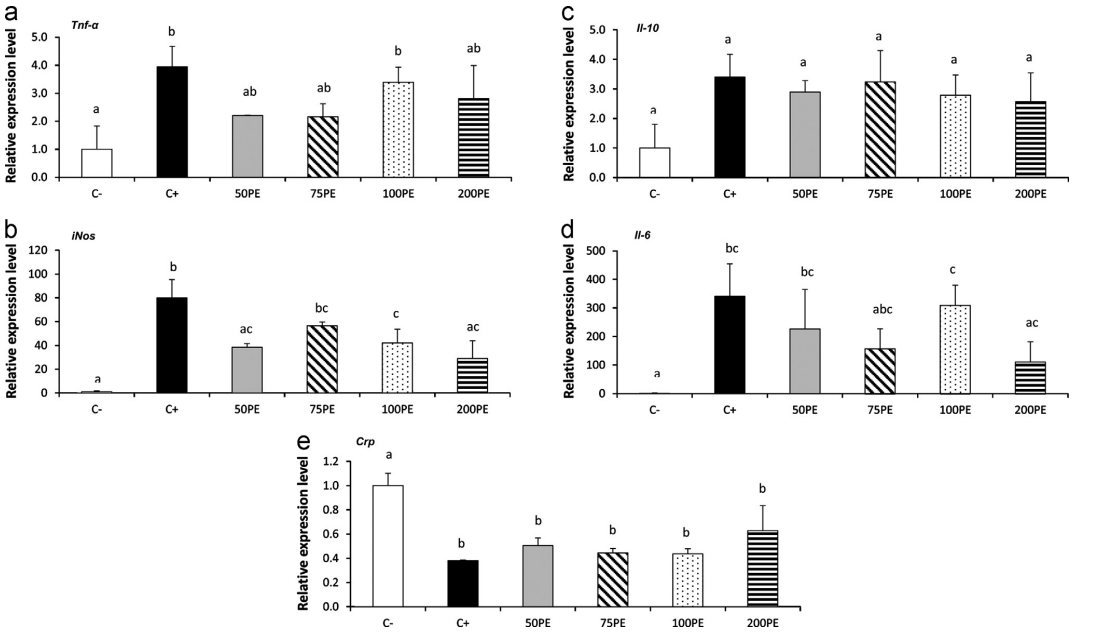

Fig. 4. Relative hepatic mRNA expression levels of the genes (a) *Tnf-α*, (b) *iNos*, (c) *Il-10*, (d) *Il-6*, and (e) *Crp*. To calculate the relative mRNA expression levels, the ΔΔCT method was used. The positive control group (C+) and groups treated with 50, 75, 100, and 200 mg/kg grape seed procyanidin extract (50PE, 75PE, 100PE, and 200PE, respectively) were normalized to the negative control group (C−). The results are presented as the mean ± SE. Different letters (a, b, c) indicate significant differences between groups. Statistical significance was determined using a one-way ANOVA, and p values <0.05 were considered statistically significant.

using the BSA conversion. Thus, 75 mg/kg GSPE can be considered a high nutritional dose. On the other hand, we consider that doses that exceed double the maximum intake in humans ($\geq$900 mg/day PA in humans, approx 90 mg/kg PA in rats) would be more difficult to implement in a human diet and would be reached only by supplemental intake (nutraceuticals). Therefore, the doses of 100 and 200 mg/kg GSPE have been considered pharmacological doses. To assess the anti-inflammatory effects of GSPE, preventive treatments for 15 days with these four doses were performed. This period was chosen because it is enough to be considered chronic and not too short to be considered acute. In addition, as a novel study we considered that 15 days were enough for GSPE to exert effects against inflammation because in previous studies using the same extract it has been shown that it exerts anti-inflammatory effects when rats were treated for 10 or 30 days with 25 and 50 mg/kg GSPE [19].

To assess the preventive effects of GSPE on acute inflammation, our experiments focused on the NOx species. NOx species (nitrate and nitrite) have been widely tested in septic shock studies [5–7]. NOx have been shown to be a relevant marker of inflammation in tissues such as the liver and spleen [4,28]. In the liver, the inducible expression of *iNos*, the enzyme implicated in the synthesis of NO, seems to play an important role in the pathogenesis of endotoxemia [4]. Therefore, NOx as well as the classical proinflammatory cytokine TNF-α and anti-inflammatory cytokine IL-10 [6] were used as markers to study the effects of GSPE in rats treated with LPS.

