UNIVERSITÀ DEGLI STUDI DI SALERNO

Department of Chemistry and Biology "A.Zambelli"

PhD in Chemical, Biological and Environmental Sciences

XXXV cycle

PhD Thesis

"Effect of environmental pollutant DDE on cell metabolism in rat tissues and liver cells in vitro: beneficial antioxidant effect of polyphenol hydroxytyrosol in liver cells"

Tutor:

Chiar.ma Prof.ssa Lillà Lionetti

Co-tutor:

Vincenzo Migliaccio

Ph.D student:

Dr.ssa Serena Penna

8801800009

Ph.D Coordinator:

Prof. Claudio Pellecchia

Academic Year 2021/2022

Abstract

Organochlorine pesticides affect cellular metabolism with effects on the onset of various metabolic diseases. Mitochondria are the main organelle involved in reactive oxygen species (ROS) production and could be involved in the adaptive response of tissular/organ to stress induced by environmental pollutants. In the present PhD thesis, it was evaluated the effects of dichlorodiphenylethylene (DDE) on the biochemical pathways involved in the onset of toxicant-associated steatohepatitis (TASH), and in the alteration of energy metabolism and thermogenesis in brown adipose tissue (BAT) in an *in vivo* experimental animal model. It was also evaluated the influence that a dietary antioxidant compound, such as hydroxytyrosol (Hty), may play against the damage induced by DDE in an *in vitro* hepatic cell model. The aim of this PhD thesis was therefore to evaluate the effects of DDE on cellular metabolism both in vivo and in vitro and of Hty in vitro on cellular metabolism. To this end, two experimental designs were used: 1) a first experimental design was conducted on an in vivo experimental model represented by male Wistar rats, treated with DDE for 4 weeks, to analyze the effects of DDE on cellular metabolism, with particular attention to mitochondrial function and oxidative stress in liver and BAT. . DDE effects on mitochondrial function and apoptosis markers were also assessed in other tissues, such as cardiac and skeletal muscle, to have a more general analysis on DDE effect on different tissues and 2) a second experimental design was conducted on an in vitro cell model, represented by liver cancer cells (Hepg2), to evaluate the protective effects of the antioxidant compound Hty, a polyphenol of olive oil, on DDE induced oxidative stress and cell damage.

The main results of the first experimental design confirmed that chronic exposure to DDE induced liver damage as well as alterations in BAT energy metabolism. Indeed, a reduction in mitochondrial function, namely cytochrome oxidase activity (COX), and in uncoupling protein 1 (UCP1) content was observed in total BAT homogenates associated with a decrease in the marker of mitochondrial biogenesis. However, COX specific activity and UCP1 content were increased in isolated BAT mitochondria, suggesting an adaptive response to counteract the impaired mitochondrial biogenesis in total tissue. These findings were associated with an increase in oxidative stress with a stimulation of the mitochondrial SOD2 antioxidant activity. As for hepatic tissue, chronic exposure to DDE induced an increase in ROS levels and SOD2 antioxidant system in both total liver homogenates and isolated mitochondria associated with the decrease in homogenate COX activity and the increase in caspase 3 activity, suggesting a cellular damage induced by mitochondrial dysfunction and ROS production. These finding suggested a key role of oxidative stress and antioxidant defense in response to DDE exposure in different tissues. Moreover, DDE induced impaired mitochondrial activity and apoptosis induction in skeletal muscle but increased mitochondrial activity and decreased in apoptosis induction in cardiac muscle, suggesting a different adaptive response to chronic DDE exposure in different tissues.

Taking into account DDE induced oxidative stress in liver , in the second experimental design the effects of the dietary antioxidant compound Hty were analyzed in vitro in liver cells treated with DDE. Cellular viability and morphology, apoptotic marker, lipid peroxidation and antioxidant systems (superoxide dismutase (SOD), Catalase (CAT),

and glutathione peroxidase (GPX) activities) were analysed in DDE-treated, Hty-treated or DDE+Hty treated cells. The results showed that DDE impairs hepatic cell viability through oxidative stress induction without adequate stimulation of antioxidant defense, mainly with high pesticide dose. Noteworthy, the simultaneous exposure to DDE and Hty, counteracts DDE induced cell damage by improving antioxidant defense. In conclusion, the results of the present PhD thesis showed the key role of mitochondrial function and oxidative stress in DDE effects on tissue metabolism in vivo, and highlighted the possible role of extra virgin olive oil (EVOO) polyphenols in maintaining liver cell health under conditions of exposure to environmental pollutants in vitro providing the basis for further mechanistic studies on this important field of research.

Abstract

I pesticidi organoclorurati agiscono sul metabolismo cellulare con effetti sull'insorgenza di diverse malattie dismetaboliche. I mitocondri sono i principali organuli cellulari coinvolti nella produzione di specie reattive dell'ossigeno (ROS) e potrebbero essere coinvolti nella risposta adattativa dei tessuti/organi allo stress indotto da inquinanti ambientali. Nella presente tesi di dottorato, sono stati valutati gli effetti del diclorodifeniletilene (DDE) sulle vie biochimiche coinvolte nell'insorgenza della steatoepatite indotta da agenti tossici (TASH), e nell'alterazione del metabolismo energetico e della termogenesi nel tessuto adiposo bruno (BAT) in un modello animale sperimentale *in vivo*. È stata inoltre valutata l'influenza che un composto antiossidante alimentare, come l'idrossitirosolo (Hty), può esercitare contro il danno indotto dal DDE in un modello di cellule epatiche *in vitro*. Lo scopo di questa tesi di dottorato è stato quindi quello di valutare gli effetti del DDE sul metabolismo cellulare sia *in vivo* che *in vitro* e dell'Hty sul metabolismo cellulare *in vitro* con contemporanea esposizione al DDE. A tal fine sono stati utilizzati due disegni sperimentali: 1) un primo disegno sperimentale è stato condotto su un modello sperimentale *in vivo* rappresentato da ratti Wistar maschi, trattati con DDE per 4 settimane, per analizzare gli effetti del DDE sul metabolismo cellulare, con particolare attenzione alla funzione mitocondriale e allo stress ossidativo nel fegato e nel BAT. Inoltre sono stati anche analizzati gli effetti del DDE in altri tessuti, quali il muscolo scheletrico e cardiaco, per avere quadro più generale degli effetti del DDE in differenti tessuti e 2) un secondo disegno sperimentale è stato condotto su un modello cellulare *in vitro*, rappresentato da cellule tumorali del

fegato (Hepg2), per valutare gli effetti protettivi del composto antiossidante Hty, un polifenolo dell'olio d'oliva, sullo stress ossidativo e sul danno cellulare indotto da DDE.

I principali risultati del primo disegno sperimentale hanno confermato che l'esposizione cronica al DDE induce danni al fegato e alterazioni al metabolismo energetico del BAT. Infatti, negli omogenati totali di BAT è stata osservata una riduzione della funzione mitocondriale, in particolare dell'attività della citocromo ossidasi (COX), e del contenuto della proteina disaccoppiante 1 (UCP1), associata a una diminuzione del marcatore della biogenesi mitocondriale. Tuttavia, l'attività specifica della COX e il contenuto di UCP1 sono aumentati nei mitocondri isolati dal BAT, suggerendo una risposta adattativa per contrastare la biogenesi mitocondriale compromessa nel tessuto totale. Questi risultati sono stati associati ad un aumento dello stress ossidativo con una stimolazione dell'attività antiossidante della SOD2 mitocondriale. Per quanto riguarda il tessuto epatico, l'esposizione cronica al DDE ha indotto un aumento dei livelli di ROS e del sistema antiossidante SOD2 sia negli omogenati epatici totali che nei mitocondri isolati, associato alla diminuzione dell'attività della COX dell'omogenato e all'aumento dell'attività della caspasi 3, suggerendo un danno cellulare indotto dal disfunzione mitocondriale e dalla produzione di ROS. Questi risultati hanno suggerito un ruolo chiave dello stress ossidativo e della difesa antiossidante in risposta all'esposizione al DDE in diversi tessuti. Inoltre, il DDE ha indotto un danno dell'attività mitocondriale ed una induzione dell'apoptosi anche nel muscolo scheletrico, mentre invece ha indotto un aumento dell'attività mitocondriale ed una minore induzione dell'apoptosi nel

muscolo cardiaco, suggerendo una differente risposta adattativa dei differenti tessuti all'esposizione cronica al DDE.

Tenendo conto di tali risultati, nel secondo disegno sperimentale sono stati analizzati gli effetti del composto antiossidante alimentare Hty in vitro in cellule epatiche trattate con DDE. La vitalità e la morfologia cellulare, marker apoptotici, perossidazione lipidica e i sistemi antiossidanti (attività di superossido dismutasi (SOD), catalasi (CAT) e glutatione perossidasi (GPX)) sono stati analizzati in cellule trattate con DDE, Hty o DDE+Hty. I risultati hanno mostrato che il DDE compromette la vitalità delle cellule epatiche attraverso l'induzione dello stress ossidativo senza un'adeguata stimolazione della difesa antiossidante, principalmente con alte dosi del pesticida. D'altra parte l'esposizione simultanea a DDE e Hty, contrasta il danno cellulare indotto da DDE migliorando la difesa antiossidante.

In conclusione, i risultati della presente tesi di dottorato hanno mostrato il ruolo chiave della funzione mitocondriale e dello stress ossidativo negli effetti del DDE sul metabolismo tissutale in vivo e hanno evidenziato il possibile ruolo dei polifenoli presenti nell'olio di oliva, quali l'Hty, nel mantenimento della salute delle cellule epatiche in condizioni di esposizione a inquinanti ambientali in vitro fornendo la base per ulteriori studi meccanicistici su questo importante campo di ricerca.

Abbreviations List

ADP - Adenosine 5'-diphosphate
AhR - Aryl hydrocarbon receptor
AKT - Akt kinase/Protein Kinase B
ALT - Alanine Aminotransferase
AMP - Adenosine 5'-monophosphate
AMPK - AMP-activated Protein kinase
ANOVA - Analysis of Variance
ARE - Antioxidant response elements
ATP - Adenosine triphosphate
BAT - Brown Adipose Tissue
BAX - BCL2 Associated X
BCL-2 - B - Cell Lymphoma-2 derived protein gene
CAT – Catalase
C-GCS - C-glutamyl-cysteine synthetase
COX – Ciclooxygenase
COX2 – Ciclooxygenase 2
CuZn-SOD - Copper/Zinc – Superoxide Dismutase 1
CYP450 - Cytochrome P450
CYTc- Cytochrome c
DDE - dichlorodiphenyldichloroethylene
DDT - dichlorodiphenyltrichloroethane
DHA - Docosahexaenoic Acid
DMSO - Dimethyl Sulfoxide
DRP1 - Dynamin-Related Protein 1
EC-SOD – Extracellular superoxide Dismutase 3
EDCs - Endocrine disruptors
EFSA - European Food Safety Authority
EPA - Environmental Protection Agency
EPA - Eicosapentaenoic Acid
ER - Endoplasmic reticulum
ETC - Electron transport chain
EVOO – Extra virgin olive oil
FIS1 - Fission Protein 1
GSH - Glutathione
GPx - Glutathione Peroxidase
GST - Glutathione S-transferase
HepG2 - Human Hepatocellular Carcinoma Cells
Hty - Hydroxytyrosol
HT-29 - Human colon cancer cell line
iNOS – Inducible nitric oxide synthase
LC3 - MAP1LC3B - Microtubule-associated proteins 1A/1B light chain 3B

LD50 – Lethal dose 50
LDL - Low Density Lipoprotein
IMM – Inner mitochondrial membrane
MCF7 – Human breast cancer cell
Mfn1 – Mitofusins 1
Mfn2 – Mitofusins 2
MMO – Outer mitochondrial membrane
MnSOD - Manganese Superoxide Dismutase 2
mtDNA – Mitochondrial Deoxyribonucleic Acid
MTT - 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
N – Normal diet
NADPH - Nicotinammide Adenine phosphoDinucleotide
NASH - non-alcoholic steatohepatitis
NCDs - Noncommunicable diseases
NCI – National cancer institute
NF-kB - Nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf-2 – Nuclear factor erythroid 2-related factor 2
NQO1 - NADPH quinone oxidoreductase-1
OCP - Organochlorine pesticide
Ole – Oleuropein
OPA1 - Optic atrophy 1
OXPHOS - Oxidative phosphorylation
PAHs - Polycyclic Aromatic Hydrocarbons
PARP - Poly (ADP-ribose) polymerase
PCBs - Polychlorinated Biphenyls
PCDD - PolyChlorinated DibenzoDioxins
PCDF - Polychlorinated DibenzoFurans
PFOS - Perfluorooctane sulfonate
PFOA - Perfluorooctanoic acid
PGC1α - Peroxisome Proliferator-Activated Gamma Coactivator 1 alpha
P62 - Ubiquitin-binding protein
pNA - para-Nitroanilide
POPs - Persistent environmental contaminants
PTP - Permeability transition pore
RIPA - Radioimmunoprecipitation assay buffer
ROS - Reactive oxygen species
SEM - Standard Error on the Mean
SOD - Superoxide Dismutase
T2DM - Diabetes Mellitus Type 2
TASH - Toxicant-associated steatohepatitis
TBARS - Thiobarbituric Acid Reactive Substances
T-BOOH - Tert-butyl hydroperoxide
TBS - Triphosphate Buffered Saline
Tfam- Mitochondrial transcription factor A

TNFα - Tumoral necrosis factor-α
UCP - Uncoupling protein
UCP1 - Uncoupling protein 1
UCP2 - Uncoupling protein 2
VDAC - Voltage-Dependent Anion-selective Channel
WAT - White Adipose Tissue

Introduction

According to the definition adopted by the European Union "an Endocrine Disruptor is an exogenous substance, or a mixture, that alters the functionality of the endocrine system, causing adverse effects on the health of an organism, or its progeny or a (sub)population. Evaluations by several international agencies indicate that Endocrine Disruptors are a large and heterogeneous group of substances that also includes persistent environmental contaminants (POPs), among which dichlorodiphenyltrichloroethane (DDT) can be mentioned. This organochlorine pesticide (OCP) was widely used for insect and wheat crops control. It was then banned by the European Union after discovering the damage they caused to humans by accumulating in the tissues. Although its use has been banned since 1986, unfortunately it is still in use in the poorest countries, and this determines its permanence in the ecosystem. In fact, there are many data in the literature that quantify the levels of dichlorodiphenyldichloroethylene, DDE, (its main metabolite) in humans. A study conducted in 2019 found the presence of DDE in human blood in more than 90% of samples of Canadians aged between 20 and 79 years, despite the fact that DDE has been banned in Canada since 1985. [1; 2] Several studies have shown that DDE is present in different tissues, such as testis (causing infertility), liver (causing steatohepatitis) [3], breast milk [4], and is associated with various diseases such as cancer[5], obesity[6], insulin resistance [7], type 2 diabetes [8], Alzheimer's disease and Parkinson. [9; 10; 11]

Moreover, it has been shown that DDT and DDE to act directly on mitochondria causing alterations in cellular respiration, compromising the complexes of the electron

transport chain, and membrane potential triggering mitochondrial dysfunction, oxidative stress, cellular dysfunction with activation of apoptotic processes. DDE also decreased membrane potential, ATP levels, and oxygen consumption rates in human HepG2 cells. [12]

Thus, mitochondrial dysfunction and oxidative stress appear to play a key role in cellular mechanisms involved in the negative effect of environmental pollutants, such as DDE, on cell health.

A first aim of the present PhD thesis was to evaluate the effect of a chronic exposure to DDE on different tissues in an animal experimental model. In particular, it was evaluated DDE effect on mitochondrial function and apoptosis markers in skeletal muscle, cardiac muscle and liver. DDE effect on brown adipose tissue (BAT) thermogenesis was also analysed by evaluating mitochondrial function, uncoupling and biogenesis, and oxidative stress. A further analysis was carried out in liver tissue to assess the damage oxidative stress and antioxidant defences.

Dietary bioactive compounds with antioxidant proprieties could be useful to mitigate environmental pollutant-induced cell damage.

Indeed, the incorporation of bioactive molecules, such as polyphenols, can represent a good starting point to develop primary and long-term prevention strategies to prevent the onset of environmental pollutant induced metabolic impairment. Several studies have shown that polyphenols, thanks to their peculiar chemical structure, are able to capture some of the environmental contaminants, including DDT / DDE, thus

"sequestering" them from the surrounding environment with nanocarrier technique. [13;14;15;16]

Polyphenols, for several years, have attracted the interest of the scientific community because they act by regulating multiple signaling pathways involved in maintaining protein homeostasis, in the regulation of inflammatory, metabolic and antioxidant responses, often recalling a caloric restriction regime that positively affects mitochondrial turnover, oxidative stress and inflammatory response, where autophagy plays a significant role. [17;18;19]

Numerous recent studies states that oleuropein and hydroxytyrosol (Hty) have important antiapoptotic and antiproliferative properties in cancer cells[20;21]thanks to the decrease in NF-kB levels (known transcription factor involved in the expression of many genes that drive the development and progression of NCDs (noncommunicable diseases such as cancer, cardiovascular disease, diabetes, and chronic respiratory diseases), cell proliferation and apoptosis. Instead, in normal cells, oleuropein (Ole) and Hty behave as normal antioxidant and anti-inflammatory molecules. [22]

Taking into account the above considerations, a further aim of the present PhD thesis was to assess the benefic antioxidant effect of Hty on DDE-induced oxidative stress and cell damage in an *in vitro* model of hepatic cells.

CHAPTER 1

Mitochondria and oxidative stress

Mitochondria are involved in many important cellular processes but in particular they perform the fundamental function of recovering the energy contained in food and then converting it into usable chemical energy in the form of adenosine triphosphate (ATP). Mitochondria move freely in the cytoplasm and tend to thicken at the points where energy demand is greatest. Mitochondria are morphologically and functionally polymorphic to meet the demands of each tissue in which they are located. In addition, they meet 90% of energy requirements and control programmed cell death (apoptosis), through complex mechanisms that could culminate with the opening of the permeability transition pore (PTP) which is a voltage-dependent transmembrane channel. Its opening causes the decoupling of the respiratory chain with the fall of the electrochemical gradient, cessation of ATP synthesis, release of mitochondrial proteins from the intermembrane space and leakage of Ca^{2+} from the matrix generating superoxide anions. All this leads to rupture of the outer mitochondrial membrane and complete depolarization.

1.1.Structure

From the typical elongated shape, the mitochondria are bordered by a double membrane, an external one in contact with the cytoplasm and an internal one that introflexes into folds called ridges; the membranes delimit an internal space called intermembrane space, while the internal membrane encloses the mitochondrial matrix. The outer membrane has a composition formed by 50% lipids and 50% proteins with reduced enzymatic or transport functions. On it we find channel proteins called porins or voltage-gated selective anion channel (VDAC). This protein

makes the outer membrane permeable to ions and molecules with molecular weight greater than 5000 Daltons. The inner membrane is structurally and functionally more complex; It folds to form mitochondrial crests and is composed of 80% proteins and 20% lipids. In its lipid component it lacks cholesterol and is replaced by cardiolipin, important because it makes the membrane selectively impermeable to ions by forming transmembrane gradients necessary for mitochondrial function. The membrane has most of the enzymes involved in electron transport and oxidative phosphorylation, dehydrogenase and various transport systems that catalyze the transfer of substrates and metabolic intermediates. The intermembrane space is crossed by structures analogous to gap junctions creating points of contact between the two membranes, and contains a specific enzyme, myokinase, which catalyzes the phosphorylation of adenosine 5'-monophosphate (AMP) to adenosine 5'-diphosphate (ADP), as well as the presence of proapoptotic proteins. Finally, the matrix contains numerous proteins, including the enzymes of the Krebs cycle, βfatty acid oxidation and amino acid metabolism.

