Università degli Studi di Salerno

Dipartimento di Chimica e Biologia

Dottorato di Ricerca in Chimica

XXXIII-Cycle

Comparative studies on the physiological adaptation of mitochondria and endoplasmic reticulum to environmental xenobiotics from invertebrate to mammals

Tesi di dottorato in Chimica

Tutor

Prof. Lillà Lionetti

Ph.D Candidate

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Co-tutor:

Prof. Caterina Faggio (University of Messina)

Dr. Rosa María Morelos-Castro (CIBNOR-México)

PhD Coordinator

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“Estudiar no es un acto de consumir ideas,
sino de crearlas y recrearlas”

Paulo Freire

Fernando, Kaleb, Valentina

Studiare non è un atto di consumare idee,
ma per crearli e ricrearli

Paulo Freire

Abstract

Mitochondria are critical for numerous cellular and biochemical processes. The reciprocal interaction between mitochondria and the endoplasmic reticulum (EnR) impacts several cellular functions. In cellular and animal models, exposure to a series of pharmaceuticals or environmental pollutants has been shown to alter energy homeostasis resulting in a predisposition to common metabolism-related pathologies. Instead, it has been shown that mitochondria are exquisitely sensitive to environmental stress and act both as a target of stress and the coordinating center for the adaptive cellular response. The present PhD thesis's first aim was to evaluate the dose-dependence effect of the endocrine disruptor DDE, the primary metabolite of DDT, on viability and mitochondrial dynamics in human liver cells (HepG2) *in vitro*, ranging between 0.5 and 100 µM. Its toxic effects on cells could be associated with mitochondrial network impairment associated with an imbalance between mitochondrial fusion and fission processes. Mitochondrial fusion and fission processes are critical to maintain the mitochondrial network and allow the cell to respond to external stressors such as environmental pollutants. Fusion processes are associated with the optimization of mitochondrial function, whereas fission processes are associated with removing damaged mitochondria. Results showed that DDE induced a decrease in cell viability in a dose-dependent manner and enhanced its effects in coincubation conditions with dietary fatty acids. The fusion protein markers Mitofusin 2 (MFN2) and Optical Atrophy 1 (OPA1) exhibited an inverted U-shape dose-response curve, showing the highest content in the 2.5-25 µM DDE dose range. On the other hand, the fission protein marker dynamin-related protein1 (DRP1) was found significantly increased, leading to an increased fission/fusion ratio, with high DDE doses. A similar trend has been observed for glucose-regulated protein 75 (GRP75), a

chaperon involved in mitochondria-endoplasmic reticulum interaction. Our results suggested that low DDE doses elicited cell adaption stimulating mitochondrial dynamics machinery to counteract the DDE effect. In contrast, high DDE doses induced cell viability loss associated with mitochondrial dynamics shifts toward fission. The PhD project's second aim was to investigate the potential effect of the pesticides endosulfan and carbofuran on the Mexican marine species Catarina scallop. We analyzed the up-and-down regulation of genes associated with mitochondrial dynamic and endoplasmic reticulum stress. Endosulfan and carbofuran are commonly used in Mexican agricultural activities with a known endocrine-disruptive action but with different persistence period. There are reports that both pesticides negatively affect mitochondrial metabolism in mammals, fish, and invertebrates. Catarina scallop adults were exposed to a non-lethal experimental single dose (0.45 μg L^{-1}) for each pesticide, and RT-PCR analyzed the expression of the mitochondrial-associated gene DRP1 and the endoplasmic reticulum-associated gene endoplasmic reticulum interferon stimulator (ERIS). Results showed that endosulfan and carbofuran could upregulate the expression of DRP1 mainly in the gonad. We can infer that they could cause a potential alteration in the reproductive process due to their endocrine disruptive action, encouraging cells to apoptosis. Up-regulation of the ERIS gene was observed in the gonad of scallops exposed to endosulfan and carbofuran. ERIS seems to play a critical role in the activation and regulation of detoxification and immune responses by regulating several cytokines/chemokines synthesis. ERIS overexpression has been associated with type I IFN up-regulation, which triggers some ERIS-dependent inflammatory processes. According to some reports, this can lead to a degree of toxicity that can be fatal in aquatic animals. Therefore, this second aim could provide us with a preliminary basis for studying marine bivalves response to short- and long-term pesticide exposure in

terms of regulated gene expression and characterizes new potential genetic markers of environmental contamination. In conclusion, results of the present PhD thesis suggested that in the research field on environmental toxicology and environmental health, it could be essential to further investigate the role of mitochondria and mitochondria-Endoplasmic reticulum interaction both in humans and wildlife to shed light on the cellular mechanisms involved in adaptive response and/or toxicity of the environmental pollutants. Moreover, present results are helpful to clarify the mechanisms underlying the cell fate towards survival or death in response to increasing doses of environmental pollutants.

Riassunto

I mitocondri sono fondamentali per numerosi processi cellulari e biochimici. L'interazione reciproca tra i mitocondri e il reticolo endoplasmatico influisce su diverse funzioni cellulari. In modelli cellulari e animali, l'esposizione a una serie di farmaci o inquinanti ambientali ha dimostrato di alterare l'omeostasi energetica con conseguente predisposizione a patologie legate al metabolismo. Infatti, è stato dimostrato che i mitocondri sono molto sensibili allo stress ambientale e agiscono sia come bersaglio dello stress che come centro di coordinamento per la risposta cellulare adattativa. Il primo scopo della presente tesi di dottorato è stato quello di valutare l'effetto dose-dipendente del distruttore endocrino DDE, il metabolita primario del DDT, sulla vitalità e sulla dinamica mitocondriale nelle cellule epatiche umane (HepG2) *in vitro*, utilizzando un vasto range di dosi di DDE comprese tra 0,5 e 100 µM. I suoi effetti tossici sulle cellule potrebbero essere collegati alla compromissione della rete mitocondriale associata a uno squilibrio tra processi di fusione e fissione mitocondriale. I processi di fusione e fissione mitocondriale sono fondamentali per mantenere la rete mitocondriale e consentire alla cellula di rispondere a fattori di stress esterni come gli inquinanti ambientali. I processi di fusione sono associati all'ottimizzazione della funzione mitocondriale, mentre i processi di fissione sono associati alla rimozione dei mitocondri danneggiati. I risultati hanno mostrato che il DDE induce una diminuzione della vitalità cellulare in modo dose-dipendente e ne potenzia gli effetti in condizioni di co-incubazione con gli acidi grassi tipicamente presenti negli alimenti. Le proteine marker dei processi di fusione, Mitofusin 2 (MFN2) and Optical Atrophy 1 (OPA1) hanno mostrato una curva dose-risposta a forma di U-invertita, raggiungendo il massimo incremento nell'intervallo di dose DDE 2,5-25 µM. D'altra parte, la proteina correlata alla fissione mitocondriale (DRP1) aumenta anche a dosi elevate di DDE

inducendo un aumento del rapporto fissione/fusione a tali dosi. Una tendenza simile è stata osservata per la proteina 75 regolata dal glucosio (GRP75), uno chaperone coinvolto nell'interazione mitocondri-reticolo endoplasmatico. I risultati di questa tesi suggeriscono che basse dosi di DDE inducono l'adattamento cellulare stimolando il meccanismo della dinamica mitocondriale per contrastare l'effetto del DDE. Al contrario, alte dosi di DDE inducono la perdita di vitalità cellulare associata a dinamiche mitocondriali che si spostano verso la fissione. Il secondo scopo del progetto di dottorato è stato quello di studiare il potenziale effetto dei pesticidi endosulfano e carbofurano sulla specie marina messicana Catarina scallop, analizzando la up e down regulation dei geni associati alla dinamica mitocondriale e allo stress del reticolo endoplasmatico. Endosulfano e carbofurano sono comunemente usati nelle attività agricole del Messico con una nota azione di disturbo endocrino ma con periodi di persistenza differenti. Ci sono rapporti secondo cui entrambi i pesticidi influenzano negativamente il metabolismo mitocondriale nei mammiferi, nei pesci e negli invertebrati. Gli adulti di Catarina scallop sono stati esposti a una singola dose sperimentale non letale (0,45 $\mu g\ L^{-1}$) di ciascun pesticida e sono state effettuate RT-PCR per analizzare l'espressione del gene associato ai mitocondri DRP1 e dello stimolatore dell'interferone del reticolo endoplasmatico associato al gene endoplasmatico (ERIS). I risultati hanno mostrato che endosulfano e carbofurano potrebbero sovra-regolare l'espressione di DRP1 principalmente nella gonade. Possiamo dedurre che potrebbero causare una potenziale alterazione del processo riproduttivo a causa della loro azione di distruttore endocrino, stimolando l'apoptosi cellulare. La sovra-regolazione del gene ERIS è stata osservata nella gonade e nel mantello delle capesante esposte all'endosulfano. L'ERIS sembra giocare un ruolo critico nell'attivazione e regolazione dei processi di detossificazione e delle risposte immunitarie regolando la sintesi di diverse

citochine/chemochine. La sovra-espressione di ERIS è stata associata all'aumento dell'IFN di tipo I, che innesca alcuni processi infiammatori dipendenti da ERIS. Secondo alcuni studi, ciò può portare a un grado di tossicità che può essere fatale per gli animali acquatici. Pertanto, questo secondo scopo della tesi potrebbe fornire una base preliminare per studiare la risposta dei bivalvi marini all'esposizione a breve e lungo termine ai pesticidi in termini di regolazione dell'espressione genica e potrebbe portare alla caratterizzazione di nuovi potenziali marcatori genetici di contaminazione ambientale. In conclusione, i risultati del presente tesi di dottorato di ricerca suggeriscono che nel campo della ricerca sulla tossicologia e salute ambientale, potrebbe essere essenziale indagare ulteriormente il ruolo dei mitocondri e dell'interazione mitocondri-reticolo endoplasmatico sia nell'uomo che nella fauna selvatica per fare luce sui meccanismi cellulari coinvolti nella risposta adattativa e/o tossicità degli inquinanti ambientali. Inoltre, i risultati attuali sono utili per chiarire i meccanismi che sono alla base del destino cellulare in termini di sopravvivenza o morte cellulari in risposta a dosi crescenti di inquinanti ambientali.