Rats treated with GSPE for 15 days showed no significant differences in food intake or in weight (Table 2). Treatment with 7 mg/kg LPS derived from *E. coli* (ip) caused acute inflammation as indicated by an increase in proinflammatory proteins such as TNF-α in the plasma at 4 h after LPS treatment (Fig. 3a). LPS treatment also caused an increase in NOx levels in the blood (Figs. 2a and b), in the liver (Fig. 2c), and in the spleen (Fig. 2d) at the 7.5-h time point after LPS treatment, and these results are similar to previous studies [4,6,29–31]. In addition to the increase in expression of proinflammatory genes such as *Il-6*, *iNos*, and *Tnf-α* and the anti-inflammatory gene *Il-10* at the transcript (Fig. 4) and protein levels (Fig. 3b), these results indicate that the dose of LPS administered was optimal to induce the proinflammatory state without causing death from septic shock within the time frame of the experiment, similar to previous studies in rats [32–34].

Additionally, the effects of GSPE on the levels of NOx in the RBCs, plasma, liver, and spleen were analyzed. The liver was included because it is one of the major endocrine organs involved in the regulation of metabolism by synthesizing cytokines and acute phase proteins during inflammation, whereas the spleen was included because it is responsible for both specific and nonspecific host defense. Furthermore, the spleen harbors a high content of phagocytes and maintains a direct connection with the bloodstream, which illustrates the importance of the spleen in the clearance of circulating microorganisms, particles, and some large molecules such as LPS [28,35]. The level of NOx species correlates with the activity of agents that cause acute inflammation such as LPS. In a global overview, several doses of GSPE reduced the levels of NOx in the plasma and RBCs as well as in the liver and spleen. Interestingly, the nutritional high dose of 75 mg/kg/day of GSPE (75PE) decreased the levels of NOx in all locations assessed (Figs. 2a–d). These results suggest that the 75 mg/kg/day dose has a stronger anti-inflammatory effect than the others, thus effectively reducing the level of NOx induced by LPS in several peripheral organs as well as at the systemic level. Significantly, high doses of GSPE also appeared to reduce the level of NOx in the liver (200PE; Fig. 2c) and the spleen (100PE and 200PE; Fig. 2d), although decreases were not observed in the circulating levels of NOx (Figs. 2a and b). These results could suggest that the

Table 2
Body weight and blood pressure measures.

Parameter	C+	50PE	75PE	100PE	200PE
Body wt (0 days)	$203.00^{a} \pm 2.28$	$202.60^{a} \pm 3.90$	$205.40^{a} \pm 4.03$	$206.00^{a} \pm 3.53$	$206.00^{a} \pm 3.70$
Body wt (15 days)	$234.00^{a} \pm 2.83$	$224.80^{a} \pm 2.68$	$243.10^{a} \pm 2.89$	$234.60^{a} \pm 3.69$	$241.12^{a} \pm 4.57$
SYS drop	$-49.65^{a} \pm 6.36$	$-44.23^{ab} \pm 6.67$	$\mathbf{-19.00^{bc} \pm 10.63}$	$\mathbf{-10.21^{c} \pm 5.08}$	$\mathbf{-7.23^{c} \pm 6.40}$
DIAS drop	$-60.82^{a} \pm 6.02$	$\mathbf{-31.15^{bc} \pm 17.85}$	$-43.56^{abc} \pm 9.65$	$-49.51^{ab} \pm 3.62$	$\mathbf{-20.66^{c} \pm 4.97}$
SYS/DIAS ratio drop	$-53.85^{a} \pm 6.19$	$-34.30^{ab} \pm 10.70$	$-38.08^{ab} \pm 13.00$	$-43.20^{ab} \pm 6.87$	$\mathbf{-21.57^{b} \pm 4.58}$

Positive control group (C+) and groups treated with 50, 75, 100, and 200 mg/kg GSPE (50PE, 75PE, 100PE, and 200PE, respectively) are shown. Body weight was measured at the initiation of GSPE treatment and after 15 days of GSPE or vehicle treatment. To calculate the SYS and DIAS drop and SYS/DIAS ratio drop, the mean value of the negative control group was subtracted from each group. The results are presented as the mean ± SE. Different superscript letters (a, b, c) indicate significant differences between groups. Values in boldface are different statistically versus C+. Statistical significance was determined using a one-way ANOVA, and p values < 0.05 were considered statistically significant. GSPE, grape seed procyanidin extract; SYS, systolic blood pressure; DIAS, diastolic blood pressure.