1.2. Mitochondria and oxidative stress

Mitochondria are a main source of cellular reactive oxygen species (ROS). Under physiological conditions, 0.2-2% of the electrons in the electron transport chain (ETC) do not follow the normal transfer order but instead directly leak out of the ETC and interact with oxygen to produce superoxide or hydrogen peroxide[23]. About 11 ROS generation sites were found on the different complexes. It is complexes I and III that generate most of the ROS in the mitochondria. In healthy physiological

conditions, cells maintain a basal level of ROS to promote the balanced redox signaling required for various processes such as cell metabolism, cell differentiation and survival, immune defense, and modulation of transcription factor activity and epigenetic state [24;25]. When ROS production increases, they start showing harmful effects on important cellular structures like proteins, lipids, and nucleic acids. A large body of evidence shows that oxidative stress can be responsible, with different degrees of importance, in the onset and/or progression of several diseases (i.e., cancer, diabetes, metabolic disorders, atherosclerosis, and cardiovascular diseases). [26]

1.3. Enzymatic antioxidant defense

The first line of defense from free radicals is represented by the superoxide dismutase (SODs) that catalyze the dismutation of superoxide in H_2O_2 and oxygen. Three isoforms of SODs have been identified, each expression of a different gene and with distinct subcellular localizations. Cu/ZnSOD (SOD1) is a cytosolic enzyme, MnSOD (SOD2) has a mitochondrial localization, and EC-SOD (SOD3) is localized in the extracellular matrix, being secreted from cells [27]. Another class of intracellular antioxidant enzyme are known as glutathione peroxidase (GPx). GPx are tetrameric enzymes containing a seleno-cysteine in their active site. In particular, GPx1 seems to be the major isoform that converts hydrogen peroxide to water and oxygen and catalyzes the reduction of peroxide radicals to alcohols and oxygen; mainly cytosolic, a small fraction is also present within the mitochondrial matrix[28]. Catalase breaks down two hydrogen peroxide molecules into one molecule of

oxygen and two molecules of water in a two-step reaction[29]. The activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) constitute a first line antioxidant defence system which plays a key and fundamental role in the total defense mechanisms and strategies in biological systems.

1.4 Mitochondrial dynamics

Mitochondria have a unique ability to regulate their morphology in response to various cellular stimuli. For example, during nutrient deprivation mitochondria fuse together and create interconnected filamentous networks to share nutrient precursors, mtDNA, ETC components and maintain OXPHOS. Conversely, mitochondrial fission produces smaller mitochondria. This mechanism is important for mitochondrial movement to regions of high energy demand or to allow equal mitochondrial distribution to daughter cells after mitosis. Mitochondrial dynamics is therefore a finely regulated phenomenon. This regulation takes place on a series of highly conserved proteins. Mitochondrial fusion is a process in which the outer and inner mitochondrial membranes of two originally distinct mitochondria physically fuse into one. The fusion process allows content exchange between mitochondria, allowing defective organelles to regain essential components of the respiratory chain and mitochondrial DNA[30]. Membrane fusion requires the fusion of two lipid bilayers that in biological systems depend on specific protein catalysts (spindles). Once the mitofusins (Mfn1 and Mfn2), of two adjacent mitochondria bind to each other, the hydrolysis of GTP allows fusion of the outer mitochondrial membranes.

Subsequent fusion of the inner mitochondrial membrane, based on membrane potential, requires OPA1. Opa1 interacts with Mfn to form a bridge between inner mitochondrial membrane (IMM) and outer mitochondrial membrane (MMO) and is necessary for lipid mixing during fusion.

The fission process is necessary for the remodelling and rearrangement of mitochondrial networks, for proper mitochondrial transport, to facilitate apoptosis and for the removal of dysfunctional mitochondria [(31)]. Alterations in the fission process, induced by DDE[(32)], increase the generation of reactive oxygen species (ROS) and lead to a heterogeneous population of mitochondria with non-uniform mitochondrial DNA distribution. Mitochondrial fission is controlled by dynamically linked protein 1 (DRP1) which recognizes, following its activation by phosphorylation, an MMO receptor protein known as Fission 1 (FIS1) [(33)].

CHAPTER 2

Pesticides DDT and DDE

2 PESTICIDES

A "pesticide" is defined as a product of natural or synthetic chemical origin used in agriculture to protect crops from harmful organisms and prevent them from being destroyed by diseases and infestations. Light pesticides (non-persistent, 1-12 weeks) are rapidly biodegradable compounds, while heavy pesticides (persistent, 2-5 years, organochlorinated) are compounds that remain in the environment for relatively long periods of time. Their persistence depends on many factors: the type of soil, humidity, pH and the extent of the crops, and is decisive for establishing the safety interval, i.e. the time that must elapse between the last treatment and harvesting. By their very nature, pesticides can be dangerous to humans or other animals, as their purpose is to kill or harm living organisms. They can be absorbed by inhalation, by skin contact or through the digestive system. Acute toxicity is classified according to the lethal dose of LD50, i.e. the dose capable of killing 50% of laboratory animals that have absorbed it. However, this system takes account only of acute and does not include long-term effects, carcinogens, mutagenic action on genetic material, teratogenic action on embryos and foetuses.

Depending on the activity carried out, they can be divided into different categories such as herbicides (herbicides), plant growth regulators, and pesticides. Pesticides can be classified into bactericides, acaricides, rodenticides, molluscicides, nematocides, fungicides and insecticides among which we find organochlorines.

2.1Organochlorine pesticides

Organochlorine pesticides are synthetic compounds that have more chlorine atoms in their molecule. Their mechanism of action is exerted at the level of neurons by opening the Na^{+}channels and triggering a continuous and uncontrollable nerve impulse in insects. Due to their massive use in agriculture, their chemical stability and poor biodegradation, these compounds are present throughout the ecosystem and are particularly toxic to aquatic organisms.

Organochlorine pesticides fall into the category of pollutants referred to as persistent organic *pollutants* (POPs). These are chemically stable organic compounds, highly toxic and with long "life times" in the environment. Among the known-best POPs are, in addition to Organochlorine Pesticides (including DDT and HexachloroBenzene), Polychlorinated Biphenyls (PCBs), PolyChlorinated DibenzoDioxins (PCDD), Polychlorinated DibenzoFurans (PCDF), Polycyclic Aromatic Hydrocarbons (PAHs).

2.2DDT and its metabolite DDE

Othmar Zeidler synthesized the first DDT molecule from Baeyer's condensation between chlorobenzene and chloral in the presence of sulfuric acid [(34)].

Historically, DDT was recreated (as an insecticide) on September 25, 1939 in the laboratories of J. R. Geigy A. G., an ancient Swiss dye company. Since they were looking for a more general contact poison, Zeidler's synthesis was repeated and found that the compound dichlorodiphenyltrichloroethane or DDT was surprisingly effective against *Calliphora* flies and a variety of other insects, including mosquitoes [(35)].

DDT was introduced as an insecticide to control malaria, a mosquito-borne disease in the 50s. In the early 60s, many toxic effects of DDT became known, for which, in the United States, DDT was banned in 1972 by the Environmental Protection Agency (EPA), due to the potential harmful effects on wildlife and humans [36; 37] .

Similarly, the use of DDT has gradually decreased in industrialized countries, including Italy. In the European Union, the use of DDT has been banned since 1986, but has increased substantially in developing countries for the control of malaria and parasites in agriculture. [38]

DDT and its primary metabolites, DDE and DDD, are synthetic substances and do not occur naturally. [39]

DDT spreads in the atmosphere following misting in geographical areas where it is still allowed to be used. The process of volatilization and deposition can be repeated many times giving rise to the phenomenon of "*global distillation"* according to which the compounds pass from warmer to cold regions and are transported to the Arctic and Antarctic regions where they are then detected in the air, sediments, snow and accumulate in living organisms [95].

In the atmosphere, about 50% of DDT is adsorbed to particulate matter and the remaining 50% is in the vapor phase, DDE and DDD are adsorbed to particulate matter to a lesser extent than DDT. [40]

DDT reaches aqueous surfaces when used as a pesticide near water basins. DDT can reach the aquatic system from industrial wastewater and sewage discharges. DDT in

water can accumulate in aquatic organisms such as plankton, and enter the food chain, reaching as far as humans [95].

Organochlorine pesticides, including DDT, in soil can have different fates: some are dispersed in the atmosphere by volatilization and transported long distances from the application sites, others are carried away by surface runoff or photo-degraded by sunlight. Once they penetrate the soil, they can be absorbed by plants and degraded, but also transported to groundwater or reach surface aquatic fauna through drainage channels. In general, the absorption of pesticides in soil or sediment is inversely proportional to the water solubility of these compounds; in fact, DDT, having a very low solubility, is strongly absorbed [95].

When DDT, DDE and DDD are deposited in the soil they are strongly absorbed, but can also re-volatilize, especially in moist soils. However, due to the strong bond with the soil, these compounds remain more in the surface layers of the soil undergoing photo/bio-degradation and only a small percentage can penetrate deeply. Biodegradation occurs both in aerobic and anaerobic conditions due to the presence of microorganisms in the soil, such as fungi, bacteria and algae. [41; 42] Among the main microorganisms capable of degrading DDT in both aerobic and anaerobic conditions are *Pseudomonas fluorescens, E. coli, Klebsiella pneumoniae,* forming 4-chlorobenzoic acid and DDE. DDE, the major metabolite of DDT, is often resistant to biodegradation under aerobic and anaerobic conditions.[43;44]

DDT can also be absorbed by plants and consequently by animals which, by feeding on it, can accumulate significant levels of the contaminant, mainly in adipose tissue and milk.

Organochlorine pesticides, being relatively stable lipophilic compounds and resistant to biodegradation, are able to progress through the food chain through the mechanism of biomagnification. In this way, these pesticides pass to higher levels of the food chain, reaching humans. In particular, humans are exposed to DDT and its metabolites through food: seafood, fish, shellfish, meat and fruit/vegetables are the food sources of these contaminants for many people in the world. This is because soil and vegetation act as reservoirs of contamination and are therefore vectors through which pesticides enter the food chain. In addition, the concentration of the pesticide in the contaminated plant also depends on the specific composition of the plant itself and its ability to bioaccumulate. In Italy, an important exposure occurred due to the presence of contaminants in the water of Lake Maggiore, where a p,p'-DDT production plant was located. Eight years after the plant closed, levels of DDT and its metabolites were still at a level that posed a risk to local flora and fauna [(95)].

Further recent analyses have been conducted in European seas on the presence of this substance in marine organisms in European seas, which have shown that in the 2005/2012 range there was still a moderate to high rate that attenuates becoming moderate in the update of data to 2019. These data show that DDE is still present in living organisms. DDT concentrations in recent years have generally been classified as "moderate" in mussels and fish from the north-east Atlantic Ocean and the Baltic Sea

and mussels from the Mediterranean Sea. It is likely that the "high" concentrations, which have been found, but to a small extent, in the north-east Atlantic Ocean pose a risk to marine organisms. In the previous assessment (2003-2014) a general downward trend was found for the North-East Atlantic Ocean. However, no general trends were detected in the current evaluation (2008-2017), indicating that the impact of abatement policies as a whole may have stabilized. The data has currently been updated (latest version 24 August 2022) and confirmed previous assessments.

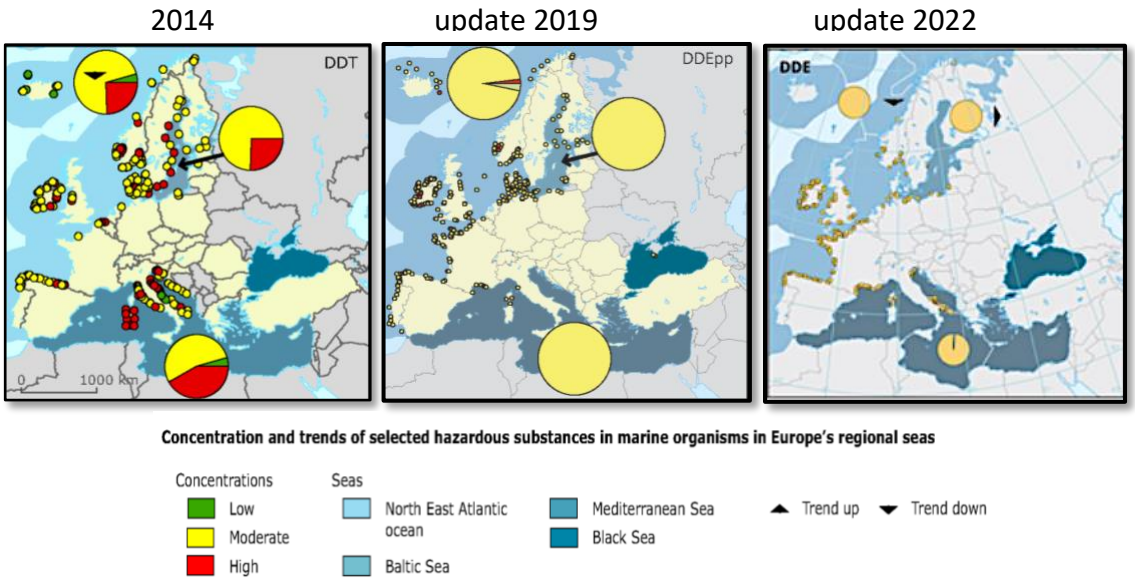

Figure 2.1 Concentration of DDT/DDE in Europe. European Environment Agency

2.3DDT and DDE physio-chemical properties

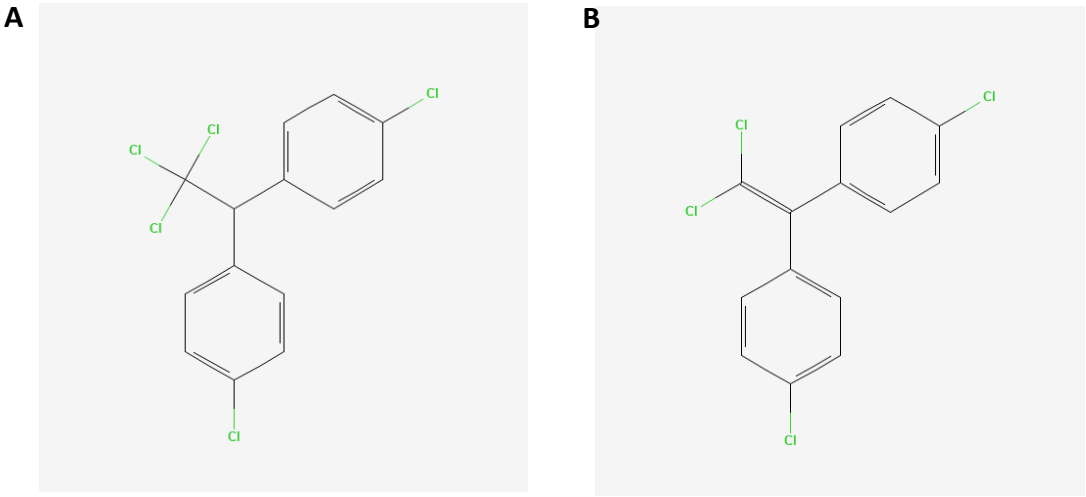

Figure 2.2 Structure of DDT (A) and DDE (B)

The term DDT, or 1,1,1-trichloro-2,2-bis (p-chloro-phenyl) ethane, chemically it is a white crystalline chlorinated hydrocarbon, odorless or slightly aromatic, tasteless, very stable and insoluble in water. It is soluble in most organic solvents, fats and oils and tends to accumulate in ecosystems.

The factors that make DDT such an effective insecticide, are its low vapor pressure, its stability to oxidation, its high solubility in fats and its low solubility in water. Due to the enormous world production and the stability of its primary degradation product, DDE (or 2,2-bis- (p-chlorophenyl)), it has become the prototype of an environmental micropollutant.

The technical mixture is an amorphous white powder that has a melting temperature ranging between 80-94 ° C and consists mainly of three forms:

- p,p'-DDT (65-80%);
- o,p'-DDT (15-21%);
- o,o'-DDT (tracce);

2.4 DDT and DDE toxicity

It is certain that DDT is a nerve poison for both insects and mammals, producing symptoms of intoxication marked by hyperexcitability, tremors of increasing severity that lead to convulsions, prostration and death.

The problem lies in the fact that DDT and its main metabolite dichlorodiphenyldichloroethylene (DDE) pervade the environment in all forms of life, humans, plants, animals, water, air and soil [45; 46]. DDT is known to have affected wildlife, causing thinning of the eggshell in bald eagles in the United States and reproductive failures in other bird species.[47]

Bioaccumulation of DDT and DDE in humans occurs in adipose tissue at much higher levels and to a lesser extent in the bloodstream and breast milk. [103; 48]A potential effect of DDE in humans is the risk of breast cancer, [49] as it is a xeno-estrogen or described as an endocrine disruptor with estrogen-like effects. [50; 51] In addition, DDT can reduce sperm count, induce testicular abnormalities, premature fetal delivery [52;53], and fetuses small for gestational age [54;55]. Other symptoms in humans are itchy tongue, perioral paresthesia, nausea, dizziness, confusion, headache, restlessness and rashes.[56] DDT has acute, non-lethal toxicity in humans at doses greater than 285 mg/kg/day[95].

DDT and DDE are subject to the phenomenon of bio-magnification in the food chain and bio-accumulated in adipose tissue and liver [57:58; 59]. According to the U.S. National

Cancer Institute (NCI), the liver appears to be the main target organ of DDE in mammals, possibly due to the high sensitivity of liver cells to exposure to xenobiotics [60;61].