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Abbreviations

AR - Androgen receptor

ATP - Adenosine triphosphate

ATP1A1 - Sodium/potassium-transporting ATPase subunit alpha-like

BAT - Brown adipose tissue

BiP, GRP-78 - Binding immunoglobulin protein

CHOP - CCAAT-enhancer-binding protein homologous protein

CYP450 - Cytochrome 450

cyt c - Cytochrome c

DDD - Dichlorodiphenildichloroetane

DDT - Dichlorodiphenyltrichoroethane

DMSO - Dimethylsulfoxide

DRP1- Dynamin-related protein 1

EDCs - Endocrine disrupting chemicals

EnR - Endoplasmic reticulum

ER - Estrogen receptor

ERIS - Endoplasmic reticulum interferon stimulator

ETC - Electron transport chain

Fis1 - Fission protein 1

GPx - Glutathione peroxidase

GSH - Glutathione

H_2O_2 - Hydrogen peroxide

HSPs - Heat shock proteins

IMM - Inner mitochondrial membrane

IRS - Indoor residual spraying

Keap1- Protein 1

MAMs - Mitochondrial-associated membranes

MDA - Malondialdheyde

MDCs - Metabolism-disrupting chemicals

Mfn1- Mitofusins 1

Mfn2 - Mitofusins 2

miRNAs - microRNAs

mRNA - Messenger RNA

mtDNA- Mitochondrial DNA

MTT - 3-(4,5-dime-thylthiazol-2-yl)-2,5-diphenylte-trazolium

NAC - N-acetyl-L-cysteine

ncRNAs - Noncoding RNAs

NO - Nitric oxide

NRF2 - Nuclear factor erythroid 2-related factor 2

OCPs - Organochlorine pesticides

PCBs - Polychlorinated biphenyl compounds

POPs - Persistent organic pollutants

PRKACA - cAMP-dependent protein kinase catalytic subunit-like isoform X3

PUFAs - Polyunsaturated fatty acids

RBP - Retinol-binding protein

ROS - Reactive oxygen species

SDS-PAGE - SDS-polyacrylamide electrophoretic gel

SODs - Superoxide dismutases

StAR - Steroidogenic acute regulatory

T_3 - Triiodothyronine

T_4 - Thyroxine

TSHr - TSH receptor

T-TBS - Tween-TBS

VDBP - Vitamin D-binding protein

WAT - White adipose tissue

WHO - World Heart Organization

$\Delta\Psi m$ - Mitochondrial potential

γ-GCS - γ-glutamyl-cysteine synthetase

INDEX

1. Introduction

The mitochondria generate most of the cell energy supply by producing adenosine triphosphate (ATP). Likewise, mitochondrial also have reactive oxygen (ROS) and nitrogen species (RNS) (Heise et al., 2003; Donaghy et al., 2012; Putti et al., 2015a; Rivera-Ingraham et al., 2016). This eukaryotic organelle morphology consists of outer and inner membranes conformed of phospholipid bilayers and proteins.

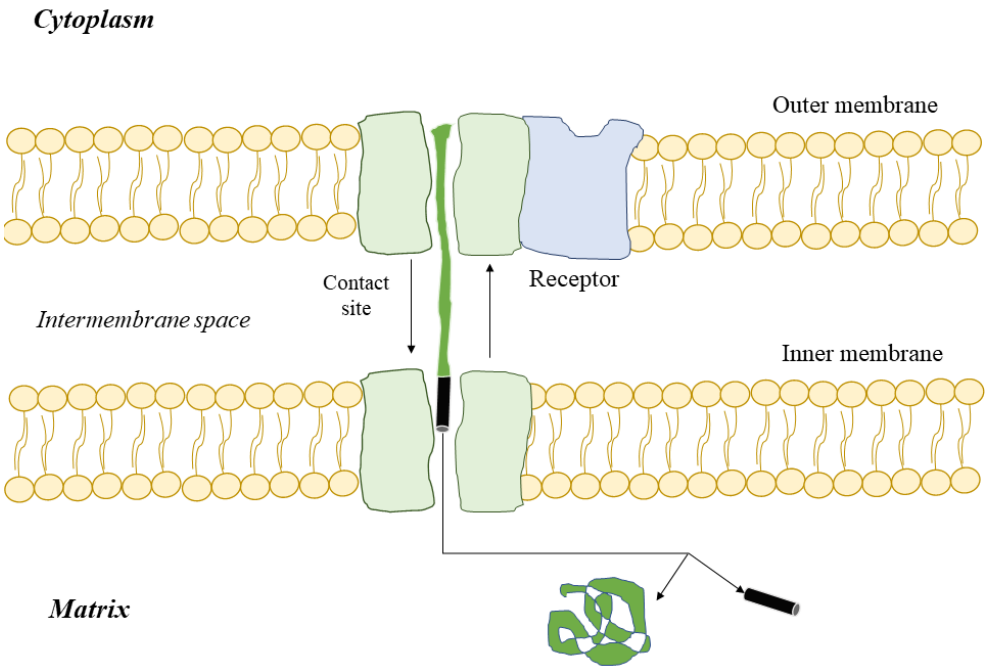

Figure 1.1. The traffic of a protein between the outer and inner mitochondrial membranes. When at this contact site, the receptor protein hands off the tethered protein to the translocator protein, channels the newly synthesized, unfolded proteins from the cytoplasm into the matrix, where folding ensues.

Through the many protein-based pores (porins) of the outer membrane, ions and uncharged molecules trafficking occur (Figure 1.1). In the inner membrane, where oxidative phosphorylation occurs, there are protein complexes involved in electron transport across the inner membrane and ATP synthesis (Figure 1.2). Due to a basic pH, it is within the matrix

that a transmembrane electrochemical gradient is created, driving the synthesis of ATP (Bayrhuber et al., 2008; Kühlbrandt, 2015).

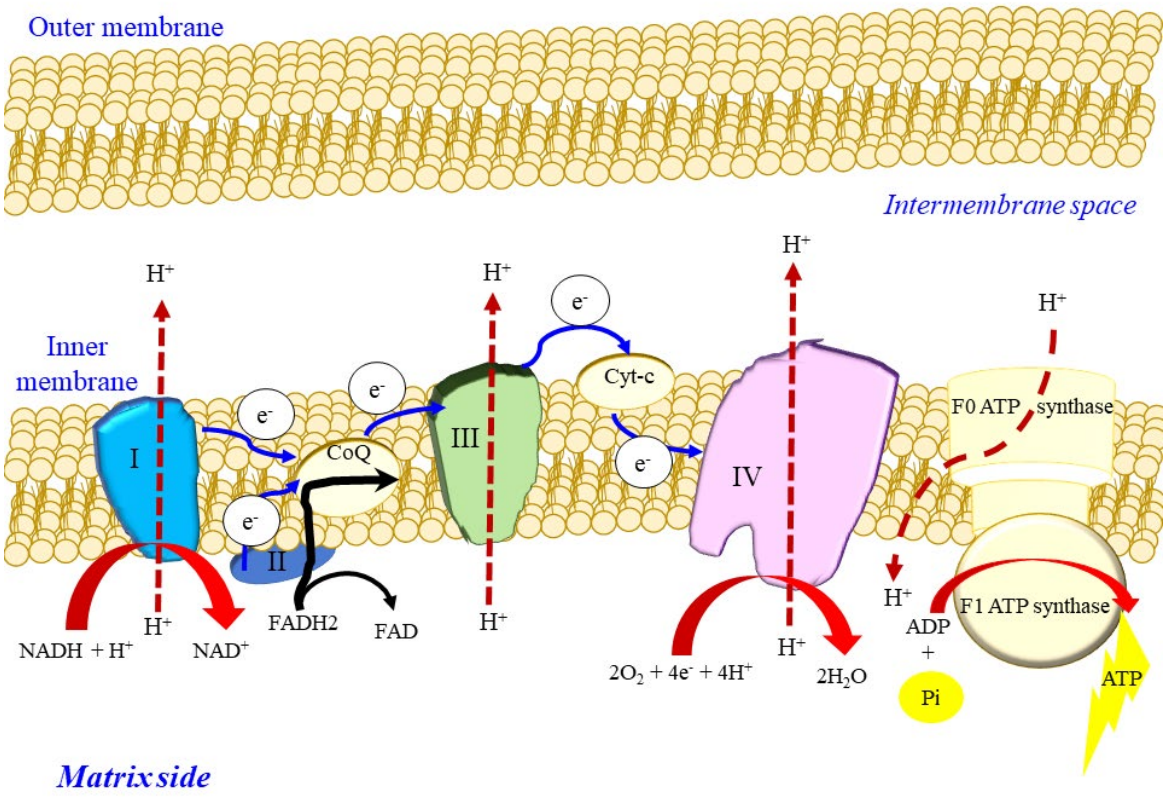

Figure 1.2. The movement of protons through the inner mitochondrial membrane leads to ATP production. The transfer of electrons through the electron transport chain releases energy through a series of oxidation-reduction reactions resulting in ATP production. The overall process is arbitrarily divided into two phases: Electron transport chain (first coupling) and Chemiosmosis (second coupling), in which proton movement is coupled with ATP synthesis by FoF1-ATP synthase. I: NADH reductase; II: Succinate dehydrogenase; III: Cytochrome reductase; IV: Cytochrome oxidase.