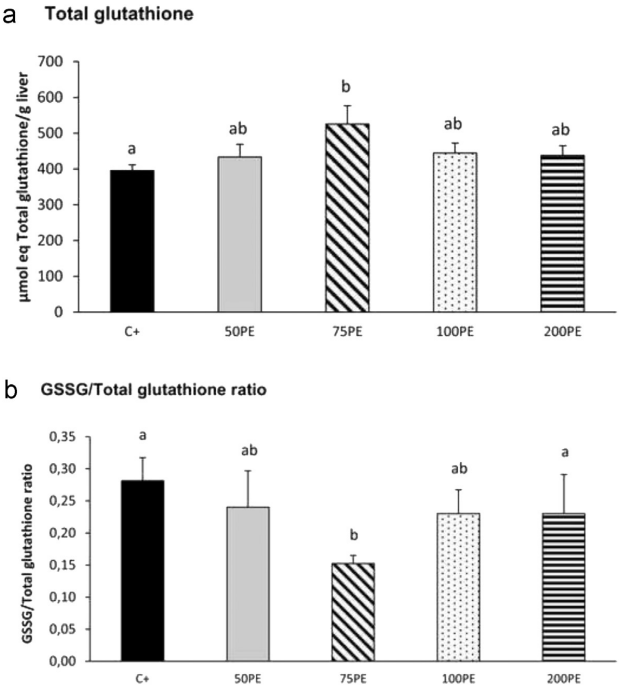

Fig. 5. (a) Total glutathione (µmol/g liver) and (b) the oxidized form of glutathione versus total glutathione (GSSG/total glutathione ratio) levels in the liver. The results are presented as the mean ± SE for each group ($n=5$): positive control group (C+) and groups treated with 50, 75, 100, and 200 mg/kg grape seed procyanidin extract (50PE, 75PE, 100PE, and 200PE, respectively). Different letters (a, b) indicate significant differences between groups. Statistical significance was determined using a one-way ANOVA, and p values < 0.05 were considered statistically significant.

highest doses may not be as effective at reducing the level of NOx in various tissues affected by LPS and may ultimately contribute to the NOx circulating levels in the blood.

On the other hand, other parameters assessed in the plasma such as the anti-inflammatory IL-10 (Fig. 3b) decreased in rats treated with either the highest dose (200PE) or the lowest dose (50PE), suggesting that GSPE could inactivate the synthesis of IL-10 to counteract the action of this anti-inflammatory cytokine. In contrast, these results could also suggest that the dose of GSPE is sufficient to reduce inflammation to the extent that IL-10 synthesis is no longer necessary, resulting in IL-10 downregulation.

Although TNF-α plasma levels were slightly increased at the highest doses (100PE, 200PE; Fig. 3a), none of the GSPE dosages could change these levels significantly, in contrast to the positive control. This suggests that none of the doses could counteract the activation of TNF-α caused by exposure to a proinflammatory stimulus.

The expression of proinflammatory and anti-inflammatory genes in the liver was analyzed to assess the effects of GSPE on this organ. The results showed that almost all GSPE dosages downregulated *iNos* expression significantly compared with the C+ group (Fig. 4b). The group receiving 200 mg/kg/day GSPE (200PE) showed the greatest downregulation of *iNos* and *Il-6* expression (Figs. 4b and d). These data indicate that GSPE exerts its effects at the level of expression; however, high doses may be necessary to achieve the full regulatory potential of GSPE. Surprisingly, *Crp*, a gene that encodes a proinflammatory acute phase protein, was downregulated in all groups treated with LPS compared with the C− group (Fig. 4e). This suggests that its expression is deactivated at 7.5 h upon LPS administration, possibly to regulate the synthesis and avoid an

excess of CRP at systemic levels, which could trigger a dangerous proinflammatory systemic state and lead to the death of the rat. On the other hand, the expression of *iNos* (Fig. 4b) correlates with NOx levels (Fig. 2c) in the liver. This result suggests that GSPE could have a direct negative transcriptional effect on the *iNos* gene resulting in lower levels of NO, which represents one of the key elements involved in liver pathology during endotoxic shock [4].