In addition, metabolic detoxification processes take place mainly in hepatocytes [62 ; 63] involving the endoplasmic reticulum (ER), mitochondria and lysosomes [64]. In fact, damage to mitochondrial bioenergetics due to exposure to DDT and DDE in humans and wildlife has been reported. [65]

As a result, DDT and DDE impair the mitochondrial respiratory chain and, consequently, the production of ATP [127]. In addition, the protein content and enzymatic activity of cytosolic and mitochondrial antioxidant enzymes are profoundly influenced by DDT/DDE exposure. [66; 67;68]

Mitochondrial dysfunction has been associated with increased ROS generation, decreased mitochondrial membrane potential, and cytochrome c (cyt c) release into the cytosol, resulting in apoptosis [69; 70] and induction of insulin resistance/diabetes. [71;72]

Chronic exposure to environmental toxic substances, has been shown to induce DNA damage in different models in vivo and in vitro. However, cells adapt their metabolic state under a stress condition by activating a series of signals that include the expression of genes involved in metabolic homeostasis, such as the up-regulation of antioxidant systems and the increase in mitochondrial activity to produce the additional ATP required by the cellular function. However, the increased mitochondrial activity is associated with increased ROS levels that, if not correctly balanced, generate oxidative stress that in turn elicits damage to macromolecules, genomic instability, and

mitochondrial/cellular dysfunction. Recent scientific findings showed that DDE induces oxidative stress, mitochondrial impairment, and cellular oxidative damage in cultured rat Sertoli cells as well as in rat liver and testis. [73;74] However, the mechanisms by which DDE induces ROS incrementation are not yet clear, but the main effects of DDE on hepatic cells involve the cytochrome P450 (CYP450) activity, used in the detoxification path. It has also been shown that pesticide can directly affect the mitochondrial electron transport chain inducing functional impairment. [136] Mitochondria play a pivotal role in the adaptation to DDE exposure by increasing fat catabolism to face the increased cellular energy needs for the hepatic detoxification processes. Together with hepatic oxidative damage, the principal antioxidant enzymes, namely SODs and GPx, were found to be stimulated in terms of protein synthesis and enzymatic activity. SOD1 is constitutively expressed, but can be induced by redox-active metals, superoxide, and xenobiotics. SOD2 is the most inducible form, raising its levels up to10-fold in presence of drugs and cytokines. Defects in SOD2 expression cause oxidative damage in liver, while the overexpression generally plays a protective role [75;57]. SOD3 does not have a significant role in superoxide detoxification in hepatocyte [76;57]. Another class of intracellular antioxidant enzyme are known as glutathione peroxidase (GPx). GPx are tetrameric enzymes containing a seleno-cysteine in their active site [77;57]. These enzymes occur in different isoforms (eight in humans), all able to degrade hydroperoxides, alkyl peroxide, and fatty acid hydroperoxides to lipid alcohols and oxygen. GPx1 seems to be the major isoform that converts hydrogen peroxide to water and oxygen and catalyzes the reduction of peroxide radicals to alcohols and oxygen;

mainly cytosolic, a small fraction is also present within the mitochondrial matrix [78;57]. GPx1 exerts its action via oxidation of reduced GSH into its disulfide form [79]. From in vivo studies, it was evident the mitochondrial role in the hepatic adaptation to pesticide-induced oxidative stress[136]. The superoxide anion produced in the matrix side of inner mitochondrial membrane has been proposed to activate the uncoupling proteins (UCPs) that in turn might reduce the generation of further superoxide anions [80;57].

2.4.1 DDE effects on liver and testis in experimental animal models

In a previous work carried out in our lab, hepatic oxidative stress and anti-oxidant systems response in rats exposed to non-toxic dose of DDE, was analyzed (Migliaccio et al 2019). The results showed an increase in lipid peroxidation associated with changes in the antioxidant SOD2 and UCP1 levels.

In fact, alongside the high SOD2 level, hepatocytes from DDE-treated rats showed high UCP2 gene expression and protein synthesis [3;57]. Probably, both SOD2 and UCP2 play a cooperative role in response to DDE at a mitochondrial level. The electron transport chain produces a superoxide anion, which is rapidly converted into H_2O_2 by SOD2. However, to avoid an excessive accumulation of H_2O_2 at a mitochondrial and cellular level, mitochondrial uncoupling is induced in order to reduce superoxide production by mitochondrial activity.

In the same experimental animal model, our research group also evaluated the effects of DDE on testis cells metabolism and oxidative stress. Several studies demonstrated that these environmental contaminants used in the last few decades alter the male

reproductive system by functioning as antiandrogenic compounds, leading, in the most serious cases, to male infertility [81;82;83;84;85]. Most studies have been carried out on cultured cells or administering moderately high doses of DDE (50–100 mg/kg body weight per day) intraperitoneally [86;87;88] or by gavage [89]. Hence, we decided to investigate the effect of a chronic lower oral DDE dose (10mg/kg b.w. per day). The results showed that DDE-treated rat exhibited higher lipid peroxidation levels associated with pronounced defect in antioxidant capacity, apoptosis, cellular proliferation, as well as with tissue damage in testis. Moreover, decreases in androgen receptor (AR) and serum testosterone levels were found in DDE-treated rats vs. control rats. Thus, DDE produced cellular stress leading to antioxidant impairment, apoptosis, and decreases in AR and serum testosterone levels associated with tissue damage. It was also observed a certain degree of adaptation to counterbalance the adverse effects of DDE in terms of increased cellular proliferation. Using this strategy, the testicular cells can generate opposite paths to maintain a pool of seminiferous tubules composed of functional cells capable of undergoing a normal differentiation and maturation to sustain spermatogenesis and fertility. Cellular proliferation could be used as an adaptation to counterbalance the occurred damage, maintaining a pool of tubules that follow physiological maturation.

2.4.2 DDE effects on BAT

BAT is characterized by brown multilocular adipocytes and is involved in the activation of thermogenesis due to the presence of the uncoupling protein 1 (UCP1), that may be useful for increasing energy expenditure [3;90;91;92] and avoiding excessive lipid accumulation in white adipose tissue (WAT), counteracting obesity and metabolic diseases development. [93;94;95;96] UCPs are a family of mitochondrial proteins formed by six trans-membrane segments into the phospholipid matrix of the inner mitochondrial membrane [97], present in animal and plant in five isoforms, from UCP1 to UCP5 [98]. UCPs isoforms are expressed in different tissues and may have different functions. The isoform1 is present in BAT, isoform 2 is expressed almost ubiquitously, isoform 3 in BAT and in skeletal muscle, isoforms 4 and 5 are present predominantly in the central nervous system [99]. Moreover, in mitochondria, UCPs functional structure is constituted by a dimer stabilized with a disulfide bridge between the cysteines present in the hydrophilic C-terminal segment [100]. These proteins are mitochondrial anion carriers [101] whose function was initially associated to uncoupling respiration from ATP synthesis performed by UCP1. Besides the adaptive thermogenesis [102], UCPs may regulate a lot of biological processes, such as the ATP synthesis and all the mechanisms directly or indirectly linked to ATP utilization, for example the inhibition of insulin secretion by UCP2 from the pancreatic beta cells([103]). The ubiquitously UCP2 is described as mitochondrial scavenger of ROS produced by mitochondria [104;105]. BAT with its thermogenic

function due to the presence of UCP1, has been positively associated with improved resistance to obesity and metabolic diseases. During recent years, the potential influence of environmental pollutants on energetic homoeostasis and obesity development has drawn increased attention. The current literature supports the hypothesis that some environmental pollutants, acting as endocrine disruptors (EDCs), such as DDT and its metabolite DDE as well as some, traffic pollutants, are associated with increased obesity risk, whereas some other chemicals, such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), had a reverse association with obesity [107]. Noteworthy, the EDCs associated with obesity and metabolic disorders impaired BAT mass and function. Perinatal DDT exposure combined with high fat feeding in adulthood has been shown to impair thermogenesis as evidenced by reductions in core temperature and in the expression of numerous RNA that promote thermogenesis and substrate utilization in the BAT of adult female mice [106]. The effect of DDT/DDE and other obesogenic xenobiotics on BAT may be mediated by the aryl hydrocarbon receptor (AhR). AhR, initially characterized as a xenobiotic sensor to mediate toxicological response, has been also shown to act as a physiological regulator of energy metabolism. Therefore, AhR activation by environmental pollutants has been suggested to promote obesity and related diseases and induces NF-κB activation and production of pro-inflammatory cytokines [107].

Ambient particulate air pollutions induced insulin resistance associated with BAT mitochondrial dysfunction. On the other hand, the environmental pollutants

(PFOS/PFOA) elicited a reduction in body weight and adipose mass associated with upregulation of UCP1 and increased oxidative capacity in brown-fat mitochondria. Further research is needed to better understand the physiological role of BAT in response to exposure to both obesogenic and anti-obesogenic pollutants and to confirm the same role in humans.

CHAPTER 3

Polyphenols of extra virgin olive oil

3 POLYPHENOLS OF EXTRA VIRGIN OLIVE OIL

Virgin olive oil is obtained only by mechanical extraction, and can be consumed directly, without further physical-chemical refining or rectification treatment. Its chemical composition is represented by a saponifiable fraction and by the "minor"

components that make up the unsaponifiable fraction. Within the non-lipid fraction, polyphenols are of particular importance, compounds that can be defined chemically as organic substances of natural origin, characterized by the presence of two or more hydroxyl groups directly linked to an aromatic ring. It is the fat component with more than 230 chemicals belonging to different classes such as aliphatic and terpene alcohols, sterols, waxes, hydrocarbons, carotenoids, pigments, vitamins, volatile compounds, tocopherols, phenolic substances and antioxidants. Antioxidants are those most correlated with the health properties of virgin oil.

These compounds originate during the process of mechanical extraction of oil from glucosidic polyphenols present in the olive. Among the most abundant secoridoids in the olive fruit we find oleuropein, demethyloleuropein, verbascoside. However, oleuropein is often degraded during olives maturations and processing and undergoes hydrolysis by producing different types of molecules. Regardless, oleuropein (Ole) can reach up to 14% of the dry weight of olives and olive oil can contain up to 2.8 mg/kg. In addition, olive leaf extracts can contain up to 61.56 g/kg. Hydrolysis of oleuropein occurs in the fruit during maturation and processing [108] and after ingestion of oleuropein, it is broken down by lipase activity and converted to hydroxytyrosol (Hty)[109]. Hty and tyrosol content of olive products can also vary. Interestingly, the hydrolysis of oleuropein during olive oil processing often results in higher concentrations of Hty present in olive oil compared to olives [(110)].

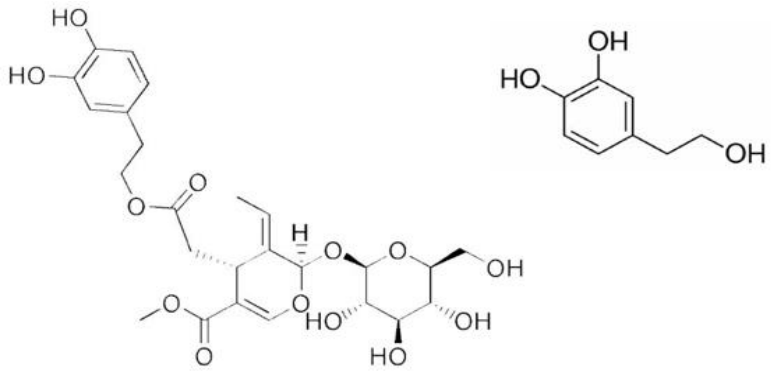

Figure 3.1. Structures of: oleuropein (left); hydroxytyrosol (middle). Int. J. Mol. Sci. 2018 , 19 (7), 1937

The phenolic substances of virgin olive oils are currently a topic of great interest this is due to:

• Definition of organoleptic characteristics

• Antioxidant action

• Health effects

The positive impact of oil on human health is associated with the synergistic effect of phenolic compounds and the high content of monounsaturated fatty acids (mainly oleic acid). In fact, the EFSA, (European Food Safety Authority) in 2011, established that an extra virgin olive oil can bear on the label the words "Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress", only if it contains at least 5 mg of Hty and its derivatives, per 20 g of olive oil. The claim must be accompanied by information that the beneficial effect is obtained with a daily intake of 20 g of olive oil (EU Reg. 432/2012 [(111)]).

3.1 Biological activity

In the past, polyphenols have been considered compounds with antinutritional action because they have the ability to complex minerals, proteins and carbohydrates, thus reducing their bioavailability. However, numerous recent studies have highlighted their important biological activity thanks to a better understanding of the biochemical processes related to the energy metabolism of the human organism.

Polyphenols alongside antioxidant power have other characteristics that make them important for our health. The mechanism of action of polyphenols strongly relates to their antioxidant activity. Polyphenols are known to decrease the level of reactive oxygen species in the human body. Other than that, health-promoting properties of plant polyphenols comprise anti-inflammatory, anti-allergic, anti-atherogenic, anti-thrombotic, anti-mutagenic effects, protection from degeneration in Alzheimer's and Parkinson. There is a body of research presenting their ability to modulate the human immune system by affecting the proliferation and activity of white blood cells, as well as the production of cytokines or other factors that participate in the immunological defense [112;113;114;115;116].

3.2Antioxidant activity

Oxidative stress consists of an imbalance between free radical production and scavenging that leads to abnormal generation of ROS, oxidative damage and

inflammation. Imbalance in this protective mechanism can lead to the damage of cellular molecules such as DNA, proteins, and lipids [117]. Reactive oxygen species are normally produced within the body in limited quantity and are important compounds involved in the regulation of processes involving the maintaining of cell homeostasis and functions such as signal transduction, gene expression, and activation of receptors [118]. Mitochondrial oxidative metabolism in cells produces ROS species and organic peroxides in the process of cell respiration [119]. ROS/RNS overproduction can cause damage to cellular structure and functions expecially if not removed quickly [120] and thus lead to cell death by the necrotic and apoptotic processes [121]. It has been proposed and demonstrated that several metabolic diseases share oxidative stress as a common feature and in this context, polyphenols can interact with ROS/RNS and thus terminate chain reaction before cell viability is seriously compromised [122].

Polyphenol antioxidant activity depends on the structure of their functional groups. The characteristic functional group of these phenolic compounds is a hydroxyl (–OH) bound directly to a carbon of a benzene ring. The substrates most affected, with pathological effects and consequences, are proteins and lipids but also amino acids, nucleic acids and nucleotides. The oxidation products of lipid substances (hydroperoxides and aldehydes) interact with DNA and some carbohydrates. The damage caused affects various aspects of primary and secondary cellular functionality (mutagenesis and increase in turnover, decrease in enzymatic activities, damage to membranes, alteration of LDL and lipoproteins in general, alteration of receptors-transmitters and finally reduction of fluid viscosity).

There are several studies in the literature, conducted on animal models, which affirm how the polyphenols of EVOO, reduce mitochondrial oxidative stress and the inflammatory phenomena associated with it due to the induction of gene expression of Nrf-2 (Nuclear factor erythroid 2-related factor 2). Nrf-2 it is considered the main regulator of redox homeostasis and its activation inhibits proinflammatory mediators such as cytokines COX2 (Ciclooxygenase 2) e iNOS (Inducible nitric oxide synthase) e NF-kB. EVOO polyphenols also activate Nrf2 in the liver resulting in the release of antioxidant enzymes. [123] Following the activation of Nrf2 and its translocation in the nucleus, the molecule binds to antioxidant response elements (ARE) and acts on the transcriptional expression of several antioxidant enzymes, including SOD (superoxide dismutase), glutathione S-transferase (GST), c-glutamyl-cysteine synthetase (C-GCS) and NADPH quinone oxidoreductase-1 (NQO1).[124;125]

EVOO polyphenols also attenuate TNF-α-induced NF-κB activation. HT showed in vitro attenuation of tumoral necrosis factor-α-induced downregulation (TNF-α) of mitochondrial biogenesis by increasing peroxisome proliferator-activated receptor (PGC)-1α coactivator-gamma, mitochondrial complexes (I and II) and myogenin expression. This indicated that Hty improves mitochondrial development and function in muscle cells under inflammatory stress [126;127]. The HepG2 treated with Hty (10 to 40 μM) for 20 hour completely prevented the effect of tert-butyl hydroperoxide (t-BOOH) in HepG2 cells. In particular, authors evidenced a significative restore in glutathione levels and a reduction malondialdehyde as markers of oxidative stress in cells In addition, Hty also significantly reducedfree radicals levels, enhancing the glutathione

peroxidase activity promoting cell integrity, antioxidant defense system and resistance to cope with a stressful situation in HepG2 cells .[128;129;130]

Ole and Hty also act on AMPK signaling, which has been reported as an interesting therapeutic target for antioxidant activity. In fact, AMPK signaling plays a key role in the cellular defense system against ROS through direct phosphorylation of human FOX01 (fork head box 01) with the consequent increase in FOX-01 dependent transcription of Mn-superoxide dismutase (Mn-SOD) and CAT (catalase).[131 ; 132] Therefore, the increase in AMPK activation by Hty is directly linked with the reduction of lipid synthesis in liver tissue.[133] Overall, different studies [134;135;136] above mentioned have shown that Hty exhibits hepatoprotective properties by increasing antioxidant activity and hepatocyte viability and decreasing apoptosis. More importantly, treatment with HT significantly reduced lipid synthesis; Accumulation of lipids in hepatocytes is often associated with the development of hepatic steatosis which subsequently leads to lower glucose utilization, liver injury and fibrosis thus exacerbating the development of insulin resistance and T2DM. These studies indicate that Hty may protect against hepatic steatosis and the development of hepatic insulin resistance.[37]

In addition, Ole and Hty have been identified as molecules capable of activating the autophagy process, useful to counteract the inflammatory process. It is a process by which cells remove damaged organelles, malformed proteins, or the accumulation of amyloid aggregates through lysosomal degradation. Autophagy is a highly conserved process and is necessary to maintain cellular homeostasis. Among the proteins involved are Beclin-1 and LC3 (important for phagophore formation), p62 (participates in load

recognition by lysosomes), mTORC-1 and AMPK (stress sensors that regulate autophagy).

MTORC-1 acts as an inhibitor, while AMPK by inducer and is stimulated under low energy conditions and bioactive molecules such as polyphenols [137].

3.3Antiproliferative activity and apoptosis

In multicellular organisms, apoptosis is a tightly regulated multiphase pathway, responsible for the process of cell death. In mammals, two mechanisms are known that directly initiate apoptosis. [138139140]

The extrinsic apoptotic pathway is activated by ligand binding to the cell surface, while the intrinsic cell death pathway is activated by UV irradiation, deprivation of growth factors, or the presence of stress stimuli, e.g. oxidative stress and DNA damage leading to induction of endoplasmic reticulum stress and pro-apoptotic members of the Bcl-2 family. Ole induces cell apoptosis through several mechanisms, such as caspase activation and suppression of the PI3K/AKT signaling pathway. Serine/threonine protein kinase B (AKT) is important in cell proliferation, apoptosis and cell survival.[141]

AKT mediates PI3K-dependent cell survival by phosphorylating a number of substrates, such as glycogen synthase kinase-3 β, 6-phosphofructo-2 kinase and kinase 1 that regulates the apoptosis signal. [142]

Changes in the biological activity of specific substrates downstream of AKT affect entire signaling cascades, including the activity of transcription factors involved in cell proliferation, apoptosis, inflammation, and metastasis [143;144]

Caspases are essential initiators or effectors in cell apoptosis. Ole induces cleavage of caspase-8, caspase-9, caspase-3 and PARP, which is a nuclear enzyme with a wide range of functions, including regulation of DNA repair, cell differentiation and gene expression. The Bcl-2 mitochondrial protein family is involved in the regulation of apoptosis [145].

Oleuropein greatly increased the expression of the BAX gene while decreasing that of the Bcl-2 gene. Alteration of the BAX/Bcl-2 ratio is related to apoptosis through a mitochondrial pathway [146].

This suggests that Ole induces apoptosis and that this progress is mediated by caspase activation, as well as regulation of mitochondrial protein expression. Thus, Ole inhibits the growth of HepG2 cells in a dose-dependent manner, particularly when the concentration of oleuropein is between 20-80 μM. In addition, changes in morphology in these cells have been observed following exposure of HepG2 with Ole, including contraction and increased cell death [45]. Both Ole and Hty have therefore shown, in different cell types (HepG2, MCF7, HT-29), anti-proliferative activity with arrest of the cell cycle in the G2/M phase, morphological changes and induction of cell death by apoptosis. [147;52; 148; 149; 150] Han et all. have demonstrated that the cells treated with Hty and oleuropein exhibited statistically significant block of G 1 to S phase transition manifested by the increase of cell number in G_0/G_1 phase. Although the oleuropein

notably induced less cell cycle arrest than Hty it was still capable to significantly promote breast cancer apoptotic cell death. [151; 152;153]

3.4 Polyphenols and mitochondria

A series of evidence show the promising role of polyphenolic compounds on mitochondrial structure and function. There are numerous literature studies that highlight how polyphenols play a vital role in the prevention and management of various chronic-degenerative syndromes. In fact, they have been described as natural antioxidants for their consolidated ability to eliminate free radicals and ROS. Hty contained in extra virgin olive oils and olives, is correlated with mitochondrial biogenesis. In *vitro* experiments described the ability of Hty to stimulate PGC-1α through deacetylation by Sirt-1[154].