The mitochondrial matrix contains organellar DNA replication, transcription, protein biosynthesis, and numerous enzymatic reactions. Mitochondrial genomes are minimal (11 and 28 kbp), and at least 37 genes are contained in the human mitochondrial genome, 13 of which produce various components of the electron transport chain (ETC) (Patananan et al., 2016). Mitochondrial functions are regulated by a complex protein encoded from both the nuclear and mitochondrial genome. Emerging evidence suggests that microRNAs (miRNAs, miRs) can control the metabolism-related genes in both physiological and pathological conditions

(Burgos-Aceves et al., 2018; Abo-Al-Ela and Faggio, 2021) through the prevention of the translation of messenger RNA (mRNA) or inducing the degradation of mRNA transcripts. The miRNAs are found primarily in the cytosol or nucleus, but a subset of ~ 150 different miRNAs (mitomiRs) have also localized in mitochondrial fractions of cells and tissues (Geiger and Dalgaard, 2017). Consequently, xenobiotic alteration could result in mitochondrial dysfunction, ROS/RNS overexpression, and liberation of apoptosis or necrosis activating proteins. Thus, there is an emerging interest in the identification and putative role of these small endogenous non-coding RNA segments in mitochondrial homeostasis (Burgos-Aceves et al., 2018).

These dynamic energy-supplying organelles are necessary to direct critical cellular processes and maintain cellular homeostasis. Mitochondria make functionally important contacts with most of the other organelles in the cell (Lackner, 2019). The physical/functional mitochondria-endoplasmic reticulum (EnR) tethering is one of the most widely studied and best-characterized connections (Marchi et al., 2014, 2017). Each organelle has its functions, and the intimate communication between them via physical contact is through mitochondrial-associated membranes (MAMs) provides another level of regulation in energy production, lipid processing, and calcium (Ca^{2+}) buffering (Theurey and Rieusset, 2017; Rieusset, 2018; Lee and Min, 2018). Besides, a series of proteins that reside within the MAMs, involved in relevant biological functions such as mitochondrial dynamics, Ca^{2+} transfer, autophagy, inflammation, unfolded protein response, cell death, among others, have been identified (Marchi et al., 2014, 2017, 2018; Filadi et al., 2018). Recent data suggest that MAMs complex provides a platform for nutrient and hormone signalling pathways and immune responses, regulating mitochondrial bioenergetics and apoptosis (Theurey and Rieusset, 2017; Rieusset,

2018; Lee and Min, 2018). Mitochondrial dynamics is a direct signal of mitochondrial-EnR tethering, and the first evidence of this was observed through the division process. During the mitochondrial fission process, the mitochondria appear to be constricted mainly at points of contact with the EnR, suggesting a crucial role for the mitochondria-EnR association in the initiation of mitochondrial fission (Marchi et al., 2014). The fission process is regulated by dynamin-related protein 1 (DRP1) and requires coordination between the recruitment of cytosolic DRP1 and its translocation to the OMM; after activation via phosphorylation, DRP1 binds to adaptor proteins (e.g., MFF, MiD49, MiD51, and FIS1). The GTPase domain of DRP1 moves away from the OMM. It connects with other DRP1 proteins forming a spiral around the mitochondria, which with the hydrolysis of GTP causes the helix's constriction and the excision of both membranes (Friedman et al., 2011; Friedman and Nunnari, 2014; Trotta and Chipuk, 2017). Thus, mitochondria-EnR contact impacts mechanisms that influence both OMM and inner mitochondrial membrane (IMM) during division (Lackner, 2019). Studies found that mitochondria-EnR contacts and mitochondrial dynamics go beyond only division. Mitochondria fuse in a process that requires the optical atrophy 1 (OPA1) GTPase present in IMM and the mitofusins1/2 (Mfn1 and Mfn2) present in OMM (Cipolat et al., 2004; Van der Bliek et al., 2014). During the mitochondrial fusion process, Mfn1 and Mfn2 assemble homo- or heterodimeric complexes with Mfn2 present in the ER membrane, within a MAM complex (de Brito and Scorrano, 2008). The fusion can only occur when mitofusins are present on opposing mitochondria, implying the formation of trans complexes during mitochondrial tethering (Koshiba et al., 2004). This fusion process is crucial for mitochondrial health, and blocking this process results in a lack of membrane potential and, ultimately, impaired ATP production. It is also vital for the successful transfer of mitochondrial proteins and

mitochondrial DNA (mtDNA) to newly synthesized mitochondria, preventing the accumulation of mtDNA mutations and endorsing normal mitochondrial function (Twig and Shirihai, 2011; Farmer et al., 2018). Then, Mfn2 is the protein that has been suggested as a direct mediator of mitochondria-EnR tethering (de Brito and Scorrano, 2008; Filadi et al., 2017, 2018). Then mitochondria-EnR contacts are closely linked to the mitochondrial network's fission and fusion dynamics (Lackner, 2019). Mitochondria-EnR coupling plays a central role in various cellular pathways, and miscommunication could involve some diseases, such as cancer, neurodegenerative, obesity, and metabolic diseases (Filadi et al., 2017, 2018).

Considering these organelles' complex and essential functions, maintaining a healthy mitochondria population is crucial for the organism's survival and fitness (Jayasundara, 2017). The growing recognition of mitochondrial aetiology in various human health disorders has led to the rise of studies on mitochondrial toxicants or mitotoxicants. However, the risk factors for mitochondrial dysfunction are not fully known. Mitochondria are vulnerable to certain anthropogenic chemicals due to the high membranous lipid content that facilitates the accumulation of lipophilic compounds and the negative charge of the matrix that promotes the assembly of cationic metals (Meyer et al., 2013). Several studies demonstrated that exposure to environmental chemicals could induce mitochondrial dysfunction *in vitro* and *in vivo* settings (Jayasundara, 2017). The most well-known and harmful synthetic molecules introduced in the environment are the persistent organic pollutants (POPs) pesticides. These anthropogenic chemicals toxicity also depends on their ability to persist in the environment, their degree of bioaccumulation, and biomagnification in the food chain (Roubicek and de Souza-Pinto, 2017). Evidence suggests that these chemicals can have the faculty to affect mitochondrial membrane potential, mtDNA copy number, mitochondrial mass, mtDNA

mutations, and ATP content (Lin et al., 2013; Kaur et al., 2014; Roubicek and de Souza-Pinto, 2017). Alteration of energy homeostasis is one of the leading direct consequences of mitochondrial dysfunction caused by mitotoxicants (Sokolova et al., 2011; Sokolova, 2018), followed by an overproduction of ROS eliciting oxidative stress response and oxidative damage, including apoptosis and cell death response (Jayasundara, 2017). Therefore, an increment in mitochondria-generated ROS production by pollutant exposure may involve the release of cytochrome c (cyt c) and other pro-apoptotic proteins (Ott et al., 2007), which can be the cause of the development of immune diseases (Burgos-Aceves et al., 2016, 2021). Evidence suggests that immune system cells are the main targets of environmental pollutants, mainly the endocrine-disrupting chemicals (EDCs). These EDCs have an apoptosis-inducing action on immune cells and erythrocytes. They could alter the transmembrane mitochondrial potential ($\Delta\Psi m$) and activate the caspase-dependent mitochondrial apoptotic pathway, leading to decreased immune cells viability and, consequently, immunosuppression. Recent studies suggest that erythrocytes also play an equivalent role in immune responses as leukocytes in organisms. These blood cells also possess complete cellular machinery with functional ribosomes and mitochondria, making them targets for EDCs. Therefore, one of the main effects of EDCs on erythrocyte cells is associated with a drop in $\Delta\Psi m$, the release of pro-apoptotic proteins. Consequently, mitochondrial pathways can trigger apoptosis, with impacts not only on immune responses but also on the alteration of haematological parameters that lead to physiological dysfunctions. Erythrocytes are often used to assess xenobiotic-induced damage in different cell compartments. Mitochondrial activities appear to be a suitable material within stress-associated haematological assessments (Burgos-Aceves et al., 2019).