GSH is the main nonenzymatic antioxidant defense within the cell, and GSH plays an important role in the protection against oxidative stress [36]. Therefore, hepatic GSH levels were measured to determine whether GSPE treatments affect the level of oxidative stress related to acute inflammation induced by LPS. Treatment with the nutritional high dose of 75 mg/kg/day GSPE (75PE) increased the total glutathione level and decreased the GSSG/total glutathione ratio (Figs. 5a and b), suggesting that GSPE also acts as an antioxidant agent reducing oxidative stress.

A recent study showed that the anti-inflammatory effects of PAs could be due to the capacity of these molecules to bind to LPS and repress the binding to Toll-like receptor 4 [37], which mediates the activation of the proinflammatory nuclear factor-κB pathway. Thus, the anti-inflammatory action of GSPE in this model could be explained by their capacity to bind to LPS, promoting an inactivation of proinflammatory pathways and decreasing the expected inflammatory response. On the other hand, it has been shown that several metabolites and molecules such as dimeric and trimeric procyanidins, as well as metabolites such as (epi)catechin glucuronides and sulfates, are found in plasma and tissues after GSPE ingestion in rats and humans [38–41]. Thus, the effects of GSPE could also be due to the ability of these molecules to repress the macrophage activation by LPS through their anti-inflammatory and antioxidant mechanisms found in studies *in vitro* [13,16,42–44].

Conclusions

In conclusion, GSPE has anti-inflammatory and antioxidant effects at the systemic and gene expression levels when administered 15 days before LPS-induced acute inflammation. In particular, the nutritional high dose (75 mg/kg/day) and the high dose (200 mg/kg/day) possess anti-inflammatory effects as shown by a decrease in the NOx proinflammatory markers in the plasma, RBCs, spleen, and liver. Furthermore, the highest dose (200 mg/kg/day) downregulated the genes *Il-6* and *iNos,* and the nutritional high dose (75 mg/kg/day) decreased the GSSG/total glutathione ratio, illustrating the antioxidant capacity of GSPE.

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Annexe II
Resum

L'obesitat és considerada un desordre metabòlic que ens els últims 20 anys està incrementant d'una manera exponencial als països desenvolupats. L'obesitat està associada amb el desenvolupament de malalties cròniques com són la hipertensió, resistència a la insulina i la dislipèmia, les quals constitueixen el Síndrome Metabòlic (MetS). Existeixen moltes evidencies suggerint que l'estrès oxidatiu (EO) juga un paper important en la fisiopatologia de les manifestacions relacionades amb el MetS. En aquest sentit, l'esteatosi hepàtica ha estat associada amb l'obesitat i, en conseqüència, l'acumulació dels lípids hepàtics i la presència d' especies reactives d'oxigen (ROS) promouen l'EO.

Cal destacar de manera important que diversos estudis han mostrat la millora de canvis metabòlics relacionats amb la obesitat utilitzant suplements nutricionals, incloent els polifenols i els àcids grassos poliinsaturats omega-3 (n-3 PUFAs). D'acord amb aquesta premissa, la hipòtesi d'aquest estudi és que els polifenols i els n-3 PUFAs poden tenir efectes additius antioxidants en diferents condicions d'EO tant en experiments *in vitro* com en *in vivo*.