Moreover, in addition to inducing PGC-1α expression, Hty has been shown to regulate Nuclear factor erythroid 2-related factor 1 (NRF-1) and Mitochondrial transcription factor A (Tfam), and to increase mtDNA content and ATP synthesis in endothelial cells [155]. Thus, mitochondria represent an important intracellular target of polyphenols.[156] Until recently, great emphasis had been placed on the antioxidant properties of these compounds, which evidently are not limited to a marked ability to eliminate ROS. Currently, it is clear that part of the antioxidant properties of polyphenols could be partly due to their ability to induce mitochondrial biogenesis and improve their functionality, which increases mitochondrial efficiency with consequent reduction of ROS production. [157] The antioxidant activity mediated by polyphenols against ROS is mainly attributed

to the presence of those hydroxyl groups linked to the benzene ring that can donate a hydrogen atom or a single electron to the ROS, stabilizing the reactive species. As a result, a phenoxyl radical of polyphenol is generated which, after reacting with a second radical, forms a stable structure of quinone. [158;90]

CHAPTER 4

Aim

Chronic exposure to pollutants could induce tissular/organ metabolic adaptation to promote cellular survival. Mitochondria are the main organelle involved in ROS production, and could be involved in the adaptive response of tissular/organ to stress induced by environmental pollutants.

The aims of the PhD thesis were: 1) to analyse DDE effects on cellular metabolism, focusing on mitochondrial function and oxidative stress, and 2) to evaluate the protective effects of dietary antioxidant compounds. To this end, two experimental designs were used, to shed light on the biological mechanisms in experimental model *in vivo* and *in vitro*.

In the first experimental design, an *in vivo* analysis was performed by using male Wistar rats, as experimental model. Rats were divided into the following two groups: 1- control group fed a standard laboratory diet (10.6% fats) (normal diet, N, group); 2- N+DDE group fed a standard laboratory diet (10.6% fats) in association with daily oral administration of 10 mg/Kg b.w. DDE. The adaptive responses activated by pesticide were mainly analyzed in liver, that functions as a central organ involved in detoxification processes, and in BAT that directly participates in thermogenesis and energy metabolisms. DDE effects on mitochondrial function and apoptosis markers were also assessed in other tissues, such as cardiac and skeletal muscle, to have a more general analysis on DDE effect on different tissues. In liver and BAT tissue, a more in-depth analysis was carried out. As for hepatic tissue, morphological analysis, oxidative stress, and apoptosis were assessed analyzing both SOD activity and content in tissue,

as well as caspase 3 activity and immunolocalization in liver sections. Mitochondrial activity was tested determined by testing cytochrome oxidase activity in enriched mitochondrial fraction or in tissue homogenate. As for BAT analyses, mitochondrial function and uncoupling were evaluated in both total homogenate and isolated mitochondria samples, together with the analysis of markers of mitochondrial biogenesis and oxidative stress. The results showed the importance of oxidative stress and antioxidant defense in response to DDE exposure in different tissues.

In the second experimental design, an *in vitro* analysis was performed to further analyse the effect of DDE on liver cells (Hepg2), and to evaluate the effects of a dietary antioxidant compound, such as hydroxythyrosol (Hty), in protecting against DDE cell damage. Hepg2 cell line was treated with different DDE doses in association with Hty focusing the analyses on cellular morphology, antioxidant activity and metabolism to detect if Hty activity could mitigate and/or inhibit the pro-oxidant and pro-apoptotic properties of DDE. Since EVOO contains a lot of phenol compounds, in vitro analyses were started investigating cell viability after treatment with two different EVOO phenols, namely Hty or oleuropein. Hty showed a lower effect on cell viability compared to oleuropein, and therefore Hty was used to perform the experiments of co-treatment with DDE. Concerning DDE doses, dose dependent experiments were performed, and two different doses were chosen to carry out the experiments in coincubation with Hty: 30μm DDE dose was chosen as a low dose able to induce hepatocytes alterations, and 100μM, as the higher experimental dose that approximate to levels of p,p-DDE found in serum, breast milk, liver, or adipose tissues in people

exposed to DDT[36]. As for Hty doses, a dose dependent experiment was performed, and 50 μM and 100 μM Hty doses with no or low effect on cell viability were chosen, in accordance with literature [159], to perform the cotreatment experiments.

Cellular viability and morphology, apoptotic marker, lipid peroxidation and antioxidant systems (superoxide dismutase (SOD), Catalase (CAT), and glutathione peroxidase (GPX) activities) were analysed in DDE-treated, Hty- treated or DDE+Hty treated cells. The results showed that DDE impairs hepatic cell viability through oxidative stress induction without adequate stimulation of antioxidant defense, mainly at high pesticide dose. Noteworthy, the simultaneous exposure to DDE and Hty, counteracts DDE induced cell damage by improving antioxidant defense.

CHAPTER 5

In *vivo* Experimental Design 1
DDE effects on liver and brown adipose tissue cell metabolism in rats

5.1 Material and methods

5.1.1 Ethics Statement

The experimental design 1 was conducted in accordance with recommendations in the EU Directive p2010/63/ for the Care and Use of Laboratory Animals. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Naples ‘‘Federico II’’ (Permit Number: 2012/0024690).

5.1.2 Materials

The chemicals to compose all buffers used for the experiments were purchased from Sigma Aldrich (St. Louis, MO, USA).

5.1.3 Experimental Design

Data showed in the present PhD thesis were obtained by working on samples of tissues from rats previously treated in the animal house of Department of Biology, University of Naples, by the research group of the PhD thesis tutor. Male *Wistar* rats, aged 60 days (Charles River Italia, Calco, Como, Italy), were caged separately in a temperature-controlled room at 24 °C with a 12 h light-dark cycle in the animal house of Department of Biology, University of Naples “Federico II”. After one week of acclimation, rats were divided into two experimental groups (eight rats for each group) with a similar mean body weight (approximately 300 g) and with the body weights normally distributed within each group: N group (standard control diet PF1915 from Mucedola, Milano, Italy, 0.6% fat J/J, 15.47 KJ/g); N+ DDE group (standard control diet, +10mg/kg b.w. of DDE). Since the environmental exposure to DDE is in general the result of the

introduction of low doses through the food, in this study we decided to administer DDE at low doses (10 mg/kg b.w.) orally. It has been reported that the oral administration of this DDE dose did not influence physical development, sexual maturation, and serum metabolic parameters in male pubertal or older rats when it was administered for 6 or 4 weeks, respectively. All rats were housed individually, acclimatized in a temperature-controlled room (24° C) and subjected to a circadian light-dark cycle (12 hours light / 12 hours dark).

At the end of the experimental period of 4 weeks, the rats were anesthetized by an intraperitoneal injection of Zoletil (40 mg/Kg body weight) and euthanized by decapitation.

Liver, white adipose tissues (epididymal and retroperitoneal WAT pads), and brown adipose tissue (BAT) were immediately removed, weighted, and processed in accordance with the experimental procedures used. Hepatic and BAT slices were either immediately processed for morphological analysis or frozen in liquid nitrogen and stored at −80 °C for later processing. Skeletal muscle (gastrocnemius) and cardiac muscle were also immediately removed and frozen in liquid nitrogen and stored at −80 °C for later processing. During my Ph.D. research period, I worked on these tissues previously taken and properly preserved.

5.1.4 Morphological analysis

Liver and BAT fragments were washed in cold ice NaCl 0.9% for few seconds and fixed in Bouin'fluid for 12 hours. Then, each fragment was dehydrated, embedded

in paraplast, and cut at 5 μm by using a microtome. The histological sections were processed by using Hematoxylin & Eosin stain or Azan-Mallory stain. Hematoxylin marks cell nuclei in purplish/blue, while eosin interacts with both extracellular matrix and cytoplasm that result as pink. Azan-Mallory staining procedure is a classical trichrome method that uses acid fuchsine followed by a solution containing phosphotungstic acid, orange G and aniline blue, provides dark red nuclei, orange/red erythrocytes, and blue collagen fibres, cartilage matrix and mucus. After staining, histological sections were examined by using a Zeiss Axioskop light microscope fitted with a TV camera.

5.1.5 Preparation of mitochondrial fraction from tissues

To obtain mitochondria from tissues, both liver and BAT fragments were washed and homogenized in extraction buffer (220mM Mannitol, 70mM sucrose, 20mM HEPES, 1mM EDTA and 0.1% fatty acid-free BSA, pH 7.4 (1:10, w/v)) with a Potter Elvehjem homogenizer (Heidolph, Kelheim, Germany) set to 500 rpm (4 strokes/min) to promote tissue and cell broken. Nex steps, specifically performed depending on tissue, were conducted as previously described by Lionetti et al. (2004) [160] for liver, or by Lombardi et al., 2015 [152] for BAT.

Then, mitochondrial suspensions were followed processed according to the experimental procedures.

5.1.6 Preparation of total tissues homogenate for western blot analysis

Total proteins from liver and BAT were obtained using RIPA buffer solution (150 mM NaCl, 50 mM Tris, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, pH 7.4) containing a cocktail of protease inhibitors (Sigma Aldrich Chemicals). Liver fragments were homogenates using a polytron (KINEMATICA Polytron Model PT10-35 GT/PT 3100D Homogenizer, Fisher Scientific) and centrifuged at 12,000g for 10 minutes. The pellet was discarded, and the supernatant was used for analysis. On the contrary, BAT homogenates were ultracentrifuged at 17,000 x g for 20 min at 4°C. Both floating fat and pellet were discarded, and the supernatants were used for protein quantification and western blot analyses.

5.1.7 Cytochrome oxidase activity

To investigate the effect of DDE exposure on mitochondrial oxidative metabolism, the activity of cytochrome oxidase (COX), a well-known marker of this metabolism, was evaluated. Cytochrome oxidase (COX) activity was measured polarographically with a Clark-type electrode at 25°C in medium containing 30μM cytochrome c, 4μM rotenone, 0.5 mM dinitrophenol, 10 mM Na-malonate, 75 mM HEPES, pH 7.4 [161;162]. To detect COX activity, tissue samples (gastrocnemius, hearth, liver and brown adipose tissue) or mitochondrial samples (brown adipose tissue) were diluted in Chappel and Perry medium (1mM ATP, 100 mM KCl, 5 mM MgCl2, 1 mM EDTA, 5 mM EGTA, 50 mM HEPES pH 7.4) containing Lubrol PX (225μg/mg protein) and incubated for 30 min in ice to unmask enzyme activity. At the end of the incubation, COX activity in the whole

homogenate was measured as oxygen consumed in the presence of 4 mM ascorbate + 0.3 mM tetramethyl-p-phenylenediammine (TMPD) [163].

5.1.8 ROS content

Total hepatic, mitochondrial and total BAT ROS levels were measured following the ROS-induced conversion of 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA, nonfluorescent compound) in dichlorofluorescein (DCF, fluorescent compound) as reported by Driver et al. [164] 30 μg of tissue or mitochondrial homogenate were dissolved in 200 μl of monobasic phosphate buffer 0.1 M, pH 7.4, and incubated for 15 min with 10 μM DCFH-DA. Then, FeCl3 was added to reach the final concentration of 100 μM; the mixture was incubated for 30 min. The Tecan Infinite 200 pro plate reader was used to detect the conversion of DCFH-DA to the fluorescent product DCF (λex 485 nm, λem 530 nm,). The conversion of DCFH to DCF in the absence of tissue homogenate was used as Background fluorescence. A standard curve of DCF was used to calculate pmol DCF formed.

5.1.9 Lipid peroxidation assay

Lipid peroxides levels were performed using TBARS assay kit (Cayman Chemical Company, No.10009055), as previously reported [3] according to the manufacturer instructions. Procedure was performed to quantify malondialdehyde (MDA) levels in both liver and BAT samples, as final product of lipid peroxidation reactions. MDA quantification was finally normalized with protein concentration in each sample.

5.1.10 Western blot analyses

Western blotting analyses were performed on total tissue homogenate by using Laemli method. Protein content in liver tissue lysates and both in BAT tissue and mitochondria lysates were determined by using Bradford method. 35µg of proteins for each sample were electrophoresed in properly performed SDS–polyacrylamide gels, together with a pre-stained protein marker (Page ruler, Thermo Scientific, 10-250KDa). After the run, the proteins were transferred onto nitrocellulose membranes (Immobilon-P, Millipore, Switzerland) at 350mA for 60 minutes. The membranes were blocked in blocking buffer solution (1×TBS/ 1% Tween-20, 5% milk) for 60 minutes at room temperature, washed in 1×TBS/ 1% Tween-20, and incubated overnight at 4°C in milk/TBS-Tween buffer (1×TBS/1% Tween-20, 2% milk) supplemented with the following antibodies of interest.

In liver lysates, I investigated: SOD2 (D3X8F Rabbit mAb #13141, 1:2000). To normalize protein levels in samples, β-Actin (Santacruz biotech, sc- sc-517582, 1:1000) was used as loading control guide.

In BAT lysates, I investigated: PGC1a (Cell signalling tech, #2178S, 1:1000) and UCP1 (Abcam, ab 10983, 1:1000); while, in BAT mitochondrial fraction, I investigated: UCP1 (Abcam, ab 10983, 1:1000) and SOD2 (Cell signalling tech, D3X8F, 1:2000). To normalize protein levels in both total tissue and mitochondrial samples, Tubulin (Cell signalling tech, #2146, 1:1000) was used as loading control guide. Antibodies preparation and dilutions were performed as indicated on datasheets.

Second day, membranes were washed 4×15 min with TBS-Tween solution and incubated for 1h at room temperature with a properly secondary antibody labeled with horseradish peroxidase, diluted in milk/TBS-Tween buffer (1×TBS/1% Tween-20, 5% milk). Finally, membranes were washed 4×15 min with TBS-Tween solution and revealed with a chemiluminescent method, using Luminol solution (final concentration 2.5 mM) supplemented with cumaric acids (final concentration 0.4 mM) and H_2O_2 (final concentration 100mM). The bands, obtained on X-Ray films, were quantified using ImageJ software.

5.1.11 Immunohistochemistry analysis in liver and BAT sections

Immunohistochemical reactions were performed using Novolink Polymer Detection Systems (RE7280-CE, Leica Biosystems). After antigen retrieval and quenching of endogenous peroxidase, sections were incubated overnight at 4°C with the following antibody of interest. In liver sections, we investigated: SOD2 (D3X8F Rabbit mAb #13141diluted 1:200 in PBS; cl-Caspase-3 (Asp175) Rabbit mAb #9661) diluted in PBS 1:100, to detect apoptotic cells. In BAT sections, we investigated UCP1 (Abcam, ab 10983 1:200/PBS). The sections were then incubated in Novolink polymer 1h at RT, and immunostaining was performed using 3,3′-diaminobenzidine (DAB) as chromogen. The sections were counterstained with hematoxylin and mounted with a coverslip. To test the specificity of the reagents, the following controls were performed: (a) omission of the primary antiserum and incubation of the sections with either non-immune serum (1:10 in PBS) or 1% bovine serum albumin (BSA; Sigma); (b) absorption of the optimally diluted primary antiserum with its specific peptide (10 nmol/ml of optimally

diluted antiserum) for 24 h at 4°C. When the specific peptide was used, the staining was abolished. Images of sections were acquired using a Zeiss Axioskop microscope fitted with a TV camera.

5.1.12 Caspase-3 activity

Tissue homogenate samples (gastrocnemius, heart and liver) were used to measure caspase 3 activity with a colorimetric kit from Sigma Aldrich (CASP3C). The assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p nitroanilide (Ac-DEVD-pNA) by caspase 3, resulting in the release of the p-nitroaniline (pNA) moiety. p-Nitroaniline has a high absorbance at 405 nm and the concentration of the pNA released from the substrate is calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined pNA solutions of known concentrations as standards.

5.1.13 Data analyses and statistics

Data analyses were obtained as mean ± standard error on the mean (SEM) and, graphically represented as fold of changes in N+DDE group vs. N. Statistical analyses were performed by using GraphPad Prism software. Differences between groups were analyzed by using student's t-test. Data were considered statistically different when P value was inferior to 0.05 ($P<0.05$).

5.2 DDE effect on body weight gain, tissues weights and serum parameters

In a previous published paper, the effect of DDE on obesity development, in terms of body weight gain and weights of visceral fat pads, and serum levels of metabolites

related to lipid metabolism (triglycerides, TG, and cholesterol levels) and hepatic injury (ALT and AST) were analysed.

At the end of the treatment period, body weight gain was calculated as difference between final body weight and initial body weight for each animal (Table 5.1, published data)[3]. No significant change in body weight gain was found in N+DDE compared to N group. In accordance, the weights of epididymal and retroperitoneal WAT pads were not significantly changed between N and N+DDE. No change was found in BAT weight. Therefore, DDE did not elicit a significant obesogenic effect under our experimental condition. However, a tendency to increase (+14%) was observed in the sum of epididymal and retroperitoneal WAT pads weights in N+DDE rats compared to control (Table 5.1).

Serum TG, cholesterol, ALT, and AST levels were assessed as markers of dyslipidaemia and liver injury, respectively. As regard serum metabolite parameters linked to body lipid metabolism, no changes in TG and cholesterol levels were found between N and N+DDE groups (Table 5.1).

The analyses of ALT activity and AST content were used to evaluate the amount of liver damage produced by DDE treatment. Increases in ALT activity (~3-fold) and AST content (3.8-fold) were found in N+DDE vs. N group (Table 5.1). This finding suggested that DDE treatment induced liver damage.

	N	**N+DDE**
Body weight gain (g)	88.9±7.5	73.1±4.7
Epididymal WAT (g)	9.5±1.4	10.6±1.0
Retroperineal WAT (g)	9.0±1.1	10.5±0.9

Total WAT pads (g)	18.5±1.2	21.10±1.7*
Liver weight (g)	12.8±0.5	13.3±1.0
ALT activity (U/mL)	52.7±11.7	152.0±11.5***
AST content (ng/mL)	1.81±0.46	6.93±1.98***
BAT weight (g)	0.83±0.12	0.83±0.10

WAT (white adipose tissue); ALT (Alanine Transaminase); AST (Aspartate Transaminase).
Data are reported as means±ES of 8 different rats for each group.
* $p<0.005$ compared vs N
*** $p<0.001$ compared vs N+DDE
N=rat fed with normal diet
N+DDE= rats fed with normal diet + 10mg/kg b.w. of DDE

Table 5.1 Body weight gain, serum metabolite parameters
Data published in Migliaccio et. al 2019[3]

Considering these previous results, in the present PhD thesis I focused mainly on DDE effects on hepatic metabolism, focusing on mitochondrial function and oxidative stress. However, I also evaluated DDE effects on other tissues, such as cardiac and skeletal muscle as well as brown adipose tissue. Indeed, DDE effects on mitochondrial function and apoptosis marker were analysed in cardiac and skeletal muscle samples. Moreover, a more in-depth analysis on the effect on mitochondrial function, uncoupling and biogenesis was carried out in BAT samples.