The potential influence of environmental pollutants on energetic homeostasis and obesity development has drawn increased attention during recent years. The current literature supports the hypothesis that some environmental POPs, acting as endocrine disruptors, are associated with increased obesity risk. Exposure to EDCs has been associated with obesity and metabolic disorders with a deterioration of brown adipose tissue (BAT) mass and function. The BAT is involved in thermogenesis, increasing energy expenditure, and avoiding excessive lipid accumulation in white adipose tissue (WAT), counteracting obesity metabolic disease development. It has been reported that a perinatal exposure to dichlorodiphenyltrichloroethane (DDT), a well-known EDCs, can impaired BAT thermogenesis and the use of the substrate, with an increased risk of susceptibility to metabolic syndrome. Likewise, some air pollutants have been shown to induce insulin resistance associated with BAT mitochondrial dysfunction (Di Gregorio et al., 2019). Then, mitochondria play a crucial role in BAT and browned WAT thermogenesis. Studies on mitochondrial bioenergetics are confirmed to be of great importance in understanding the etiopathogenesis of diet-induced (Putti et al., 2015a,b; Lepretti et al., 2018) and toxicant-induced (Llobet et al., 2015; Burgos-Aceves et al., 2018) obesity and in discovering new therapy for obesity-related diseases (Di Gregorio et al., 2019).

Insulin resistance is a metabolic condition associated with the risk of cardiovascular diseases, obesity, or diabetes, where mitochondrial and EnR seem to play a crucial role (Martin and McGee, 2014). Data indicate the mechanisms causing insulin resistance initiation are multifactorial and complex. They could produce directly or indirectly an increased metabolic inflammation associated with EnR stress, mitochondrial dysfunction (Rheinheimer et al., 2017), fatty acid oxidation, and increased lipolysis (Guilherme et al., 2008). Exposure

to POPs, including DDT, seems to be one factor causing insulin resistance, as observed in an *in vitro* assay with a rat myoblast (L6) cell line. The L6 cells treated for 18 h with 30 mg L^{-1} and 60 mg L^{-1} of DDT showed a dose-dependent reduction of antioxidant proteins and insulin-stimulated glucose uptake, thus targeting insulin resistance in muscle cells (Singh et al., 2019). While, rat pancreatic beta-cells (INS1E) exposed to non-lethal concentrations of p,p′-DDT, and p,p′-DDE (10 μM) for one month expressed a reduction in the intracellular level of insulin mRNA, proinsulin, and insulin monomer associated to overexpression of vitamin D-binding protein (VDBP). This protein serves as a transporter for vitamin D in the blood (Pavlíková et al., 2019). The variation in the VDBP gene expression has been associated with several types of diabetes (Blanton et al., 2011; Wang et al., 2014; Wang et al., 2015; Yu et al., 2018). Moreover, in an *in vivo* assay, female BALBc mice exposed to 50 mg Kg^{-1} and 100 mg Kg^{-1} p,p′-DDT also showed dose-dependent oxidative stress, with changes in the expression of some genes associated with insulin resistance pathways (Arroyo-Salgado et al., 2016). Emerging evidence indicates that the nutritional status can influence the toxicity of DDT, which can affect the insulin signalling pathway (Zhang et al., 2020). Therefore, nutrients appear to be another of these factors associated with the induction of insulin resistance and consequently in the pathogenesis and progression of many chronic diseases (Weickert, 2012; Ekpenyong, 2018; Nunes Couto et al., 2019), where a diet rich in saturated fat can be associated with insulin resistance onset. Then, in overnutrition conditions, the mitochondrial and EnR can experience stress, activating the unfolded protein response, and in turn, the principal inflammatory pathways are triggered. Such fat-induced insulin resistance could be counteracted by a diet rich in omega 3 polyunsaturated fatty acids (PUFAs) by modulating mitochondrial bioenergetics and EnR stress. Thus, Omega 3 PUFA stimulates mitochondrial

function and fusion processes reducing ROS production, ameliorating mitochondrial function, and maintaining MAM integrity necessary for insulin sensitivity (Lepretti et al., 2018).

Mitochondria are on the front lines in supplying energy to cellular mechanisms involved in defence against xenobiotics, such as chemicals, pharmaceutical molecules, and environmental pollutants. For this reason, they are considered the main target of numerous drugs and environmental toxins, so it is essential to develop new research to evaluate whether these endpoints provide valuable tools to indicate stress due to contamination or drug use. Therefore, mitochondria are good predictors of acute toxicity. The evaluation of mitochondrial bioenergetics could be used as a toxicological indicator as a first-line economic model to detect xenobiotic possible effects (Mota et al., 2011; Pshenichnyuk and Modelli, 2013; Yeh et al., 2017).

2. Objective

Conduct a comparative analysis of mitochondrial dynamic behavior adaptation and endoplasmic reticulum stress induction in response to the presence of a xenobiotic in mammal and non-mammal experimental models.

3. Review

Persistent environmental pollutants impact on cellular stress and mitochondrial function: focus on 1,1-Dichloro-2,2-bis(p, p'-chlorophenyl) ethylene (DDE)

3.1 Abstract

Cells are involved in various biological functions, including detoxification of xenobiotics. As the "powerhouse of the cell," mitochondria provides the energy supply to defend against them. Several xenobiotics, such as environmental pollutants, act as an endocrine disruptor and may cause mitochondrial toxicity and cellular stress, but their action mechanisms are not well clarified. In the present review, we summarized current knowledge concerning the effects of the environmental pollutant dichlorodiphenyldichloroethylene (DDE), a persistent metabolite of dichlorodiphenyltrichloroethane (DDT), on cellular stress and mitochondrial function. We chose to focus on DDE given that its effects on cells and mitochondria have been recently analyzed in several research works. In the first part of the review, we introduced DDE characteristics as a predominant organochlorine pesticide, its action as an endocrine disruptor, and obesogenic agent. Then, we addressed DDE effects on mitochondrial function and oxidative stress involved in the onset of metabolic disorders induced by this environmental pollutant. The increased incidence of diseases and disorders of the thyroid, immune, and metabolic system suggests that EDCs, including DDT or its metabolites, could affect these systems through nuclear receptors and have led to further research in these areas. We will discuss antioxidant utilization for treating pollutant toxicity in the last part. Understanding DDE action mechanisms on cellular physiology could help find the cellular target for preventing and metabolic therapy disorders induced by DDE and other environmental

pollutants. This review provides an overview of how exposure to DDT/DDE may interfere in human and wildlife health with an estrogen-disrupting action. We will discuss the crucial role of mitochondria in DDT/DDE impact on health.

3.2 Introduction

As the "powerhouse of the cell," Mitochondria play a key role in cellular homeostasis and adaptive response to environmental stimuli by providing energy to cellular metabolism. They also provide energy to cellular mechanisms involved in the defence against xenobiotics, such as chemicals, pharmaceutical molecules, and environmental pollutants. Many of these xenobiotics activate mitochondrial toxicity pathways. Dykens and co-workers showed that 35% of pharmaceutically relevant molecules tested were mitotoxic (Dykens et al., 2007, 2008; Dykens and Will, 2007; Chromcova et al., 2015; Plhalova et al., 2018; Fiorino et al., 2018; Burgos-Aceves et al., 2018a). Moreover, several recent works focused on the evidence that many pollutants affect mitochondria, even if they are not the only subcellular targets (Kovacic et al., 2005; Shaughnessy et al., 2010; Moreira et al., 2011; Meyer et al., 2013, 2017; Bestman et al., 2015; Roubicek and de Souza-Pinto, 2017; Weinhouse, 2017). Meyer and Chan underlined that pharmaceuticals are extensively texted for toxicity, whereas the tens of thousands of pollutant chemicals to which individuals in the modern society may be exposed are much less well-tested for toxicity, including mitochondrial effects (Meyer and Chan, 2017).