El nostre grup de recerca ha estat estudiant principalment els efectes beneficiosos dels polifenols. Per aquesta raó, el primer objectiu d'aquest recerca va ser avaluar els efectes de l'extracte de proantocianidines de pinyol de raïm (GSPE) en condicions d' EO associada a l'obesitat genètica que desenvolupen les rates Zucker fa/fa. Un cop confirmat que els animals control d'obesitat van desenvolupar hipertrigliceridèmia, hiperinsulinemia, EO i esteatosis hepàtica o fetge gras no alcohòlic (NAFLD), els animals van ser tractats diàriament amb 35 mg GSPE/kg pes corporal durant 10 setmanes, i vam estudiar els efectes del GSPE en l'EO, així com en la esteatosis hepàtica. Els resultats mostren que el GSPE va millorar el metabolisme hepàtic del glutatió (GSH), evidenciat per un augment del ratio GSH/GSSG i per la disminució dels nivells de GSSG. A més, el GSPE va augmentar significativament la capacitat total antioxidant de la cèl·lula. Degut a aquest efecte, els enzims GPx, GR i GST no es van activar. En concordança, ha estat demostrat que animals tractats amb compostos antioxidants mostraven menys activitat enzimàtica antioxidant, degut a la inhibició de la generació de radicals superòxids i hidroxils.

Un cop demostrat els efectes antioxidants del GSPE davant de l'EO relacionat amb la obesitat genètica, vam voler estudiar si els n-3 PUFAs eren capaços de mitigar l'EO en combinació amb el GSPE.

Primer de tot, vam avaluar dos compostos purs pertanyents al grup dels flavonoides i dels n-3 PUFAs, fent servir cultius cel·lulars d'hepatòcits de rata. Malgrat els beneficis

relacionats amb el consum de n-3 PUFAs, ha estat mostrat que aquests àcids grassos poden incrementar la peroxidació lipídica degut a la susceptibilitat que tenen a la oxidació davant dels radicals lliures, incrementat així l'EO. Vam estudiar si el tractament amb l'omega-3 àcid docosahexaenoic (DHA), podia incrementar l'EO dels hepatòcits induït amb *tert*-BHP, i si, pel contrari, el polifenol majoritari del té verd, l'epigalocatequin-3-galat (EGCG), podia protegir la cèl·lula d'aquest efecte perjudicial. Vam observar que el tractament amb DHA augmentava el dany oxidatiu relacionat amb el *tert*-BHP, augmentant la producció de ROS i la peroxidació lipídica, i disminuint el ratio GSH/GSSG. En conseqüència, la translocació nuclear del factor de transcripció Nrf2, considerat com el principal regulador de la defensa antioxidant, va ser activada, augmentant l'expressió de HO-1, un enzim antioxidant. En aquest sentit, és ben sabut que el DHA s'incorpora fàcilment als fosfolípids de les membranes augmentant al fetge la susceptibilitat a la peroxidació lipídica. Quan les cèl·lules van ser tractades amb la combinació de DHA i EGCG, es va veure que el polifenol era capaç de protegir la cèl·lula del dany oxidatiu induït pel *tert*-BHP i el DHA. D'aquesta manera, l'EGCG va inhibir la translocació nuclear de Nrf2 i va disminuir l'expressió de HO-1 com a indicatiu d'una millora en el balanç redox, degut a (a) la disminució de la producció de ROS; (b) disminució de la peroxidació lipídica; (c) augment de la viabilitat cel·lular; (d) augment del ratio GSH/GSSG. Aquest estudi suggereix que podria ser útil considerar l'estat d'estrès oxidatiu quan s'administrin n-3 PUFAs, encara que és important considerar que són necessaris estudis complementaris en altres línies cel·lulars per clarificar els efectes del DHA en l'EO cel·lular.

Després d'estudiar els efectes *in vitro*, vam decidir continuar estudiant els efectes dels polifenols i n-3 PUFAs en models *in vivo*.

El primer va ser estudiar aquests efectes en condicions d'EO postprandial en rates mascles sanes. L'EO postprandial es caracteritza per un increment en la susceptibilitat a l'organisme cap all dany oxidatiu després del consum d'un menjar ric en lípids i/o carbohidrats. En aquest estudi, l'estat postprandial va ser induït amb una càrrega oral de greix saturat (mantega de porc). Vam estudiar els efectes sobre l'EO de GSPE i d'un oli ric en DHA, incloent el metabolisme del GSH i la ruta de senyalització Nrf2. Els resultats vam mostrar que després de 5 h de l'administració de GSPE els nivells de AST, marcador de dany hepàtic, i de la CRP, marcador d'inflamació, estaven reduïts igual que l'expressió del gen p22phox, subunitat de la major font de ROS, la NADPH oxidasa. A més, el tractament amb DHA-OR va disminuir els nivells de TG i colesterol hepàtics, així com la CRP i expressió de p22phox. El resultats més importants en aquest estudi van ser que el tractament amb DHA-OR i GSPE+DHA-OR van reduir els nivells del GSH hepàtic. Aquesta disminució es correlaciona amb l'increment de l'activitat de l'enzim

GST al fetge particularment en el grup de GSPE+DHA-OR, suggerint que la disminució dels nivells de GSH és degut a la conjugació del grup S amb els hidroperòxids lipídics.