5.3 DDE effect on mitochondrial function and apoptosis markers in cardiac and skeletal muscle tissues

To evaluate the effects of DDE in cardiac and skeletal muscle (gastrocnemius) tissues, cytochrome c oxidase and caspase 3 activities were analysed in tissue homogenates as

markers of mitochondrial function and apoptosis, respectively. Interestingly, DDE was shown to have opposite effects on skeletal and cardiac muscle.

As for cytochrome c oxidase activity, DDE elicited an increase (+89%) in COX activity in cardiac muscle, whereas a decrease (-22%) in its activity was observed in skeletal muscle (figure 5.1A).

As concerns caspase 3 activity, DDE elicited a significant decrease (-54%) in cardiac muscle, whereas an increase (+35%) in its activity was found in skeletal muscle (figure 5.1B).

Therefore, DDE exhibited different effects in the two types of muscle tissues. In skeletal muscle, DDE induced apoptosis onset associated with an impairment in mitochondrial function. On the other hand, DDE did not induced apoptosis in cardiac muscle where mitochondrial function was found increased. This finding confirmed the importance of mitochondrial function to preserve cell viability. It also suggested that in cardiac muscle DDE elicited an adaptive pro-survival response by improving mitochondrial function and avoiding apoptosis onset, whereas it damaged skeletal muscle cell viability by impairing mitochondrial function. Further experiments are needed to clarify the cellular mechanisms and physiological importance of the opposite effects of DDE in skeletal and cardiac muscle.

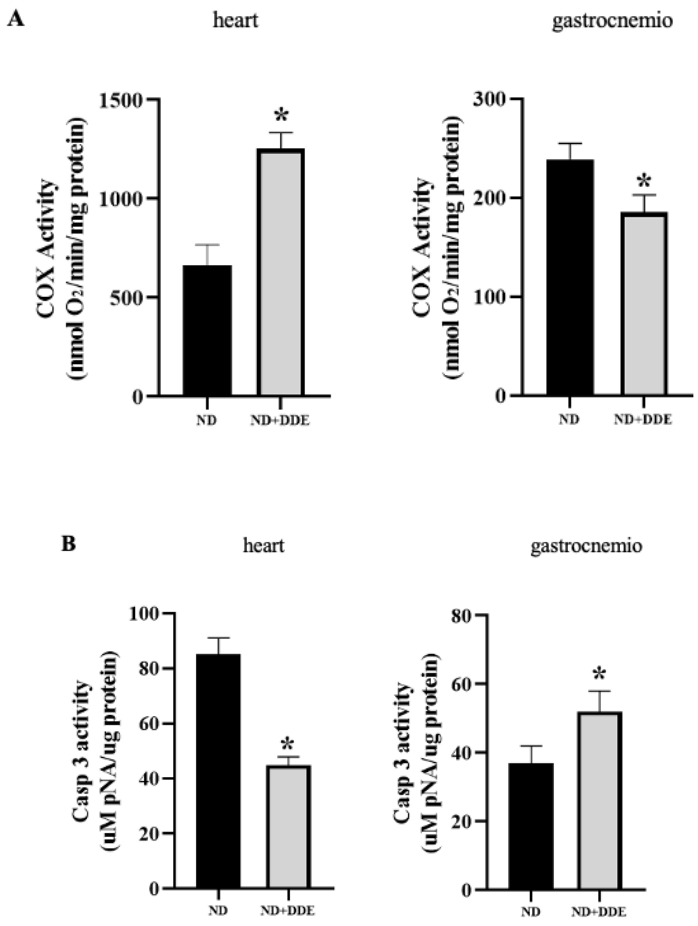

Figure 5..1 (A) Cytochrome oxidase (COX) activity in gastrocnemius and heart homogenates. (B) Caspase 3 activity in gastrocnemius and heart homogenates.

Data are represented as media ± SEM for 8 different rats per group. The student t-test was applied. Statistical differences are reported *P<0.05 compared to N rats.

5.4 Effects of DDE on BAT

BAT is specialized in thermogenesis. The brown adipocytes contain a lot of lipid droplets and many mitochondria expressing uncoupling protein-1 (UCP1). The tissue produces heat by dissipating mitochondrial proton motive force, generated by respiratory chain, through the activation of UCP1. Therefore, the activation of BAT, by promoting an increase in energy dissipation in the form of heat, plays an important role in metabolic and energy homeostasis.

The potential influence of environmental pollutants on energetic homoeostasis by altering BAT thermogenic activity has recently drawn increased attention [165;97].

In the present PhD thesis, the metabolic adaptation of BAT in response to chronic exposure to DDE was evaluated by analysing tissular morphology, mitochondrial function, uncoupling and biogenesis.

5.4.1 Effects of DDE on tissular morphology

Histological sections did not show alterations in tissue parenchymal morphology and structure, as well as fatty depots. Indeed, adipocytes in DDE-treated animals were found similarly characterized of small lipid droplets that fill cell cytosol (figure 5.2).

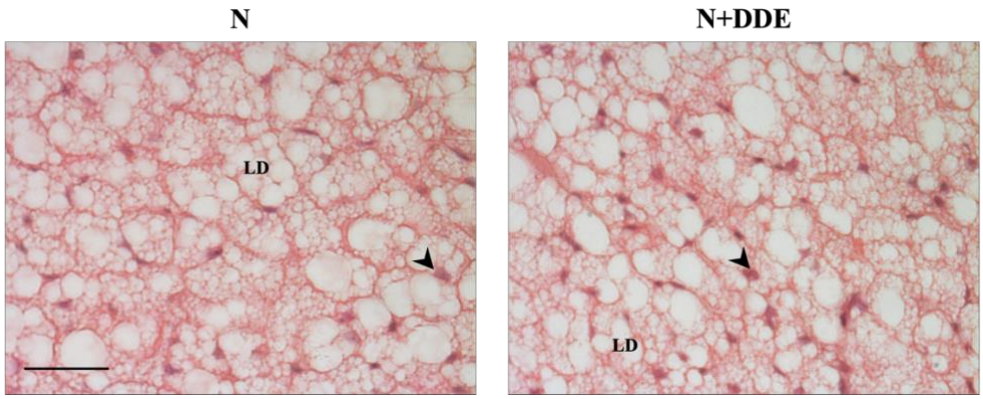

Figure 5.2 BAT morphology
Haematoxylin & eosin staining. Morphological analyses showed small lipid droplets in DDE-treated rats.

5.4.2 Effects of DDE on mitochondrial function, uncoupling and biogenesis in BAT

Considering that mitochondria play a key role in the main function of BAT, namely thermogenesis, mitochondrial cytochrome oxidase (COX) activities and uncoupling

protein 1 (UCP1) content were analysed as markers of mitochondrial function and efficiency, respectively.

COX activities were evaluated both in total BAT homogenate and enriched mitochondrial fraction. The analyses showed a reduction of about 33% in the maximal tissue oxidation capacity in N+DDE vs N in total homogenate, while an increase of about 40% was detected in isolated mitochondria in N+DDE group vs. N (figure 5.3).

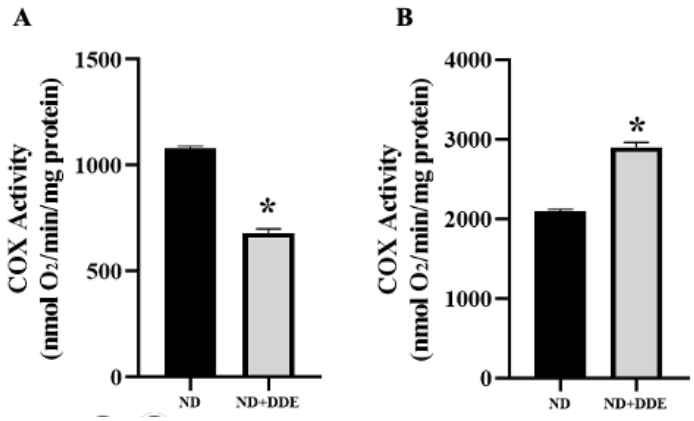

Figure 5.3 Cytochrome oxidase activity in BAT.
A) COX activity in total tissue homogenate; B) COX activity in isolated mitochondria. Data are represented as media ± SEM for 8 different rats per group. The student t-test was applied. Statistical differences are reported *P<0.05 compared to N rats.

The content of UCP1, the protein specifically involved in thermogenesis, was found significantly reduced in BAT homogenates (~50%) (figure 5.4A). Data was also confirmed by immunohistochemical analyses (figure 5.4C). Interesting, UCP1 protein levels were found increase of about 2-fold in isolated mitochondria (figure 5.4B).

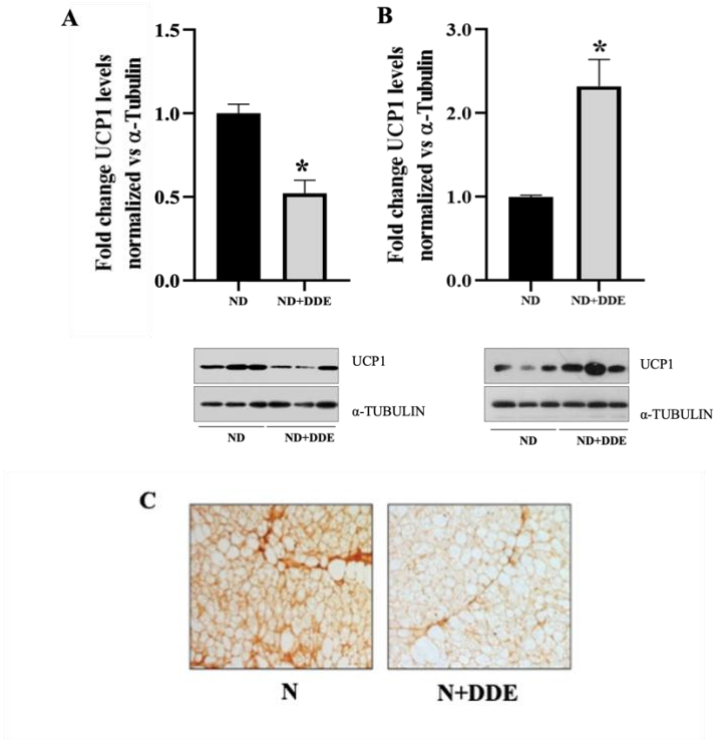

Figure 5.4 UCP1 protein content.
A) UCP1 content in total tissue homogenate; B) UCP1 content in isolated mitochondria. Data are represented as media ± SEM for 6 different rats per group. The student t-test was applied. Statistical differences are reported *P<0.05 compared to N rats. C) UCP1 immunostaining. Magnification used 20X; scale bar applied 50µm.

Noteworthy, the effect of DDE on mitochondrial function and uncoupling showed a significant decrease of the respective markers in total tissue homogenates, whereas the activity of COX and the content of UCP1 were increased in enriched mitochondrial fraction. This finding suggested that DDE elicited an adaptive response by increasing mitochondrial function and uncoupling evaluated in isolated mitochondrial fraction, whereas the decrease in these parameters in total BAT homogenates suggested a decrease in mitochondrial content. To evaluate this hypothesis, the content of PGC1α, as a master regulator of mitochondrial biogenesis, was analyzed. The results showed a

significative reduction in PGC1α content in tissue, suggesting a possible decrease in mitochondrial mass due to DDE administration (figure 5.5).

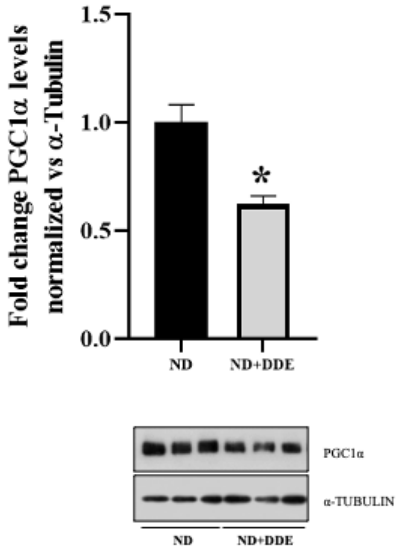

Figure 5.5 PGC1α levels in total tissue homogenate.
Data are represented as media ± SEM for 6 different rats per group. The student t-test was applied. Statistical differences are reported *P<0.05 compared to N rats.

5.4.3 Effect of DDE on BAT oxidative stress and damage

To assess the possible pro-oxidant effects of DDE on BAT, different parameters were analyzed. In particular, our results evidenced both ROS and lipid peroxides accumulation in N+DDE group, that were found increased 4-fold and 3-fold vs. N group, respectively. Interesting, testing mitochondrial SOD2 levels as antioxidant protein that functions as sensor of stress, protein levels were found significantly up-regulated of about 2-fold in N+DDE group vs. N

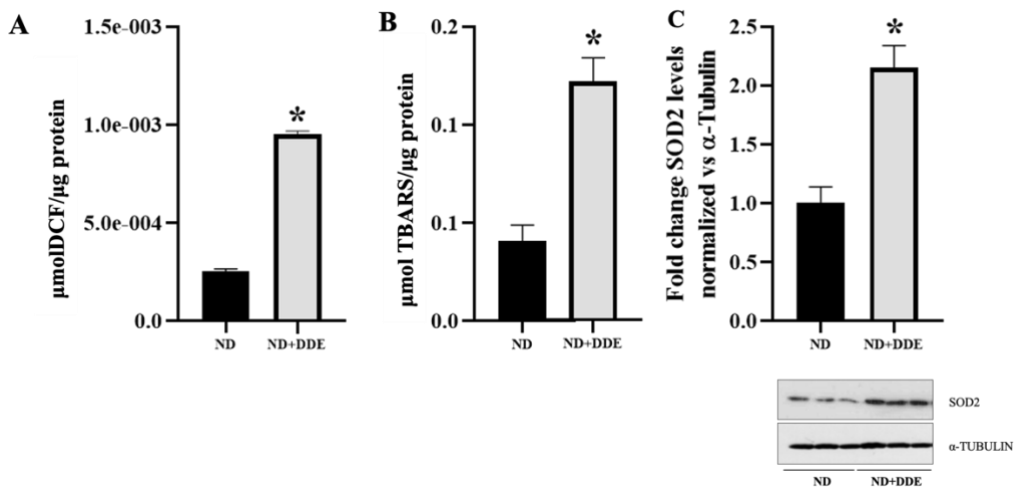

Figure 5.6 oxidative stress and damage.
Total homogenate ROS levels (A); Total homogenate lipid peroxidation (B); SOD2 levels in isolated mitochondria (C). Data are represented as media ± SEM for 6 different rats per group. The student t-test was applied. Statistical differences are reported *P<0.05 compared to N rats.

In the present PhD thesis, we reported novel data concerning the metabolic adaptation observed in BAT following the chronic exposure to DDE. DDE induced a reduction in the maximum tissue oxidative capacity and the number of mitochondria, plausibly through PGC1alpha down-regulation. This finding is in accordance with data reported in the literature concerning the ability of the pesticide to reduce PGC1 alfa mRNA in BAT. [97]

Noteworthy, mitochondria isolated from DDE-treated animals, despite being reduced in quantity, were also more active than controls, since DDE induces an increase in specific cytochrome oxidase activity and UCP1 protein levels. This increase could be considered an adaptive response to counteract thermogenesis impairment induced by the chronic exposure to DDE. DDE also induced oxidative stress, as shown by the increase of total ROS and lipid peroxidation. It should be noted that the antioxidant

defense was stimulated by activating mitochondrial antioxidant system SOD2 that could contribute to counteract further increase in oxidative stress.

5.5Effects of the DDE on liver.

The liver is the main organ involved in xenobiotic detoxification as well as in dietary lipid metabolism, and hepatic steatosis is the most common pathologic liver responses to both high fat diet and chemical exposures, non-alcoholic and toxicant-associated steatohepatitis (NASH, and TASH), respectively. [166]

Oxidative stress plays a key role in steatohepatitis induced by both xenobiotic agents and high fat diet. In a previous work, we analysed hepatic oxidative stress and anti-oxidant systems response in rats exposed to HFD and/or non-toxic dose of DDE [3]. The results showed malondialdehyde accumulation in livers of all groups, confirming the pro-oxidant effects of both HFD and DDE, but with a greater effect of DDE in absence of HFD. In addition, DDE mainly induced UCP2 and SOD2 as anti-oxidant defense response to limit excessive ROS damage, mainly in condition of xenobiotic exposure.

In the present PhD thesis, I further analysed the effect of DDE on hepatic morphology and oxidative stress in association with mitochondrial function and apoptosis markers analysis.

5.5.1 Effects of the DDE on liver morphology

Morphological analysis was performed by haematoxylin & eosin and azan-mallory stains. Hemallum & eosin evidenced tissue disorganization around lobular veins, with a lot of vacuoles or droplets in cell cytosol (Figure 5.7). Interesting, using Azan-Mallory staining, our treatment did not alter collagen deposition and cellular lipid depots. However, many peri-vessel cells showed acidophilic phenotype that suggests cellular stress or increased protein synthesis and accumulation.

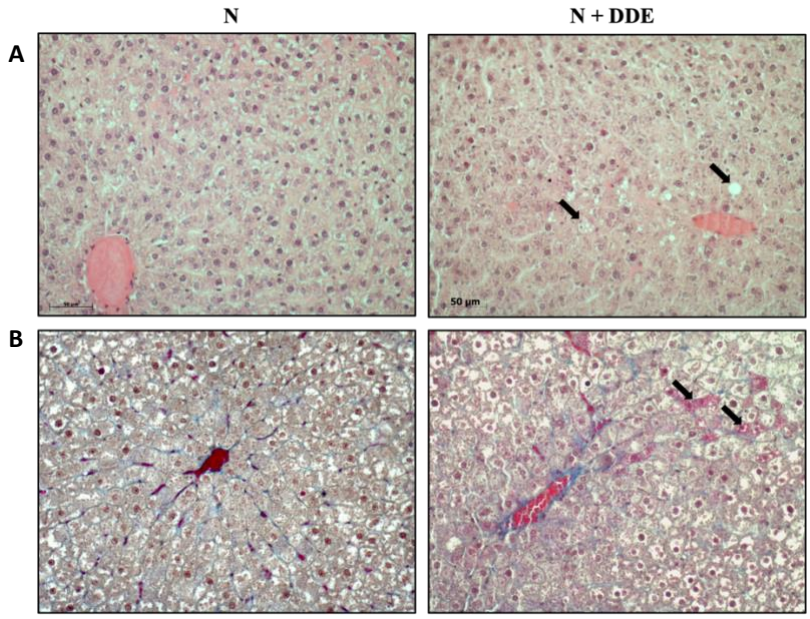

Figure 5.7: Liver morphology
A) Haematoxylin & eosin (H&E staining; B) Azan-Mallory staining.
Morphological analyses showed alteration of liver parenchyma around lobular vessels (black arrows in panel B, H&E). Some eosinophilic cells were also detected in N+DDE (arrowheads in panel D, Azan-Mallory). Magnification used 20X; scale bar applied 50µm.