Over the last decades, vast ubiquitous man-made compounds have been linked to hormone-related alterations. Most of them classified as endocrine-disrupting chemicals (EDCs) with adverse effects on the physiology of organisms (Burgos-Aceves et al., 2016;

Chromcova et al., 2015; Plhalova et al., 2018; Blahova et al., 2020; Pagano et al., 2020; Petrovici et al., 2020; Stara et al., 2019, 2020). The EDCs include pesticides and herbicides (such as dichlorodiphenyltrichloroethane, DDT, or its metabolites), plastic contaminants (e.g., bisphenol A), pharmaceuticals (i.e., diethylstilbestrol; 17alpha-ethinylestradiol), or dietary components (such as phytoestrogens) (McKinlay et al., 2008; Alimba and Faggio, 2019; Stara et al., 2019). They produce their effects by mimicking, antagonizing, or altering endogenous steroid levels (androgens or estrogens) (Jenssen, 2006; Diamanti-Kandarakis et al., 2009). Exposure to EDCs is more dangerous if it occurs during specific "critical periods" of life (intrauterine, perinatal, juvenile, or puberty periods) when organisms are more sensitive to hormonal disruption, but they can also alter physiology in adulthood too (Frye et al., 2012). EDCs can act as "obesogens" and increase the risk of developing metabolic disorders such as insulin resistance and diabetes (Newbold et al., 2009; Newbold, 2010; Janesick and Blumberg, 2011; Schaefer et al., 2013; Annamalai and Namasivayam, 2015; Marroqui et al., 2018). Noteworthy, mitochondrial dysfunction has been suggested to play a crucial role in both obesity and associated metabolic disorders (Gastaldi et al., 2008; Bournat and Brown, 2010; Patti and Corvera, 2010; Mitchell and Darley-Usmar, 2012; Mabalirajan and Ghosh, 2013; McInnes, 2013; Montgomery and Turner, 2015; Jha et al., 2017; Rovira-Llopis et al., 2017; de Mello et al., 2018). Therefore, the specific effect of EDCs on mitochondrial function may be involved in the etiopathogenesis of these metabolic diseases. The researches focused on clarifying the impact of EDCs on mitochondrial function may help understand the aetiology of obesity and related metabolic disorders.

DDT is a potent organochlorine insecticide initially used to combat malaria, typhus, and other insect-borne human diseases. Afterward, it has been used as an agricultural and

household pesticide and even as antifouling paint (Xin et al., 2011). DDT is one of twelve chemicals identified as a persistent organic pollutant (POP), remaining in the soil 10-15 years after application with a wide-reaching dispersion facility (Stockholm convention secretariat). DDT has been banned since the 1970s worldwide, essentially for ecological reasons. Therefore, its only remaining legal use is in indoor residual spraying (IRS) with disease vector control purposes (Table 3.1) for those countries registered in the Stockholm convention (WHO, 2018).

Table 3.1 Courtiers with current production and/or use of DDT. Currently Indoor Residual Spraying (IRS) application.

Country	Production	IRS	Observations	Reference
Botswana	No	Yes	The Ministry of Health has not banned the use of DDT for public health purposes	WHO, 2018
Eritrea	No	Yes	From 2010	Stockholm Convention
Eswatini	No	Yes	Last reporting period 2009-2011	WHO, 2018; UNEP, 2019
Ethiopia	Until 2009	Yes	Production record in 2006	UNEP, 2017
Gambia	No	Yes	Last reporting period 2011-2012	WHO, 2018
Madagascar	No	Yes	Reused from 2009	Stockholm Convention
Mauritius	No	Yes	DDT is being sprayed as a residual insecticide at the port and airport. Stocks of DDT product	UNEP, 2019
Morocco	No	No	Use in limited to areas	Stockholm Convention
Mozambique	No	Yes	Planned as from 2005 up to 2008. Used only for malaria prevention	WHO, 2018
Namibia	Until 2009	Yes	Last reporting period 2012-2013	WHO, 2018
Senegal	No	Yes	Used in case of persistent paludism	Stockholm Convention
South Africa	No	Yes	From 2004. Stocks of DDT product	WHO, 2018; UNEP, 2019
Swaziland	No	Yes	Use restricted	Stockholm Convention
Uganda	No	Yes	Reintroduction and use of DDT in 2008	Stockholm Convention
Zambia	No	Yes	Restricted use until an effective and affordable alternative insecticide	WHO, 2018; UNEP, 2019
Zimbabwe	No	Yes	Reintroduction and use of DDT in 2018. Stocks of DDT product	WHO, 2018
China	Until 2014	No	Only in case of malaria's emergency	Stockholm Convention
India	Present	Yes	DDT manufactured by Hindustan Insecticides Limited-HIL. Stocks of DDT product	UNEP, 2019
Myanmar	No	No	Recent use of alternative insecticides	Stockholm Convention
Yemen	No	Yes	Efforts to replace DDT with other safe chemicals	Stockholm Convention
Venezuela	No	Yes	Only in case of malaria's emergency	Stockholm Convention

The toxic effect of DDT and its principal metabolite dichlorodiphenyldichloroethylene (DDE) on wildlife was corroborated by pointing out a several bird populations decline because of eggshell thinning over the past century (Nakamaru et al., 2003). Simultaneously, possible adverse effects of DDT/DDE in human health are less clear-cut. While short-term acute effects of DDT/DDE on humans are limited or unknown, long-term exposures have been associated with chronic health effects (Stockholm convention secretariat). Findings evidence DDT/DDE may increase the risk of developing cancer in the breast (Soto and Sonnenschein, 2015; Parada et al., 2019) as well as in other organs (Song et al., 2014a, b). There is even cumulative evidence suggesting that DDT/DDE exposure has long-term adverse effects on development (Strong et al., 2015), bone mineral density (Beard, 2006), nervous system (Eskenazi et al., 2009), immune system (Vine et al., 2001; Daniel et al., 2002), and metabolism (Heindel et al., 2017; Morales-Prieto and Abril, 2017).

Noteworthy, it has been suggested that DDE act as an endocrine disruptor in different animal species (WHO/UNEP, 2013; Monteiro et al., 2015), acting as a potent androgen receptor (AR) antagonist (Xu et al., 2006), an activator of androgen-sensitive cells proliferation (Tessier et al., 2001), stimulator of estrogen receptor (ER) production and, ER agonist and progesterone receptor (PR) antagonist (Tapiero et al., 2002; Bulayeva et al., 2004). Moreover, recent experimental evidence suggests that DDE may also be a causative or mediating factor in the development of metabolic diseases such as obesity (Maisano et al., 2016; Cano-Sancho et al., 2017; Liu et al., 2017) and type 2-diabetes (Turyk et al., 2009; Son et al., 2010; Airaksinen et al., 2011). It was associated with mitochondrial dysfunction, alteration in reactive oxygen species (ROS), impairment of the antioxidant defence system, and apoptosis pathway activation (Harada et al., 2016; Morales-Prieto et al., 2017; Morales-

Prieto and Abril, 2017). A prospective role of DDT/DDE in mitochondria-associated diseases (Migliaccio et al., 2019a, b; Elmore and La Merrill, 2019). However, the mechanisms underlying DDT/DDE mitotoxicity remain poorly understood.

The present review's goal was to clarify the effects of EDCs on mitochondrial function by focusing on DDE. It is auspicial that other pesticides and environmental pollutants increase the number of research works. Our review may help researchers focus on other environmental contaminants to check if they have similar or different effects compared to DDE. Moreover, discussing the impact of an EDC molecule that has already been banned but persisting in the environment can reflect the importance of reducing or banning other pollutants as soon as possible. In the first part of the review, we focused on DDT and DDE characteristics as persistent organic pollutants. Then, the DDE proprieties as EDCs and the ability to induce obesity and metabolic disorders will be discussed and its capacity to impair the immune system in human and wildlife. In the last part of the review, we addressed the impact of DDE on mitochondrial function and oxidative stress and antioxidants' utility in preventing and therapy environmental pollutant injury. It should be noted that the details of how mitochondria respond to environmental stressors and how different environmental exposures affect mitochondrial function are an area of research that needs to grow and to be further analyzed.

3.3 DDT and DDE: predominant organochlorine pesticides

The DDT is a well-known synthetic pesticide created in 1874 by Othmar Zeidler. However, only 65 years later, in 1939, the chemist Poul Herman Müller discovered the power of DDT as poison (Berry-Cabán, 2011). At room temperature, DDT is a white crystalline powder containing 65-80% p,p'-DDT (or DDT), 15-21% o,p'-DDT, and up to 4% of

dichlorodiphenildichloroetane (DDD). It was extensively used during the second world war and post-world war periods to control parasites principal vectors such as mosquitoes, phlebotomine, and lies (Conis, 2017). The use of DDT has been effective in containing cases of mosquito-borne malaria. It has been estimated that millions of peoples worldwide have been saved in the last century due to pesticide use in eradicating malaria in many countries. Given its insecticide activity, DDT has also been used extensively in agriculture. However, its uncontrolled use elicited negative environmental consequences, such as a thinning of eggshell and reduction in hatchings at the end of brood in birds (Mitra et al., 2011; Bouwman et al., 2019; Kamata et al., 2020). Moreover, a negative correlation between DDT and reproductive capacity has also been reported in higher animals (Marouani et al., 2017; Bornman et al., 2018). These effects worried the international scientific community, and DDT was banned first in North America and then in Europe, starting in the 1970s. Nowadays, World Heart Organization (WHO) recommends DDT utilization by health advice in the world zones where malaria is still endemic but prohibits its use for agriculture purposes (Rehwagen, 2006).