Concloent, aquest estudi evidencia que la combinació de GSPE+DHA-OR modifica l'estat antioxidant hepàtic en el context d'un sol àpat alt en greix, promovent un efecte beneficiós a través de mecanismes genòmics (translocació de Nrf2) i no genòmics (activitats enzimàtiques antioxidants).

Finalment, es va dissenyar un experiment animal d'obesitat induïda per una dieta rica en greix. Aquesta dieta, anomenada dieta de cafeteria (CD), consisteix en bacon, galetes, paté, formatge, magdalenes, pastanaga i llet amb sucre, i està molt demostrat que és un bon model per induir obesitat i desordres relacionats en rates. Molts estudis suggereixen que l'obesitat i la esteatosis hepàtica induïda per CD poden causar EO a través de diversos mecanismes incloent la disfunció mitocondrial, esgotament del GSH i la reducció d'activitats d'enzims antioxidants. En aquest sentit, ha estat descrit que els polifenols i els n-3 PUFAs són capaços de frenar el desenvolupament de malalties hepàtiques com NAFLD. Així, rates mascles van ser alimentades amb CD, i un cop confirmat que patien obesitat i esteatosis hepàtica, se'ls hi va administrar una dosi diària de GSPE, DHA-OR i GSPE+DHA-OR durant tres setmanes, per avaluar el efectes d'aquests compostos bioactius de manera individual o combinada en l'EO induït per la CD.

S'ha descrit que els polifenols prevenen la obesitat induïda per la dieta millorant l'EO. En aquest sentit, el tractament de GSPE va reduir els nivells de GSSG i, en conseqüència, va millorar la capacitat antioxidant cel·lular. A més, al cap de tres setmanes de tractament amb GSPE, els animals van reduir el guany de pes corporal significativament comparat amb les rates control.

Per una altra banda, el DHA-OR va augmentar el ratio GSH/GSSG encara que aquest tractament no va ser capaç de millorar la capacitat total antioxidant, així com tampoc va influir en el pes corporal dels animals. En aquest sentit, es troben resultats oposats a la literatura. Alguns autors demostren efectes beneficiosos del DHA en el pes corporal, mentre que altres autors informen que la ingesta d'oli de peix no es capaç de reduir el pes corporal.

Finalment, el tractament amb els dos compostos junts, GSPE+DHA-OR, encara que va reduir els nivells de CRP, no va ser capaç de millorar els marcadors d'EO evidenciat per una disminució del ratio GSH/GSSG. Així doncs, GSPE+DHA-OR van activar la síntesi de

la proteïna HO-1 com a resposta contra l'EO. En aquest cas, GSPE+DHA-OR podria millorar l'EO via l'activació de Nrf2 i no directament reaccionant amb el GSH.

Tenint tot en compte, els resultats obtinguts en *in vivo* suggereixen que els flavonoides, sota un ambient d'EO incloent l'obesitat genètica, un estat postprandial lipidic i obesitat induïda per la dieta, són capaços de modular el metabolisme del GSH. Per tant, les proantocianidines augmenten la capacitat antioxidant total de la cèl·lula. Quan els animals van ser tractats amb n-3 PUFAs en un estat postprandial lipidic o en un model d'obesitat induïda per la dieta, els resultats mostren que també milloren l'EO, tal i com mostren els resultats del metabolisme del GSH i els marcadors d'estat antioxidant.

A més, la combinació de GSPE amb DHA-OR modifiquen l'estat antioxidant hepàtic en un estat postprandial lipidic i podria ser una interessant opció per la prevenció del desequilibri redox transitori relacionat amb un estat postprandial lipidic. Per una altra part, GSPE i DHA-OR mostren diferents efectes en l'EO en un model d'obesitat induïda per la dieta, encara que la combinació del dos productes naturals augmenten l'expressió de la proteïna HO-1 per combatre l'EO relacionat amb l'obesitat.