5.5.2 Effects of the DDE on hepatic oxidative stress generation

ROS levels in liver tissue were analysed in both mitochondrial fraction and total tissue homogenate. Data evidenced that DDE induced an increase in mitochondrial ROS levels (2-fold), associated with total ROS accumulation in tissue (+50%) compared to control

rats. As effect, DDE produced tissue alterations confirmed by testing lipid peroxides levels that were found to increase of about 2-fold in N+DDE vs. N group.

In accordance with the finding of the previous work [3], chronic exposure to DDE induced an increase in oxidative stress in rat liver.

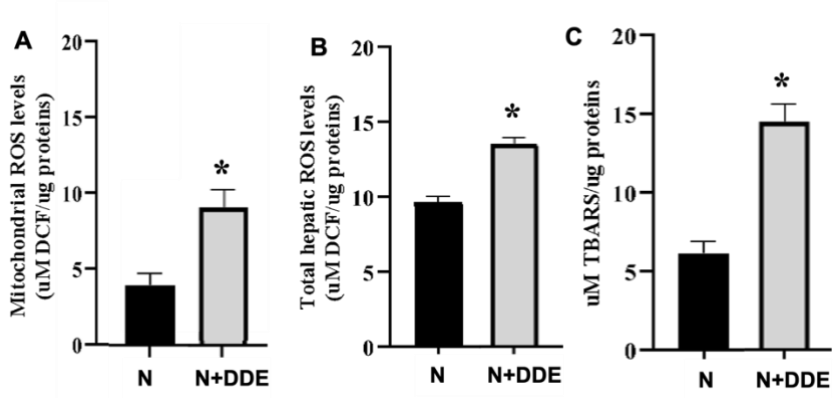

Figure 5.8. Hepatic oxidative stress.
A) ROS accumulation in liver mitochondria; B) ROS accumulation in total tissue homogenate; C) lipid peroxides in total tissue.
Data are represented as media ± SEM for 6 or 8 different rats per group. The student t-test was applied. Statistical differences are reported *P<0.05 compared to N rats.

5.5.3 Effects of the DDE mitochondrial antioxidant defense

Associated with ROS accumulation and oxidative damage in tissue, both SOD2 enzyme activity and protein content were evaluated. This enzyme, that function as sensor of stress, directly participate in mitochondrial superoxide scavenging. Our analyses evidenced that both protein content and enzyme activity, were increased of about 50% in N+DDE vs. N, suggesting that liver produces adaptive responses to limit organelle impairment and disfunction. In addition, concerning protein tissue localization, a lot of immunoreactive cells around centrilobular veins were detected in N+DDE (Figure 5.9C)

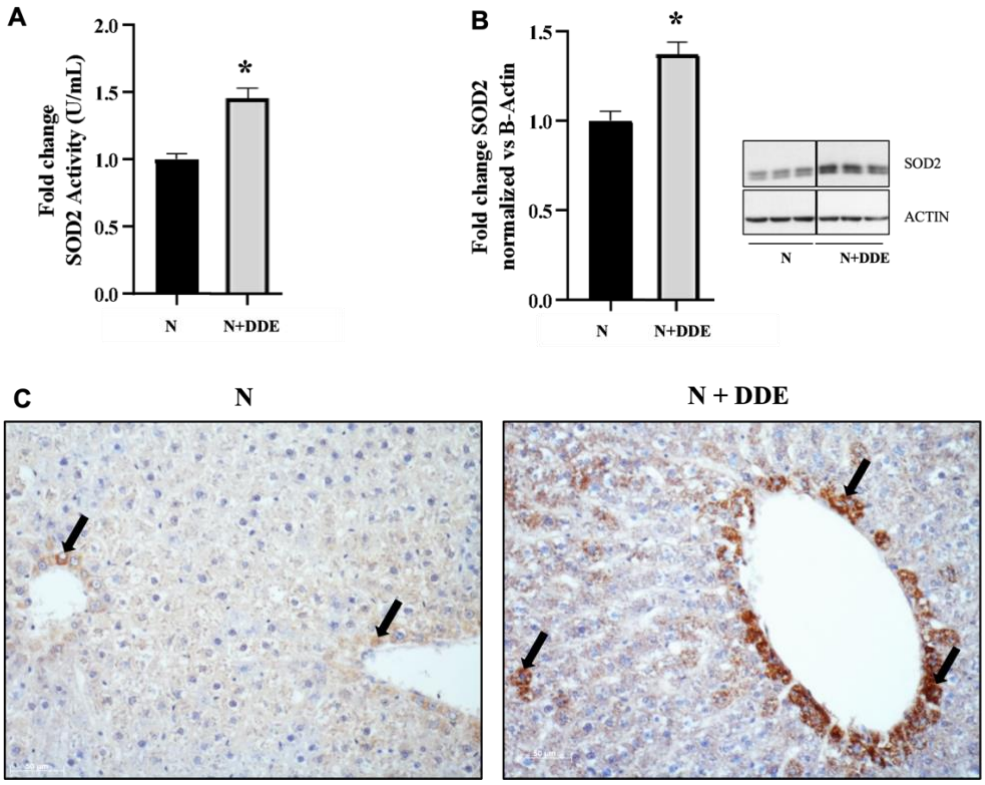

Figure 5.9 Antioxidant enzyme SOD2 levels.

A) enzymatic activity in total tissue homogenate and B) SOD2 protein content. Data are represented as media ± SEM for 6 or 8 different rats per group. The student t-test was applied. Statistical differences are reported *P<0.05 compared to N rats. C) SOD2 immunolocalization. Immunoreactive cells (black arrows) were particularly detected around vessels. Magnification used 20X; scale bar applied: 50µm.

5.5.4 DDE effect on mitochondrial function and apoptosis markers in liver

To further evaluate the effects of DDE in hepatic tissues, cytochrome c oxidase (COX) and caspase 3 activities were analysed as markers of mitochondrial function and apoptosis, respectively.

To evaluate mitochondrial function, COX was measured in liver homogenates. Results showed that DDE elicited a decrease (-25%) in COX activity in liver homogenates compared to control group (figure 5.10).

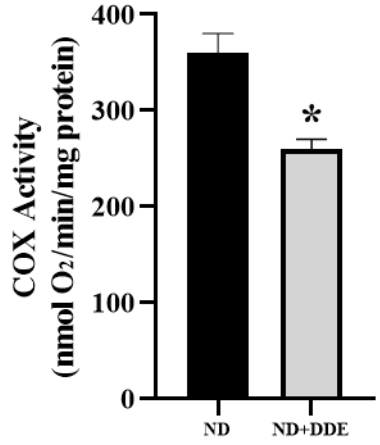

Figure 5.10 Cytochrome oxidase (COX) activity in liver.
Data are represented as media ± SEM for 8 different rats per group. The student t-test was applied. Statistical differences are reported *P<0.05 compared to N rats.

To analyze the possible association among DDE induced oxidative stress, mitochondrial dysfunction and cell damage, we analyzed the activity and the immunolocalization of caspase 3 as a marker of apoptosis. In particular, caspase 3 activity and immunolocalization in tissue were assessed. In agreement with the oxidative and mitochondrial damage induced by DDE, the analysis performed in total tissue homogenate showed an increase in caspase 3 activity of about 30% in N+DDE group vs. N.

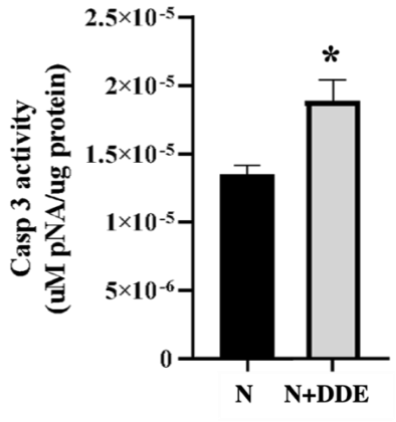

Figure 5.11 Caspase 3 activity in liver. Data are represented as media ± SEM for 6 different rats per group. The student t-test was applied. Statistical differences are reported *P<0.05 compared to N rats.

Protein immunolocalization of caspase 3 cleaved form was also performed. According with enzymatical activity, immunolocalization analysis evidenced enhanced protein immunoreactivity in N+DDE group where liver nuclei and cells were stained.

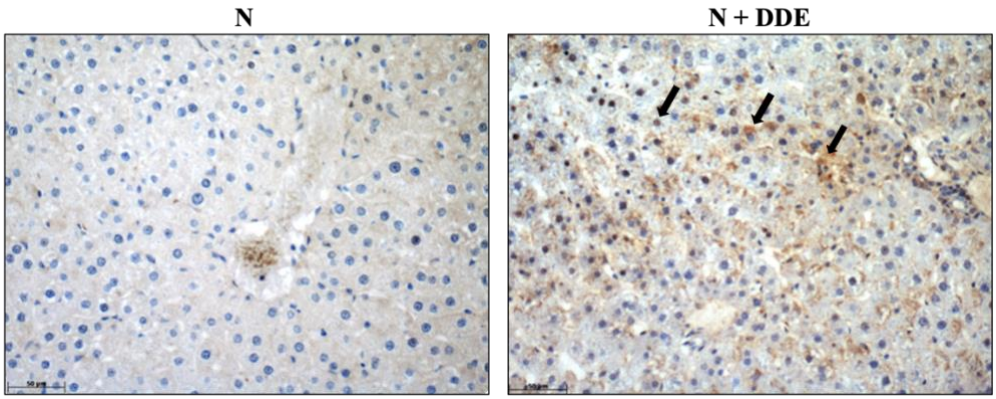

Figure 5.12 cl-Caspase 3 immunoreactivity in liver. Nuclei and cell cytosol in N+DDE group were found strongly stained (black arrows). Magnification used 20X; scale bar applied: 50µm.

Therefore, DDE induced apoptosis onset associated with an impairment in mitochondrial function and oxidative stress associated with alterations of hepatic morphology.

5.6 DDE effects on liver and brown adipose tissue cell metabolism in rats: Discussion and conclusion

The aim of the first experimental design of the present PhD thesis was to study the effect of chronic oral administration of DDE on different tissue and organs in animal experimental model. This experimental design was conceptualized considering the previous experimental results obtained in our laboratory on the same experimental model, mainly in liver and testis tissue [3], [74], [167]. DDE-treated rats exhibited higher lipid peroxidation levels associated with pronounced defect in antioxidant capacity, apoptosis, cellular proliferation, and tissue damage in testis [167]. In hepatic tissue, DDE-treated rats showed a pro-oxidant effect by increasing lipid peroxidation with an increase in antioxidant defense, mainly UCP2 and SOD2, suggesting that UCP2 induction could be an adaptive response to limit excessive ROS damage in condition of chronic xenobiotic exposure [3]. In this thesis, we have further investigated the effects of DDE in liver tissue, and we have also performed analyzes in other tissues, mainly in BAT.

DDE chronic exposure induced impairment in BAT function by acting at the mitochondrial level, without alteration in tissue morphology. In tissue homogenates, DDE induced a reduction in mitochondrial activity and thermogenesis, as shown by the decrease in COX activity and UCP1 content compared to control group. The impairment in homogenate mitochondrial function and uncoupling was due to a decrease in mitochondrial biogenesis, as shown by the decrease in PGC1alpha content. However, COX specific activity and UCP1 content were increased in isolated BAT mitochondria, suggesting an adaptive response to counteract the impaired mitochondrial biogenesis in

total tissue. These findings were associated with an increase in oxidative stress, as shown by the increase in ROS and lipid peroxidation levels, with a stimulation of the mitochondrial SOD2 antioxidant activity.

As for hepatic tissue, in accordance with previous finding [74], an increase in ROS levels was also found in both total liver homogenates and isolated mitochondria from DDE-treated rats. The increase in SOD2 antioxidant system was confirmed by our results on SOD2 protein content, enzymatic activity and immunolocalization.

The increase in ROS levels and SOD2 antioxidant defence was associated with an impairment in mitochondrial function and apoptosis onset, as suggested by the decrease in homogenate COX activity and the increase in caspase 3 activity, respectively, in DDE-treated rats. Mitochondrial impairment and oxidative stress could play a key role in liver injury and TASH development.

The results of this PhD thesis shed further light on the effects of chronic oral exposure to environmental pollutants, such as DDE. The main result is that DDE induced several damage and/or adaptive response in different tissues. In particular, DDE impaired BAT thermogenesis, and induced oxidative stress and cellular apoptosis in liver, associated with alteration in serum transaminase (ALT and AST) suggesting hepatic injury. The effect on BAT thermogenesis could be associated with obesity development, whereas DDE-induced liver injury could develop in TASH with longer period of exposure to DDE.

Moreover, present results also showed impaired mitochondrial activity and apoptosis induction in skeletal muscle. It is well known that skeletal muscle metabolism

impairment is associated with insulin resistance onset. Recent evidence shows that environmental pollutants exposure could induce insulin resistance [168] and the mitochondrial dysfunction could play an important role as underlying mechanism. The increase in mitochondrial activity and decrease in apoptosis induction in cardiac muscle could be an adaptive response to induce a stimulation in cardiac function to counteract systemic damage induce by DDE exposure.

The results obtained in liver tissue suggested that DDE-induced oxidative stress could play a key role in the onset of toxicant associated steatohepatitis (TASH).

To shed further light on the mechanisms underlying DDE toxic effect in liver, the second experimental design of the present PhD thesis focused on the effect of DDE in a model of hepatic cell in vitro (HepG2). A further aim of the second experimental design was to investigate the possible protective role of antioxidant dietary compound, such as Hty, on liver injury development.

CHAPTER 6

In *vitro* Experimental Design 2-
DDE effects on hepatic cells in vitro: beneficial antioxidant effect of hydroxytyrosol, a polyphenol from olive oil

6.1 Material and methods

6.1.1 Cell Culture and Treatments

HepG2 cell line was cultured in Minimum Essential Medium supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) non-essential amino acids, 0.2 mM L-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin (Invitrogen SRL, Milan, Italy). Cells were maintained at 37 °C in a 5% CO2, 95% air-humidified atmosphere and passaged twice a week. To perform treatments with DDE and Hty, cells were seeded at the density of 4.0 × 104/cm2 and cultured for 24 h before stimulation. The two different doses of DDE (Merck KGaA, Darmstadt, Germany) 30 and 100 mM stock solution were prepared dissolving pesticide in DMSO as reported by manufacturer. For each treatment, final concentration of DMSO in the medium was about 0.1%, which represents a non-toxic level for cells as reported in literature (Nikolaou et al., 2016), while, DDE final concentration used were 30 and 100µM. Concerning extra virgin olive oil polyphenols, both Hty and oleuropein stock solutions were prepared in distillated water and used in a large range of doses (0-500µM) to assess the cytotoxic effect of phenol compound. Subsequently, we performed experiments cotreating HepG2 with DDE doses above described with Hty final concentrations 50 and 100 µM in the medium starting from a stock solution 162mM Hty prepared dissolving powder in distilled water. To assess phenol effects, Hty was also used alone, supplementing medium with DMSO 0.1%. A control group of cells (control group) was stimulated with DMSO 0.1%. The experiments were conducted at 24h of exposure.

6.1.2 Cell Viability assay

Compound toxicity was assessed by performing the 3-(4,5-dime-thylthiazol-2-yl)-2,5-diphenylte-trazolium (MTT) assay. This technique is based on the enzymatic conversion of MTT in mitochondria [169]. After treatments, 0.5 mg/mL of MTT was added to the cell medium and plates were incubated for 1.5 h at 37 °C, 5% CO2. During time, living cells metabolize MTT. The resulting formazan crystals were dissolved in 100 μL/well DMSO, as previously reported[170], and the absorbance of the resulting cell suspension was measured at 595 and 655 nm to subtract the background in each sample. Then, the percentage of cytotoxicity in treated cells was calculated according to the following equation: cytotoxicity (%) = (OD control group − OD treatment group)/OD control group × 100%. [171]

6.1.3 Western blot

As already described in section 5, western blotting analyses was performed by using Laemli method. Briefly, at the end of the treatment, cell medium was removed and adherent cells were washed with PBS1X. After washing, cells were mechanically scraped, collected in centrifuge tube, and lysed in RIPA buffer solution supplemented with both protease and phosphatase inhibitors (Sigma Aldrich). The lysis was started mechanically using an insulin syringe equipped with thin needle to facilitate cell broken. Protein concentration was determined using the Bio-Rad Protein Assay. The primary antibodies used for in vitro analyses were SOD2 (Cell signalling tech, D3X8F, 1:2000)

and PARP (Rabbit mAb, #9542, 1:1000). Antibodies preparation and dilutions, were performed as indicated on datasheets.

For protein normalization, each membrane was also analyzed for the corresponding loading control Beta-Actin (Santacruz biotech, sc- sc-517582, 1:1000).

6.1.4 Oil Red-O Staining

Oil red stain quantitative (spectrophotometric) and qualitative (microscopic) analyses were performed using a classic protocol to detect lipid droplets in cells [172]. Briefly, after treatment, cells were washed in PBS at 37 °C, fixed in 4% PFA for 45 min at room temperature, washed two-time with distilled water and stained as indicated by Oil-Red-O conventional protocols. To quantify oil-Red O accumulated in cells, the stain was extracted from lipid droplets by using isopropanol 100% and measured by using a 96 well plate reader at 490 nm. Quantitative and qualitative analyses were compared to non-stimulated cells.

6.1.5 Caspase-3 activity

Caspase 3 activity was measured with a colorimetric kit from Sigma Aldrich (CASP3C). The assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p nitroanilide (Ac-DEVD-pNA) by caspase 3, resulting in the release of the p-nitroaniline (pNA) moiety. p-Nitroaniline has a high absorbance at 405 nm and the concentration of the pNA released from the substrate is calculated from the absorbance values at 405 nm

or from a calibration curve prepared with defined pNA solutions of known concentrations as standards.

6.1.6 Total Antioxidant enzymes activities

The activity of the main antioxidant enzymes in cells were tested by using conventional kits from Cayman Chemical. Superoxide Dismutase (SODs) assay kit (No. 706002), Glutathione peroxidase (GPX) assay kit (No. 703102), and Catalase (CAT) assay kit (No. 707002) were used. Samples and procedures were prepared and performed as indicated by manufacturer.

6.1.7 Lipid peroxidation assay

Lipid peroxides were tested in DDE-Hty coincubation experimental series. Procedure was performed to quantify malondialdehyde (MDA) levels in total cell lysates, as final product of lipid peroxidation reactions. After treatments, Hepg2 were lysed in RIPA buffer solution and processed as described by De Leon and Borges (2022)[173]. MDA quantification in samples were finally normalized with protein concentration in each sample.

6.1.8 Statistics

Statistical analyses were performed by using GraphPad Software Inc., San Diego, CA, USA. Each experiment was performed in biological triplicates or more. One-way ANOVA test followed by Bonferroni's post hoc test or t-test were used. Differences were considered statistically significant with a P value was < 0.05.

6.2 Dose-dependent effect of DDE and EVOO polyphenols, hydroxythyrosol and oleuropein, on cell viability

In the first period of my PhD thesis, I collaborated to the lab research project which aimed to analyse the dose-dependent response to the DDE focusing on cell viability and mitochondrial fusion/fission proteins in HepG2 cells [32]. To analyze the dose-dependent effect of DDE on Hepg2 cell viability, MTT assays were performed.