Despite its ban decades ago, DDT exposure remains through its primary metabolite, DDE. DDT/DDE has been detected in river water and sediments worldwide (Peris et al., 2005; Xue et al., 2006; Chiesa et al., 2016) due to a geological process known as global distillation. DDT and its metabolites evaporate in the hot zone, rise to the upper layers of the atmosphere, and are transported worldwide. It has been reported that about 50% of DDT in the atmosphere is present in the form of gas, whereas the remaining part is adsorbed into particulate matter (PM) (Chattopadhyay and Chattopadhyay, 2015). As a gas, DDT's half-life in the atmosphere was found to be extremely low and estimated in about 37 hours because it reacts with radical species produced by solar radiation that chemically modify the original molecule. Instead, the

other part of DDT adsorbed on PM can be transported by wind at a long distance from the utilization site contaminating soils and water surface (EFSA, 2006). DDT has been estimated to have a half-life between 2 and 15 years in the ground, which will depend on the time elapsed since the first irroration and the populations of microorganisms in the soil that influence the chemical degradation (Ortíz et al., 2013; Pan et al., 2016). Photochemical degradation and dehydrochlorination represent the principal reactions by which DDT is converted to DDE (Thomas et al., 2008).

3.3.1 DDT production

According to information gathered from secretariat reports, by 2005, India, China, and the Democratic People's Republic of Korea (North Korea) were the only countries producing DDT. After 2009, India was the only country officially recognized as a producer. China discontinued its production in 2007 (UNEP, 2015), withdrawing all uses of DDT since 2014 (UNEP, 2015). As of January 2015, Ethiopia, India, and Namibia are currently registered for acceptable production of DDT (Table 3.1). However, while registered, Ethiopia has not reformulated or produced DDT since the cessation of production in China. Similarly, Namibia, while recorded, never produced or reformulated DDT. In 2015, the only production facility was in India (UNEP, 2015). Historically, the most significant production of DDT was in 1962, about 85,000 tons (Van den Berg et al., 2017). For 2005, its production was estimated at around 6,269 tons (i.a.), with a declining annual output reporting nearby matched the reports of global yearly use of DDT (Figure 3.1).

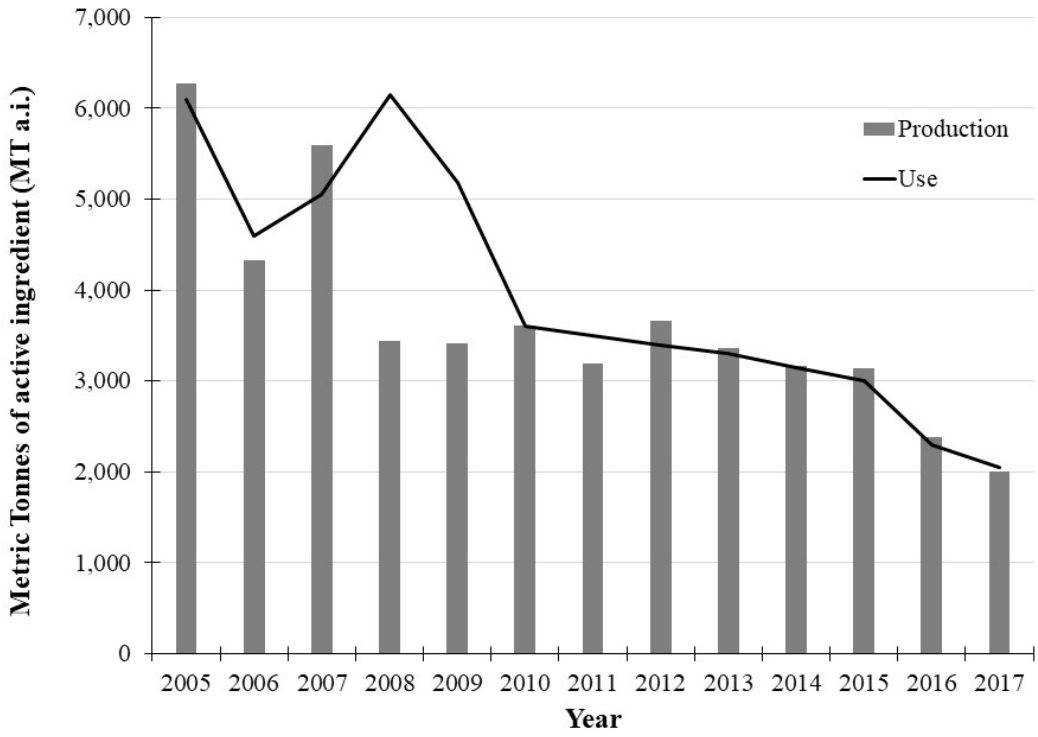

Figure 3.1. Global production and trend use of DDT in Metric Tonnes is an active ingredient (MT a.i.). It is supplemented with data from the Secretariat of the Stockholm Convention (UNEP, 2015, 2017, 2019).

3.3.2 DDT/DDE exposure routes

In contrast to what happens in areas where DDT is still produced and used, recent data showed a decline in DDT exposure in the general public since its ban, depending on geographical location and environmental conditions (ATSDR, 2002, 2019). Three routes of exposure have been identified in the general population, either through dermal (deposition onto plants, objects, spaces, or clothing), ingestion (contaminated food, water), or inhalation of DDT vapor. Epidemiological studies do not provide enough data to describe exposure-response relationships for potential health outcomes associated with inhalation exposure to DDT or DDE (ATSDR, 2019). Exposure to DDT from drinking water is considered negligible due to the hydrophobicity of DDT and the purification processes of water (ATSDR, 2019).

However, oral exposure to DDT/DDE seems to be the predominant route and, to a lesser extent, dermal exposure, with pesticide-manufacturing workers, commercial pesticide applicators, and farmers being the most vulnerable sector (ATSDR, 2019). According to the U.S. Department of Health and Human Services, food consumption is considered the main route of entry of DDT or DDE into the body, even in areas where DDT remains in use (ATSDR, 2019). Previous studies showed that more than 90% of the DDT body burden came from dietary intake of fish and other fatty foods of animal origin, with a concentration of DDT that can range between 0.74 and 131 ng g^{-1} with an average of 12.4 ng g^{-1} (Wang et al., 2011a,b, 2013). It has been reported that humans tolerate doses of DDT as high as 285 mg Kg^{-1} without a fatal result. However, the absorbed quantity is unknown because vomit occurred at these concentrations. Besides, there are not documented reports of fatal human poisoning occurring exclusively from ingestion of pure pesticide (Faroon and Harris, 2002).

3.3.3 Distribution and storage of DDT into the body

After absorption, mainly by consuming contaminated foods, DDT and much more its metabolite DDE, are readily distributed via the lymph and blood to all body tissues and accumulated mainly in fatty tissue, breast milk, and placenta, showing a half-life of about eight years (National Children's Study Placenta Consortium et al., 2014; Fisher et al., 2016; Van den Berg et al. 2016). In animals and humans, DDE is generally more persistent (EFSA, 2006), resistant to degradation, and liposoluble than DDT (Kezios et al., 2013). DDE has a high fat:water partition coefficient and shows a tendency to accumulate in different tissues, especially in adipose tissue and liver fatty depots. Given its hydrophobicity, DDE undergoes a progressive biomagnification process from aquatic species, such as fishes, where it tends to

accumulate in adipose depots (Harshbarger et al., 2000; Storelli et al., 2006; Binelliet al., 2008; Pshenichnyuk and Modelli, 2013; Maisano et al., 2016; Sehonova et al., 2017; Faggio et al., 2018). Therefore, DDE can be concentrated through the food chain (Kim, 2020), and it may represent a significant risk for human and wildlife health (Rodgers et al., 2018). In a recent paper, Sun and collaborators (2020) investigated the DDT and its metabolite concentration in yellowfin tuna *Thunnus albacares*, one of the most consumed fish species worldwide, collected from the South China Sea (SCS). They detected a DDT concentration of around 92.1-221.8 ng g^{-1} in yellowfin tuna, while a DDT concentration from 54.9 to 93.5 ng g^{-1} was found in their prey. DDT levels were lower than those in previous studies and higher in adult yellowfin tuna than in young ones. These results suggested that DDTs' contamination status in the SCS has been dramatically mitigated in past decades since DDTs' banning for agriculture use. Higher DDT levels were observed in abdominal fat muscle than lean dorsal muscle in the adult yellowfin tuna. The composition profile of DDT and its metabolites suggested that DDTs in fish from the SCS were mainly from technical DDTs' historical discharge. The authors also evaluated the cumulative carcinogenic and non-carcinogenic risks of DDT exposure from lifetime consumption of these fish for humans, showing a minimal health risk to coastal residents (Sun et al., 2020).

3.3.4 Presence of DDT/DDE in blood

DDE has been detected in human blood around the world (Mishra et al., 2011; Orta-García et al., 2014; Arrebola et al., 2015; Schettgen et al., 2015; Elbashir et al., 2015; Flores-Ramírez et al., 2017; Fry et al., 2017; do Nascimento et al., 2017; Rosenquist et al., 2017; Singh et al., 2017; Lim et al., 2018; Parada et al., 2019). It can accumulate and reach toxic

levels causing health problems (Liu et al., 2017). Therefore, recent studies were conducted to monitor DDE levels in serum and blood human samples in different world zones (Figure 3.2).

Figure 3.2. Worldwide detection of DDE in human blood. Recent reports showed DDE detection even in countries where DDT was banned more than four decades ago.