Per una altra part, l'experiment *in vitro* suggereix que el DHA no sempre és beneficiós per les cèl·lules, especialment en un ambient d'EO. No obstant, la combinació amb l'EGCG podria ser una estratègia per reduir els riscos relacionats amb el suplement de DHA.

Les principals conclusions d'aquesta recerca son:

A) Experiment *in vitro*

1) El DHA augmenta l'estrès oxidatiu en un ambient amb espècies reactives d'oxígen.

En cèl·lules d'hepatòcits tractats amb n-3 PUFAs, el DHA s'oxida per les ROS produïdes per l'agent oxidant *tert*-BHP, i és capaç de produir més dany oxidatiu. Aquests danys oxidatius (increment de ROS i MDA, i disminució del ratio GSH/GSSG) indueix la translocació al nucli del Nrf2, el qual activa la defensa antioxidant (proteïna HO-1 i CAT)

2) L'EGCG protegeix les cèl·lules del "dany del DHA" en un ambient d'estrès oxidatiu.

Quan les cèl·lules son pretractades amb la combinació d'EGCG i DHA, el n-3 PUFA es conserva degut a l'efecte del polifenol, i es genera menys estrès oxidatiu. En conseqüència, la translocació nuclear del Nrf2 i l'expressió de HO-1 es reprimeix. A més, EGCG+DHA disminueix la peroxidació lipídica i restaura els ratio GSH/GSSG.

4) El DHA no sempre és beneficiós per les cèl·lules i s'ha de considerar com una "arma de doble tall" en termes de beneficis i riscos, especialment en situacions d'estrès oxidatiu.

B) Experiments *in vivo*

- **Model d'obesitat genètica (Zucker)**

1) El GSPE minora l'alteració del glutatió en estrès oxidatiu induït per l'obesitat genètica.

L'administració del GSPE en rates obeses genèticament disminueix el contingut de GSSG i, conseqüentment, augmenta el ratio GSH/GSSG, el que suggereix una millora de l'estrès oxidatiu. Aquesta millora provoca la

disminució de l'activació del enzims antioxidants GPx, GR i GST i un augment de la capacitat total antioxidant de la cèl·lula.

2) El GSPE no va canviar l'expressió genètica d'enzims antioxidants, encara que és capaç de modificar l'activitat de diversos enzims antioxidants.

- **Model postprandial**

3) La combinació del GSPE i del DHA-OR inhibeix la translocació nuclear del Nrf2 i l'expressió de HO-1 en un estat postprandial lipídic.

La inhibició de la translocació nuclear del Nrf2 podria ser un indicador d'una millora en l'estrès oxidatiu per la combinació de proantocianidinas i n-3 PUFAs.

4) La combinació del GSPE i del DHA-OR modifica l'estat antioxidant en un estat postprandial lipídic.

La combinació del GSPE i del DHA-OR disminueix el contingut GSH hepàtic i augmenta l'activitat enzimàtica GST, suggerint que la destinació de GSH és la conjugació entre el grup S del GSH i els hidroperòxids lipídics.

5) La combinació del GSPE i del DHA-OR podria representar una opció interesant per la prevenció del desequilibri transitori entre els hidroperòxids lipídics i l'estat antioxidant associat a un estat postprandial lipídic.

6) La dieta de cafeteria és un bon model per induir estrès oxidatiu associat a l'obesitat.

La ingesta de dieta de cafeteria augmenta la producció de ROS al fetge i, en conseqüència, augmenta el contingut hepàtic de GSSG i disminueix el ratio GSH/GSSG.

7) El GSPE redueix el contingut hepàtic de GSSG i augmenta la capacitat total antioxidant al plasma en un model d'obesitat induïda per la dieta.

8) La combinació del GSPE i del DHA-OR mitiga l'estrès oxidatiu a través de la via de Nrf2 i no interaccionant amb el GSH.

Encara que GSPE+DHA-OR disminueix els nivells hepàtics de GSH, la combinació dels dos compostos augmenta l'expressió proteica de HO-1 per combatre l'estrès oxidatiu.