DDE induced a decrease in cell viability in a dose-dependent manner (figure 1A)[32]. Indeed, no significant effect was found in the presence of low DDE doses up to 5 μM, whereas significant decreases in cell viability with values of 64% and 46%, compared to control cells (100%), were observed with high DDE doses (50 μM and 100 μM, respectively) (figure 1A)[32]. Given the lipophilic propriety of DDE and its ability to accumulate in lipid depots, we also analysed cell viability and intracellular fat depots under conditions of cotreatment with diverse dietary fatty acids, namely palmitate (as a saturated fatty acid), oleate (as a monounsaturated fatty acid) and EPA + DHA (1:1) mixture (as polyunsaturated fatty acids). Regarding the effect of fatty acids on cell viability, cells treated with 250 μM of palmitate or oleate reduced their viability values to 71% and 76%, respectively (Figure 1B,C), whereas cells treated with 250 μM EPA + DHA mixture did not show any changes in viability (Figure 1D) compared to control.

Noteworthy, fatty acids induced an increase in DDE toxicity. Cells co-treated with fatty acids and DDE showed a significant reduction in cell viability even with low DDE doses, which did not elicit any significant decrease in cell viability in the absence of fatty acids. Dose-dependent decreases in cell viability values were less marked, mainly

with high DDE doses when cells were cotreated with EPA + DHA mixture (Figure 1D)[32].

These data suggested that DDE associated with fatty acids was particularly detrimental for cell viability and that mainly saturated fatty acids could exacerbate environmental pollutants' toxicity.

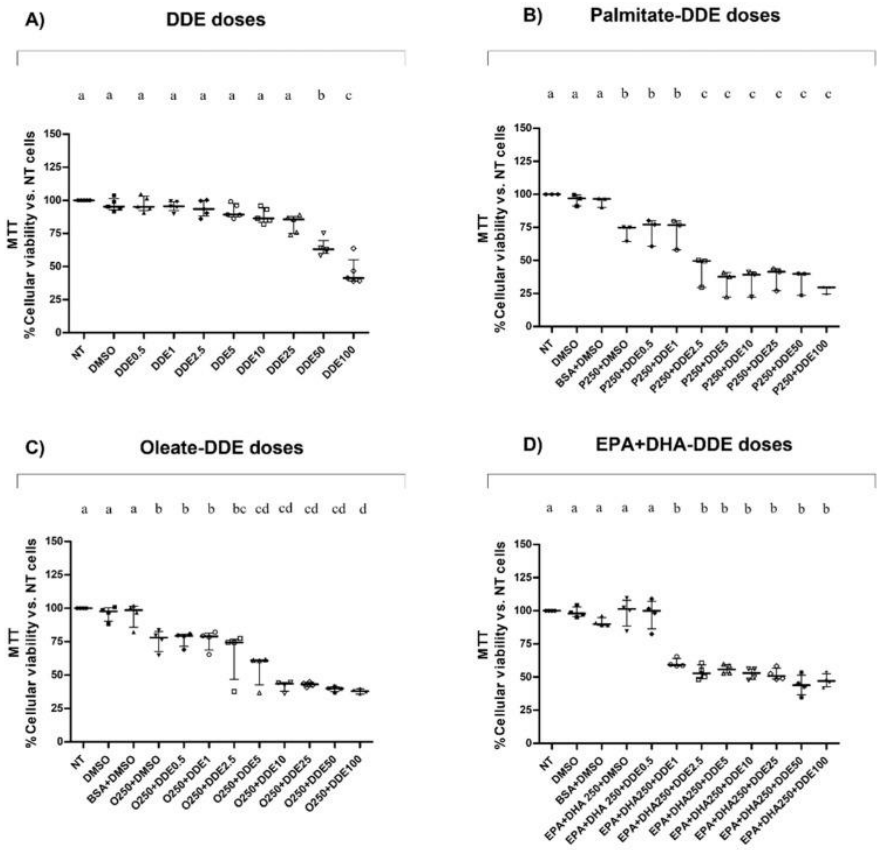

Figure 6.1 HepG2 cellular viability. Data were obtained stimulating cells with different doses of DDE (0.5 µM, 1 µM, 2.5µM, 5 µM, 10 µM,25 µM, 50 µM and 100 µM) (A) or with DDE in association with palmitate (250 µM) (B), oleate (250 µM) (C), or EPA + DHA (1:1) mixture (250 µM) (D). Results were graphically represented as median with range (B) or with interquartile or range (A,C,D) for 3 or 4 biological replicates. Statistical analyses were performed using GraphPad Prism software. One-way ANOVA analysis was followed by Bonferroni's post-hoc test: different letters indicate statistically different values. (Data published)[32]

In the present PhD thesis, I further analysed the simultaneous effect of DDE with other dietary compounds on Hepg2 cell viability. Considering the induction of oxidative stress by DDE, I focused on the effect of antioxidant bioactive compounds, such as polyphenols from olive oil. The dose-dependent effects of two main polyphenolic compounds of EVOO, namely Ole and Hty, on cell viability were analysed. The combined effects of Hty with DDE on cell viability were also tested to evaluate its possible beneficial effects.

Concerning Ole, a significative drop in living cells was detected starting from 10 μM (-15%) becoming increasingly evident with the highest dose of 500 μM, where cell viability was found strongly reduced of about 90% (figure 6.2).

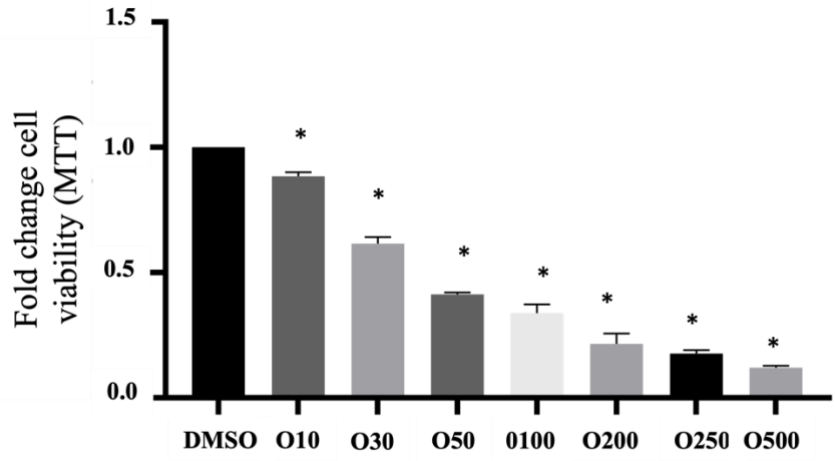

Figure 6.2 Effect of Ole on HepG2 cellular viability. Data were obtained stimulating cells with different doses of Ole (10 μM, 30μM, 50 μM, 100 μM,200 μM, 250 μM, 500 μM), and are represented as media ± SEM for 3 independent experiments, performed in biological replicate each. One-way ANOVA analysis followed by Bonferroni's post-hoc test was applied. Statistical differences are reported vs. DMSO-treated cells *P<0.05

Similarly, Hty produced a significative dose-dependent reduction in cell viability. However, as also reported in literature [149] the effect of Hty were detectable only starting from 100 μM where the effect was lower (-10%) if compared to the highest dose tested (500 μM) that produced a drop in living cells of about 30% (figure 6.3).

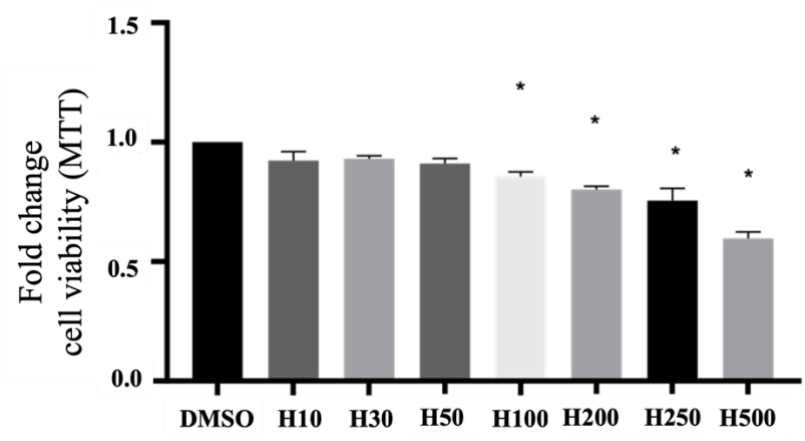

Figure 6.3 Effect of Hty on HepG2 cellular viability.
Data were obtained stimulating cells with different doses of Hty (10 μM, 30μM, 50 μM, 100 μM,200 μM, 250 μM, 500 μM), and are represented as media ± SEM for 3 independent experiments, performed in biological replicate each. One-way ANOVA analysis followed by Bonferroni's post-hoc test was applied. Statistical differences are reported vs. DMSO-treated cells *P<0.05

The data reported herein showed that Hty produced lower effects on cell viability than oleuropein. For this reason, we decided to use Hty to study the combined effects of simultaneous treatment with the pesticide DDE and a polyphenol with antioxidant capacities. This choice was also supported by the fact that Hty is considered the most powerful antioxidant compound after gallic acid, and one of the most powerful antioxidant compounds between phenolic compounds from olive tree followed by oleuropein, caffeic and tyrosol [174].

To perform the co-treatment experiments, two doses of DDE were used, namely 30 and 100 μM, to compare the effects of a dose with a low effect and a dose with a significant

reducing effect on cell viability, respectively (in accordance with literature, figure 6.1A) [32].

To study the effect of Hty on DDE-treated cells, the doses of 50 and 100 μM of Hty were chosen among the doses used, as the last dose without effect and the first dose with reducing effect on cell viability, respectively. Both Hty doses were tested on cell treated with 30 or with 100 μM DDE.

Noteworthy, the combination of Hty with DDE treatment partially reverted pesticide effect. In particular, referring to 30 μM DDE, the association with 50 μM Hty induced a slight restoration of cell viability of about +10% vs. DDE, but keeping this parameter lower than in control cells (-20%). On the other hand. the association with 100 μM Hty completely reverted the effect of pesticide (figure 6.4A)

Similarly, the same recovery trend was observed in the coincubation of Hty with the 100 μM DDE dose. In this experimental condition, co-treatment with 50 μM Hty and 100 μM DDE resulted in no change in cell viability compared to the DDE-treated cell (figure 6.4B). However, treatment with 100 μM DDE associated with 100 μM Hty showed a partial recovery of living cells of approximately 50% compared to DDE-treated cells (figure 6.4B). This data showed that Hty attenuated pesticide-induced cytotoxicity, suggesting a possible protective effect of this phenol compound against toxic-induced steatohepatitis.

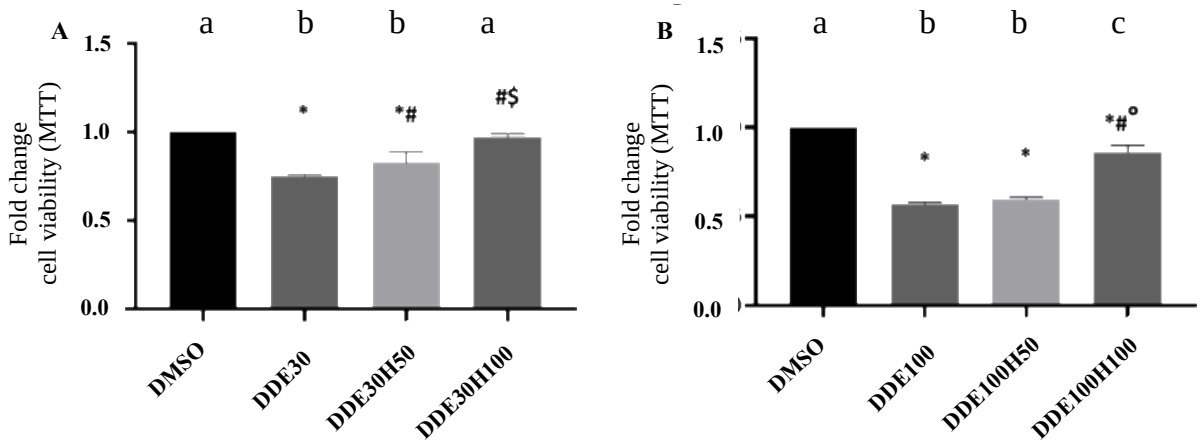

Figure 6.4 Effect of the simultaneous exposure to DDE and Hty on HepG2 cellular viability.

Data are represented as media ± SEM of 3 independent experiment performed in biological replicates. (A) 30 μM DDE combined with 50 μM and 100 μM Hty. (B) 100 μM DDE combined with 50 μM and 100 μM Hty. Different letters indicate statistical difference among groups analyzed by using One-way ANOVA followed by Bonferroni's post-hoc ($P<0.05$). In addition, student T-test were applied. *$p<0,05$ vs. DMSO; #$p<0.05$ vs. 30 μM DDE and vs. 100 μM DDE; §$p<0.05$ vs. 30 μM DDE + 50 μM Hty; °$p<0.05$ vs. 100 μM DDE + 50 μM Hty and vs. 100 μM DDE + 100 μM Hty.

6.3Effect of DDE and Hty on cells morphology

According to MTT analyses, morphological alterations of cells were detected in presence of DDE. Indeed, pesticide induced a progressive dose-dependent change of cellular shape evidencing intracellular droplets/vacuoles accumulation (red arrows), compared to the control cells (DMSO) (figure 6.5). On the contrary, the simultaneous treatment with DDE and Hty improved cell morphology compared to DDE treatment alone, restoring physiological structure of cells and reducing cytosol vacuolation and debris. No changes in HepG2 structure, number and morphology were detected in Hty treated cells (figure 6.5).

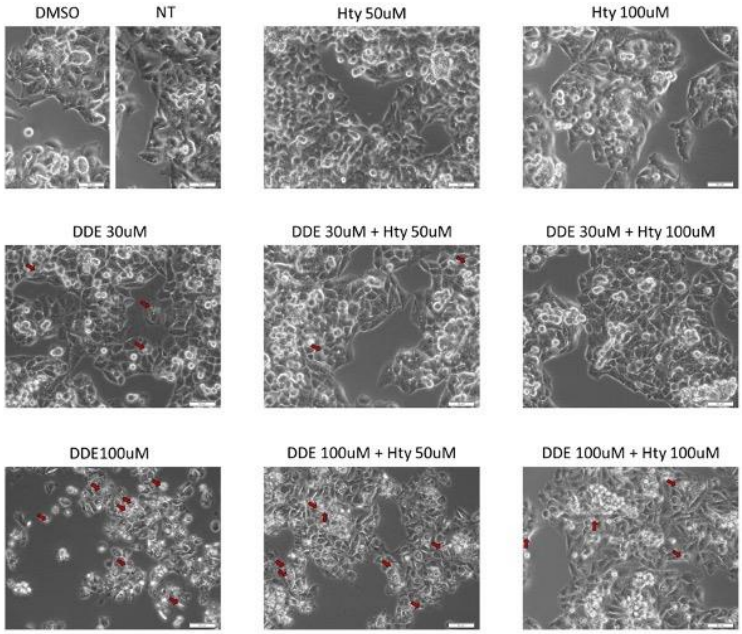

Figure 6.5 Morphological analysis. Cell morphology, assessed by optic microscope, evidenced cytosolic vacuolization of cells (red arrows) that were particularly evidenced at 100µM pesticide. HTy-DDE coincubation improve cell structure and chape. Magnification used 20X; Scale bar applied 50µm.

6.4 Dose-dependent effect of DDE and Hydroxytyrosol on lipid accumulation

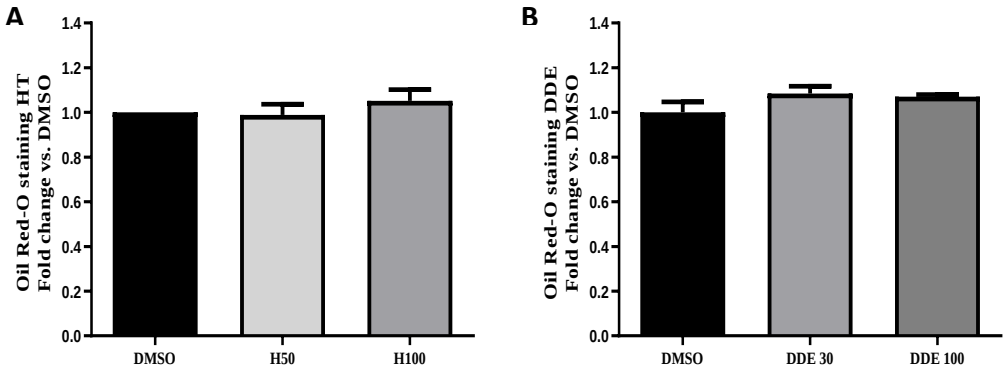

Figure 6.6 Total lipid content in response to different doses of Hty (A) and DDE (B). Data are represented as media ± SEM of 3 independent experiments performed in biological triplicates.

To study the possible implication of stimuli on lipid accumulation in hepatocytes, Oil red-O quantitative analyses were performed. Our results did not evidence alteration of total lipid content in cells measured in presence of Hty and DDE alone or in combination between them suggesting that, in our experimental conditions, treatments are not steatogenic.

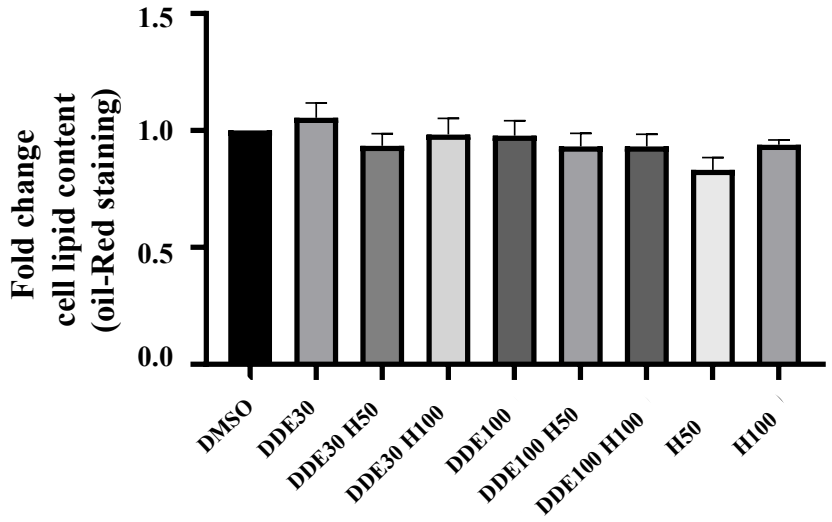

Figure 6.7 Total lipid content in response to different doses of DDE co-incubated with Hty.
Data are represented as media ± SEM of 3 independent experiments performed in biological triplicates.

6.5 Effect of DDE and Hydroxytyrosol on lipid peroxidation

To detect the possible oxidative damage induced by DDE, lipid peroxidation assay was performed measuring MDA levels in total cellular homogenates. Concerning DDE, results showed no changes in MDA content at the lowest dose used (figure 6.8). On the contrary, DDE 100µM produced a significative increase of MDA level of about 2.5-fold vs control cells. Instead, coincubation with 50 µM and 100 µM Hty reduced

lipid peroxidation to a value of about 2-fold and 1.5-fold in 100 µM DDE + 50 µM Hty and 100 µM DDE + 100 µM Hty treated cells vs control cells, respectively (figure 6.8).