Schettgen et al. (2015) reported organochlorine pesticides (OCPs) serum plasma levels in the German population. In their study, the authors divided the population into seven groups based on different ages between 6 and 65. The results showed increased OCPs accumulation with increasing age, probably because groups of individuals with lower age were born after the prohibition of the use of these substances. DDE serum levels were measured using gas chromatography coupled with mass spectrometry (GC/MS). The results showed a total average level in the population of 0.37µg L^{-1} plasma in females and 0.35 µg L^{-1} in males in 2010-2014, showing the highest mean plasma level (0.94 µg L^{-1}) in older subjects. Noteworthy, DDE levels in the German population were 0.60 µg L^{-1} in 2003-2004 (Schettgen

et al., 2011). These data showed a tendency to decrease serum concentration over time following the reduction of these environmental pollutants. In previous work, Turci et al. (2010) found detectable levels of different POPs in the Italian population (Pavia), demonstrating that DDT's breakdown products are still detectable in the general population long-banned time. In particular, the authors showed that DDE plasma levels measured by using GC/MS high resolution were about 0.27µg L^{-1}. In 2014, Orta-García and collaborators demonstrated exposure to POPs in people living in different sites in Mexico by testing total POPs in blood samples using GC/MS. They found a range level of POPs between 8.2 to 91 ng g^{-1} lipids. In 2015, in areas of intensive pesticide use in Sudan, DDE and other POPs levels in blood samples were assessed using gas-liquid chromatography equipped with an electron capture detector (Elbashir et al., 2015). The highest DDE range level monitored was 69-618 ng mL^{-1} in the Wad Medani area, with the lower levels in young people (69 ng mL^{-1} in the age group 20-25 years) and higher levels in older people (618 ng mL^{-1} in the age group 46-70 years) (Elbashir et al., 2015), confirming the tendency to decrease in a serum concentration over time following the reduction of DDT use (Figure 3.1). A study by Koureas et al. (2019) shows a global spatial distribution of DDE blood levels with elevated concentrations in India, China, Mexico, Greenland, Slovakia, and Ukraine. The highest levels have been reported in Sudan, Vietnam, Gambia, India, and Romania. Increasing DDE levels were reported in Southwest Asia and Eastern Europe compared to global average levels (Koureas et al., 2019) despite a worldwide gradual declining use of DDT (van den Berg et al., 2012, 2017; Bouwman et al., 2013).

3.4 DDE action as an endocrine disruptor in human and wildlife

DDT and its metabolite DDE are considered xenoestrogen compounds. They can interfere with the endocrine system physiology due to their endocrine disruptor properties (Van den Berg et al., 2003; WHO/UNEP, 2013). A link between exposure to DDT metabolites and an increasing trend in endocrine-related disorders in humans and wildlife has been suggested (WHO/UNEP, 2013). This section will focus on the effect of DDE on reproductive system physiology and thyroid function in both wildlife and human.

DDE has been indicated as a potent androgen receptor (AR) antagonist (Xu et al., 2006), and it may interact in additive or multiplicative ways with other endocrine disruptive environmental pollutants. It has been shown that DDE induced alterations in male reproductive hormones balance with an excessive estrogen synthesis (Thibaut and Porte, 2004; Bornman et al., 2018). It also caused some types of reproductive dysfunction (Kristensen et al., 2006; Monteiro et al., 2015; Tavares et al., 2015; Quan et al., 2016; Hoffmann and Kloas, 2016) and, in the worst case, prostate cancer progression in human (Wong et al., 2015). Moreover, it has been reported that parental exposure to some types of OCPs could adversely affect male reproductive health by altering several physiological processes. In a case-control study, Damgaard and co-workers investigated the human association between maternal exposure to different OCPs present in mothers' breast milk, including DDE and cryptorchidism among male children in Finland and Denmark (Damgaard et al., 2006). Cryptorchidism represents an incorrect or incomplete testicular descending in the scrotum after born. The study indicated that more than one chemical detectable in mothers' breast milk at low concentrations represents a risk factor for congenital cryptorchidism (Damgaard et al., 2006). Testicle descending is a physiological process characterized by two

different phases: transabdominal and inguinoscrotal phases (Virtanen and Adamsson, 2012). Given that the inguinoscrotal stage is dependent on androgens, endocrine disruptors can be directly or indirectly implicated in the alteration of testicular descent at the scrotal level. Our research group recently reported that chronic oral exposure to 10 mg Kg^{-1} DDE induced alteration in serum testosterone levels and testicular AR content, causing spermatogenesis defects in rats (Migliaccio et al., 2019c). At the cellular level, Xiong and colleagues (2006) hypothesized a relationship between Sertoli cell survival rate and DDE concentration associated with a down-expression level of transferrin mRNA, which is necessary for the proper function of Sertoli cells. As described in mammalian species, in the Zebrafish model, DDE sublethal exposure in a concentration ranging from 0.01 to 2.0 µg L^{-1} induced endocrine disruption during gonad differentiation in juvenile specimens and reduced the number of mature oocytes in exposed females (Monteiro et al., 2015). However, no correlation between DDE exposure and reproductive system impairment was observed in a study performed in crocodiles that live near irrigation areas in the northeast of Western Australia, where DDT was applied in the cotton crop from 1964 to 1974 (Yoshikane et al., 2006). The results showed that despite the high DDE concentrations observed in liver tissue (>250 µg g^{-1}), no histological differences were observed in testes and ovaries of exposed vs control animals. Also, the concentration of sex steroids, including testosterone and β-estradiol, varied according to the individual animals' age and breeding state. There was no real relationship with DDE concentration (Yoshikane et al., 2006).

DDE exposure has been associated with pubertal and menopause timing alteration (Den Hond et al., 2011; Grindler et al., 2015) and endocrine-related breast cancer (Demers et al., 2000; Ingber et al., 2013). According to Aubé and colleagues, DDE conferred a

proliferative advantage to precancerous hormone-dependent cells by blocking the AR-signalling pathway that represses cell growth (Aubé et al., 2008). It has been reported that androgens could reduce estrogen-driven cell proliferation (Jonsson et al., 2014) by regulating the expression of estrogen receptor (ER)-alpha and ER-beta (Kurebayashi et al., 2000; Khan et al., 2000). Epidemiological evidence also suggested that exposure to DDE could be associated with the development of polycystic ovaries and impaired fertility in animals and humans by alteration in the expression of vascular endothelial growth factor and insulin-like growth factor (Gotz et al., 2001; Holloway et al., 2007), which seem to be essential for ovarian follicular development and corpus luteum function (Geva et al., 2000).

A recent study in the bovine model showed that DDT and DDE could alter hormonal secretion by increasing oxytocin, estradiol, progesterone synthesis, and secretion from the bovine placentome (Wojciechowska et al., 2017). Besides, decreased expression levels of structural proteins connexins and placenta-specific 1, responsible for the maternal-fetal connections' integrity, were found. It has been shown that DDT and DDE could interfere with the barrier function and secretory activity of the placenta by enhancing uterine contractility, affecting blood flow in the foetal part of the placenta, and impairing the pregnancy course and foetal development (Wojciechowska et al., 2017). In teleost fish, DDE has been suggested to alter the steroid pathway by suppressing mRNA expression of steroid receptors, including the glucocorticoid receptor (GR), along with the expression of heat shock proteins (HSPs). HSPs, namely hsp70 and hsp90, seemed to play a chaperon role in protecting the steroid receptors (Thibaut and Porte, 2004). In female carps *Cyprinus carpio*, DDE enhanced ovarian synthesis of maturation-inducing hormones 20α- and 20β-hydroxysteroid dehydrogenase associated with final oocyte maturation (Kwong et al., 2009). In juveniles of zebrafish *Danio rerio*, DDE

could cause endocrine disruption during gonad differentiation. Moreover, it promoted a reduction in the number of mature oocytes in females, and an overproduction of vitellogenin (vtg) protein content in males, impairing reproductive success (Bornman et al., 2018). In the largemouth bass *Micropterus salmoides*, DDE up-regulated vtg and ER-alpha expression in the liver, suggesting that it could act as a weak estrogen mimic. The steroidogenic acute regulatory (StAR) protein involved in cholesterol transportation in the mitochondria for steroid biosynthesis can also be up-regulated mainly in the gonad of males (Kristensen et al., 2006).

The endocrine disruption function of DDT/DDE and many other endocrine disruptors seemed to be regulated through agonist or antagonist interaction with nuclear receptors. Eliciting gene expression changes, modulating both coding mRNAs and more recently discovered noncoding RNAs (ncRNAs), namely microRNAs (Burgos-Aceves et al., 2018a,b; Kalinina et al., 2018; Seong et al., 2019; Abo-Al-Ela and Faggio, 2021).