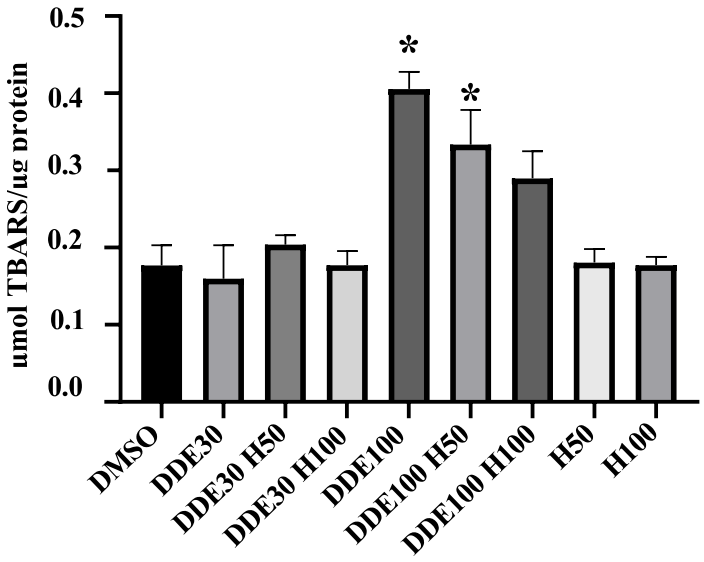

Figure 6.8 Lipid peroxidation (MDA levels). Data are represented as media ± SEM performed in biological triplicates. One-way ANOVA analyses and Student's t-test analysis were applied. *p<0.05 vs. DMSO

6.6 Hydroxytyrosol reverts DDE-induced cell apoptosis.

Considering cell viability and cell morphology results, apoptotic markers were analysed in our experimental conditions.

Regarding the reduction of cell viability induced by Hty, the analysis of Poly-Adenyl Ribose polymerase (PARP) content in cells stimulated with 50 and 100 µM Hty showed that the phenolic compound does not produce the cleavage of PARP in cells which, in turn, lead to non-activation of caspase 3 in hepatocytes (figure 6.9).

This data, supported by literature evidence [149], suggests that Hty-induced viability reduction is not due to phenol toxicity, but could be explained by its well-known anti-proliferative activity on cancer cells.

Further experiments are needed to confirm anti-proliferative effect of Hty in our experimental conditions. However, the association of decreased cell viability with no effect on cell morphology supported the hypothesis of a nontoxic effect of Hty.

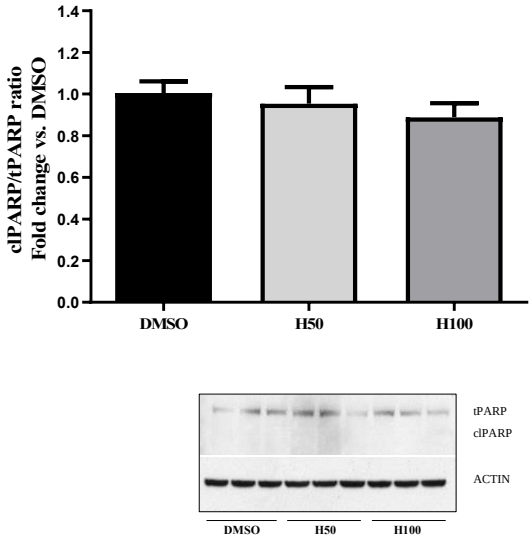

Figure 6.9 PARP protein levels in presence of Hty. Data are represented as media ± SEM of 3 independent experiment performed in biological triplicates. Representative images of bands were shown (PARP and its cleaved form P89) and the loading control guide beta-Actin.

I further analysed apoptotic markers by measuring caspase-3 activity. The nontoxic effect o Hty was confirmed by the finding of no significative changes in caspase 3 activity in Hty treated cells (figure 6.10).

As regard DDE treatment, results showed that 30 and 100 μM DDE increased the caspase 3 enzymatic activity of about 6-fold and 8-fold, respectively (figure 6.10),

confirming that DDE induced apoptosis and cell damage in accordance with the finding of reduced cell viability and morphology alterations.

Noteworthy, combinations of 30 μM DDE with 50 μM or 100 μM Hty showed attenuation of caspase 3 activity that appears to be fully restored compared to DMSO-treated cells (figure 6.10). A similar trend was also seen in cells treated with 100 μM DDE in which coincubation with 50 μM or 100 μM Hty reduced caspase activity to values similar to those found in control cells (figure 6.10).

These results are in line with the findings on cell viability and morphology in which co-treatment with Hty and DDE restored living cells and morphological changes compared to DDE-treated cells.

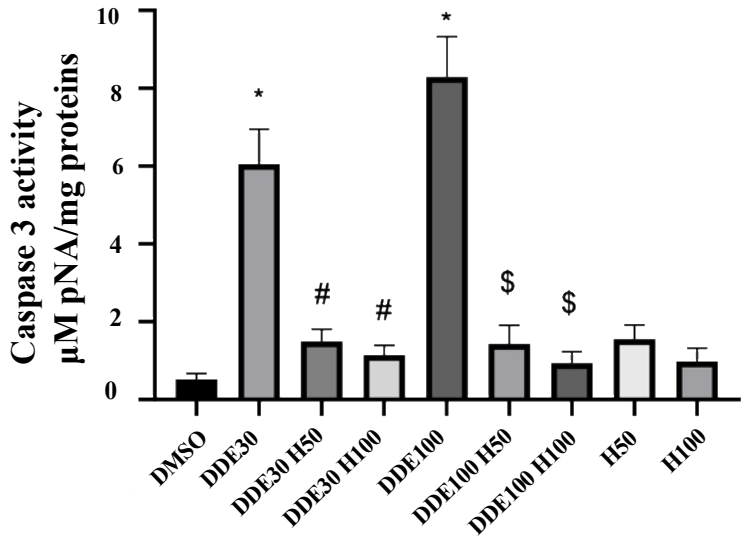

Figure 6.10 Caspase 3 activity. Data were graphically represented as mean ± standard error on the mean (SEM). One-way ANOVA analysis followed by Bonferroni's post-hoc test was applied. Statistical differences are shown: * $p < 0.05$ vs. DMSO; #$p < 0.05$ vs. DDE 30 μM; $ $p<0.05$ vs. DDE 100 μM.

6.7 Effect of DDE and Hydroxytyrosol on antioxidant system defence

Considering the antioxidant effects of polyphenols, it was interesting to evaluate the effect of Hty co-treatment with DDE to test the hypothesis that the beneficial effect of Hty on cell viability and morphology found in co-treated cells could be due to the activation of the antioxidant defense. To this end, SOD2 protein levels, total SOD activities, catalase and GPX1 activities were analysed.

6.7.1 Effect of DDE and Hydroxytyrosol on SOD2 protein levels and total SOD activities

Western blotting analysis evidenced that DDE alters protein levels in total cell homogenates. Indeed, both 30 and 100 μM induced a reduction of about 20% of protein levels compared to DMSO (figure 6.11). On the contrary, 50 and 100μM Hty did not change SOD2 content. Interestingly, combining 30 or 100 μM DDE with 50 or 100 μM Hty restored protein levels found decreased by DDE exposure (figure 6.11A).

Moreover, total SOD activity was also evaluated. Treatment with DDE at both 30 μM and 100 μM dose showed a no significant trend to decrease in total SODs enzymatical activity (figure 6.11B). In contrast, treatment with both 50 and 100 μM Hty induced a significant increase of about 1.5-fold vs. control cells. This trend was also observed in the co-treatment experiments, where the enzymatic activity was found to increase reaching up to 2-fold higher values in 30μM DDE + 100μM Hty vs. control cells.

6.7.2 Effect of DDE and Hydroxytyrosol on Catalase activity

Concerning catalase activity, DDE produced different effects depending on the dose. At 30μM, pesticide induced an increase in enzymatic activity of about 1.8-fold. No significative changes were detected with the 100 μM DDE dose.

Regarding Hty treatment, CAT activity was found to increase approximately 1.5-fold with both doses used (figure 6.11C). Interestingly, it was observed a Hty dose dependent decrease in CAT activity in 30 μM DDE + Hty cotreated cells compared to 30 μM DDE treated cells. No significant changes were found among 100μM DDE + Hty treated cells and 100μM DDE treated (figure 6.11C).

6.7.3 Effect of DDE and Hydroxytyrosol on total Glutathione peroxidase

Considering glutathione peroxidase activity, DDE produced different effects depending on the dose. An increase of about 30% was found in 30 μM DDE treated cells, whereas no change in GPX activity was found in 100 μM DDE treated cells compared to control cells (figure 6.11D).

As for Hty treatment, it has been found an increase of about 25% in both 50 and 100 μM Hty treated cells vs. control (figure 6.11D).

In cells cotreated with Hty and DDE, an increase of about 40% was observed in 30 μM DDE +Hty treated cells vs. control cells, in line with the increase of about 30% found in 30 μM DDE treated cells. A no significant tendence to increase (+25%) was also found in 100 μM DDE +Hty treated cells vs. control cells (figure 6.11D).

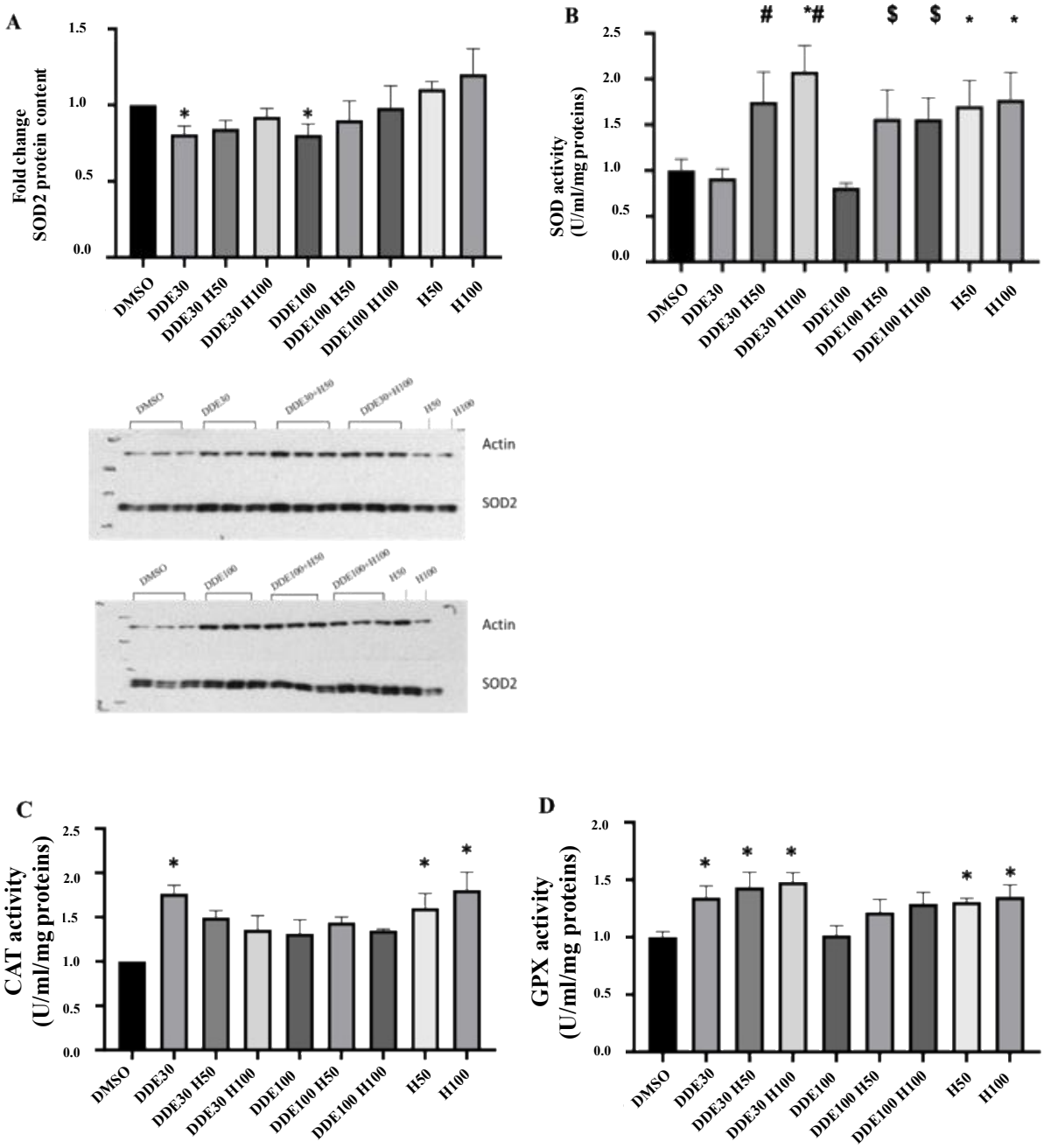

Figure 6.11 Activity and content of antioxidant enzymes. SOD2 protein levels (A); Total superoxide dismutase (SODs) activity (B); Catalase (CAT) antioxidant activities (C); Glutathione peroxidase (GPX) activity (D). Data are represented as media ± SEM of 3 independent experiment performed in biological replicates. Representative images of bands were shown for SOD2 and the loading control guide beta-Actin. Student's t-test analysis was applied. * $p<0.05$ vs. DMSO.

6.8 Beneficial antioxidant effect of hydroxytyrosol on hepatic cells exposed to DDE: discussion and conclusions

Exposure to environmental contaminants can produce liver damage by inducing oxidative stress and cell death progression. Thus, dietary antioxidant compounds may be useful for promoting liver cell health under conditions of exposure to environmental pollutants.

In the present PhD thesis, it was evaluated the effect of the environmental pollutant DDE, the main metabolite of the insecticide DDT, on cell viability, morphology, and oxidative stress in an *in vitro* model of hepatic cell culture (HepG2). The aim of the present experimental design was to evaluate the protective effects of Hty against DDE-induced oxidative damage in liver cells. Hty is considered one of the most powerful antioxidant compounds between phenolic compounds from olive tree.

In the first part of the experiments, cell viability, morphology and lipid peroxidation were evaluated in cells treated with increasing doses of DDE or Hty as well as in cells co-treated with both DDE and Hty. In the second part of the experiments, the main antioxidant enzymatic activities were analysed to evaluate the antioxidant properties of Hty, under the above-mentioned experimental conditions.

A dose-dependent effect of DDE on cell viability and morphology was found with a significant decrease in cell viability, as also reported in a previous work (Burgos-Aceves et al. 2021), associated with a progressive cell vacuolization. Furthermore, an increase in lipid peroxidation was also found with the highest dose of DDE used. No changes in lipid accumulation were evidenced. Therefore, DDE treatments induced hepatic cellular

damage and lipid peroxidation, without steatogenic effect. Analysis of the apoptosis marker caspase 3 was performed to evaluate the induction of apoptosis by DDE. Results showed a DDE dose-dependent stimulation of caspase 3 activities. The increase in caspase 3 activities was associated with a decrease in SOD2 protein levels and with a no significant tendency to decrease in total SOD activity. An increase in catalase and GPX1 activity were found mainly at the lowest dose of DDE (30 μM), but not with the highest doses. These increases in antioxidant system activities could be an adaptive response to avoid an excessive oxidative stress under conditions of exposure to low dose of the pesticide. On the other hand, the exposure to high DDE dose did not induce this adaptive response, and thus an increase in oxidative stress (lipid peroxidation) associated to a significative increase in apoptosis markers and cell viability was observed. Thus, it can be suggested that DDE-induced oxidative stress without adequate stimulation of antioxidant defense could play a key role in inducing cell damage and apoptosis.

The dietary antioxidant compounds Hty was used to test its proprieties in counteract oxidative stress induced by DDE.

Firstly, dose dependent effect of Hty was analysed to evaluate its effect on cell viability and morphology. Results showed a decrease in cell viability starting by the dose of 100μM of Hty, with no impairments in cell morphology and lipid peroxidation. These findings were not associated with changes in PARP cleavage and caspase 3 activity, suggesting that the Hty-induced reduction in cell viability could be due to its known anti-proliferative effect in tumor cells, rather than its toxicity. Our results confirmed the

antioxidant effect of Hty. Indeed, the activities of the antioxidant enzyme analyzed, namely SOD, catalase and GPX1, were increased in both 50 and 100 μM Hty treated cells.

Noteworthy, simultaneous treatment with Hty and DDE resulted in benefits for cells compared to cells treated with DDE alone. Indeed, Hty cotreatment reverted DDE-induced cell viability reduction, morphology alterations and caspase 3 activity induction. Moreover, Hty also decreased the stimulation of lipid peroxidation induced by the highest dose of DDE. These Hty effects were associated with the increase in both SOD2 protein content and total SOD activity found in DDE + Hty cotreated cells compared to DDE-treated cells. A similar trend was found in GPX activity.

The results of the present PhD thesis confirmed that environmental pollutants may cause Toxicant-associated steatohepatitis (TASH) by impairing cell viability through oxidative stress induction without adequate stimulation of antioxidant defense. Thus, the antioxidant effect of polyphenolic compounds, such as Hty, may be useful to counteract DDE induced cell impairment by improving antioxidant defense.

Further experiments are needed to better elucidate the cellular mechanism involved. However, the results of this PhD thesis have highlighted an important role of EVOO polyphenols in maintaining liver cell health under conditions of exposure to environmental pollutants and provide the basis to promote further mechanistic studies on this important research field.

CHAPTER 7

Conclusion

In conclusion, one of the main achievements of the present PhD thesis is to shed light on the mechanisms underlying DDE-induced damages in different metabolic tissues, especially BAT and liver, in an experimental animal model, focusing on the involvement of mitochondrial function and of oxidative stress. Indeed, DDE chronic exposure induced impairment in total BAT function by inducing a reduction in mitochondrial activity and thermogenesis, as shown by the decrease in COX activity and UCP1 content, and was due to a decrease in mitochondrial biogenesis, as shown by the decrease in PGC1alpha content in BAT homogenates. However, COX specific activity and UCP1 content were increased in isolated BAT mitochondria, suggesting an adaptive response to counteract the impaired mitochondrial biogenesis in total tissue. These findings were associated with an increase in oxidative stress with a stimulation of the mitochondrial SOD2 antioxidant activity. As for hepatic tissue, the increase in ROS levels and SOD2 antioxidant system was found in both total liver homogenates and isolated mitochondria associated with the decrease in homogenate COX activity and the increase in caspase 3 activity, respectively, in DDE-treated animals.

A further main result of the present PhD thesis concerns the protective effects of antioxidants compounds, such as Hty, on cellular health in a model of hepatic culture cells treated with DDE in vitro. A dose-dependent effect of DDE on cell viability and morphology was found associated with a significant progressive increase in lipid peroxidation and caspase 3 activities, as well as with a decrease in SOD2 levels. Noteworthy, simultaneous treatment with Hty and DDE resulted in benefits for cells compared to cells treated with DDE alone. Indeed, Hty cotreatment reverted DDE-

induced cell viability reduction, morphology alterations and caspase 3 activity induction. Hty also decreased the stimulation of lipid peroxidation induced by the highest dose of DDE, and induced an increase in both SOD2 protein content and total SOD activity.

Taken together, the results of this PhD thesis highlighted the role of mitochondrial function and oxidative stress in DDE tissue damage *in vivo*, and shed light on the key role that the EVOO polyphenol Hty may play in maintaining liver cell health under conditions of exposure to environmental pollutants in an *in vitro* experimental model. Further studies are needed to verify the protective effect of Hty on DDE induced tissue damage in the experimental animal model in vivo, and to shed further light on the cellular and molecular mechanisms involved in the protective effects.

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