Thyroid-disrupting effects by EDCs, including DDT, have also been reported more in wildlife than in humans (Boas et al., 2012). In wildlife, exposure to DDT or its metabolite DDE was associated with thyroid disorders. In adult humans, it did not seem to have a significant adverse effect on thyroid function (Benson et al., 2018). Turyk and collaborators (2007) did not find any significant associations between DDE exposure and thyroid hormone production in both sexes. However, there was a dose-dependent relationship with total thyroxine (T_4) concentration, but only in young women (Turyk et al., 2007). In a study on young people in Akwesasne Mohawk, a higher concentration of DDE was reported in breast-fed adolescents compared with nonbreast-fed adolescents. However, alterations in thyroid functions were not reported (Schell et al., 2008). In adult men of Venda ethnicity, long-term

DDT uptake appeared to decrease serum retinol-binding protein (RBP) concentration which could have deleterious effects on thyroid function and vitamin A nutritional status (Delport et al., 2011). In wildlife, severe interfollicular fibrosis in harbour porpoises from the German and Norwegian coasts was observed in the presence of DDT and other compounds. However, a cause-effect relationship could not be established, but the hypothesis of contaminant-induced thyroid fibrosis has been suggested raising the question of the long-term viability in highly polluted areas (Das et al., 2006). DDT could also alter the thyroid homeostasis by inhibiting TSH receptor (TSHr) internalization, as observed in Chinese hamster ovary cells. This seemed to happen through DDT goitrogenic effects altering the membrane rafts hosting the TSHr itself (De Gregorio et al., 2011). Rossi et al. (2018) proposed that DDT/DDE-induced development of extracellular vesicles containing the TSH receptor could be directly involved in developing autoimmune responses against the TSH receptor. On the other hand, DDT did not induce a significant change in plasma thyroid hormones in Gambel's white-crowned sparrow, *Zonotrichia leucophrys gambelli* (Scollon et al., 2004). In the same way, despite the high concentrations of DDT (28 ± 19 mg Kg^{-1} lipid weight) in the serum of juvenile California sea lions, no thyroid hormone impairments were reported (Debier et al., 2005). On the other hand, in a dose-dependent way, DDT altered the serum concentration of thyroid hormones (T_3 and T_4). Histologically, it induced a hypothyroidism state with a colloid goiter in an experimental rat model (Tebourbi et al., 2010). Exposure to DDE can alter thyroid hormone-dependent genes at the genetic level, leading to some possible physiological and endocrine consequences during smoltification in Atlantic Salmon (*Salmo Salar*) (Mortensen and Arukwe, 2006).

The above findings suggested that DDT and DDE play a role as endocrine disruptors by impairing reproductive system physiology and thyroid function in wildlife and humans, even if some differences can be due to the different animal species and exposure time in the further experimental studies. Therefore, it is necessary to develop assays to help identify endocrine disruptors mechanism, coming nearby and supporting animal studies or epidemiological inquiries.

3.5 DDE action in obesity and metabolic disorders

In the last decade, persistent organic pollutants such as PCBs and/or OCPs have been cataloged as obesogens (Salihovic et al., 2016). In an integrated systematic review, Cano-Sancho and co-workers showed a significant positive association between DDE exposure and adiposity (Binelli et al., 2008). Increased adipose tissue mass is associated with metabolic dysfunctions, energy imbalance, and decreased energy expenditure due to a decrease in brown adipose RNA involved in thermogenesis regulation (La Merrill et al., 2014; Di Gregorio et al., 2019). The risk of obesity development seems to be increased when pesticide exposure occurs during the prenatal stage (Turyk et al., 2009; La Merrill et al., 2014). Moreover, it has been suggested that obesity may be associated with a perturbation of adipose tissue homeostasis through macrophage function modulation (Mangum et al., 2016). DDE exposure impairs macrophage reactivity to inflammatory and polarization stimuli, and it may enhance macrophage recruitment and/or proliferation in adipose tissue. Increased macrophage infiltration and a shift toward chronic low-grade inflammation are hallmarks of obesity-associated diseases. It can suggest an association between DDE and metabolic disorders due to the DDE effect on macrophage accumulation (Mangum et al., 2016).

Obesity is associated with alteration in lipid serum profile and hepatic lipid metabolism. Salihovic and colleagues used a non-targeted metabolomics approach to examine the associations between DDE exposure and human serum metabolic profiles. They established a link between DDE exposure and serum metabolites related to human lipid metabolism (Salihovic et al., 2016). The liver plays a crucial role in regulating lipid metabolism and dyslipidemia onset. It is also involved in detoxification processes and can be a potential DDE target. DDE can alter the mechanisms that regulate lipid metabolism and secretion in hepatocytes by inducing lipogenesis, increasing fatty acid secretion, and decreasing lipid degradation. (Jin et al., 2016; Ward et al., 2016; Liu et al., 2017). Moreover, a non-toxic dose of DDE (10 mg Kg^{-1} b.w. for 4 weeks) enhanced both hepatic β-oxidation rate and carnitine palmitoyltransferase system activity in rats. This mechanism may represent a metabolic adaptation to produce energy in ATP to cope with the excessive energy demand for DDE-stimulated detoxification pathways (Migliaccio et al., 2019a).

It was also evidenced as different environmental pollutants can induce cellular toxicity acting on PPARs family members altering gene expression (Arciello et al., 2013). Reduction in PPARs produces cellular toxicity and EnR stress (Laing et al., 2010). However, DDT was found involved in PPARγ stimulation in human mesenchymal stem cells with alterations in cellular proliferation, differentiation, and gene expression, suggesting a role of EDCs in the homeostatic imbalance and increased cancer incidence in consequence to environmental exposition (Huang et al., 2017). On the contrary, another study showed that adult male C57BL/6 mice exposed to a low dose of p, p'-DDE for eight weeks exhibited an increase in the expression of genes involved in the liver's fatty acid synthesis. Also, DDE can decrease mitochondrial fatty acid β-ox and impairment in mitochondrial morphology as observed by

electron microscopy (Liu et al., 2017). In the same study, DDE induced in HepG2 cells a significant decrease in enzymes responsible for mitochondrial fatty acid β-oxidation, mitochondrial membrane potential, oxygen consumption rate, and ATP production (Liu et al., 2017). DDE induced lipid accumulation in mice liver and HepG2 cells, suggesting a pivotal role of mitochondrial dysfunction in steatosis onset.

Hepatic lipid accumulation may lead to lipotoxicity and insulin resistance development (Kahn and Flier, 2000; Greenberg and McDaniel, 2002; Guilherme et al., 2008; Lara-Castro and Garvey, 2008; Perry et al., 2014). A relationship between DDE concentration and diabetes development has also been suggested (Singh and Chan, 2017). DDE can stimulate type 2 diabetes at high concentrations through a pathway involving insulin resistance. Howell and colleagues evaluated the metabolic effects of this xenoendocrine metabolite in the male mice model's blood and liver (Howell et al., 2015). The results indicated that chronic exposure to DDE could directly affect systemic blood glucose and hepatic lipid metabolism; however, the degree of alteration is a diet-dependent factor. At low exposure, DDE endorses other metabolic abnormalities such as dyslipidemia, insulin resistance, and obesity among people free of diabetes (Lee et al., 2011; Burgos-Aceves et al., 2018a). Given the critical role of mitochondria in obesity-induced metabolic diseases, such as hepatic steatosis and insulin resistance (Migliaccio et al., 2019a; Sergi et al., 2019). The effects of DDE on obesity and metabolic disorder onset may also be mediated by the impact of such pollutants on mitochondrial function and reactive oxygen species (ROS) production due to mitochondrial dysfunction.

3.6 DDE action on the immune system in humans and wildlife.

In the last decades, it has been hypothesized that the immune system constitutes one of the targets of EDCs action based on literature that demonstrated the relationship between the endocrine and immune systems. Nowak et al. (2019) recently reviewed EDC effects on human immune cells' development and function. In line with this finding, DDT and its metabolites have been shown to have a modulatory action on the immune system by affecting the activation state and the activity of some immune cells in both human and wildlife (Cuesta et al., 2008; Dutta et al., 2008; Shimada et al., 2015; Mangum et al., 2016; Martyniuk et al., 2016). In wildlife, DDE showed a higher genotoxicity potential than DDT or DDD on haemocyte cells. In zebra mussel *Dreissena polymorpha*, DDE caused massive and irreversible DNA damage in haemocyte by the appearance of micronuclei, even at low concentrations (Binelli et al., 2008). Under chemical stress, the immune cells must divide rapidly, but during cell division, DDE seemed to cause chromosomal damage resulting in micronuclei formation (Binelli et al., 2008), as also reported in *Mytilus galloprovincialis* (Fernández et al., 2011). Moreover, Centelleghe et al. (2019) showed that DDT exhibited an immunosuppressive effect by inhibiting lymphocyte proliferation in a dose-dependent approach in the bottlenose *Tursiops truncates* and striped *Stenella coeruleoalba* dolphins. On the other hand, in gilthead seabream, *Sparus aurata*, acute exposure to DDE did not seem to have any adverse effects on leucocyte viability. It produced very slight variations in the immunological parameters with an up-regulation in immune-related genes, such as IL-1b, TNFα, MHCIα, MHCIIα, Mx, TLR9, IgML, and TCRα , suggesting an anti-inflammatory response (Cuesta et al., 2008). Also, chronic exposure to DDE ingestion at high concentrations

(120 mg Kg^{-1}) altered gene networks related to the immune system, such as leukocyte cell adhesion in the largemouth bass *Micropterus salmoides* (Martyniuk et al., 2016). DDT also showed immunomodulatory properties in mouse macrophage cells, influencing the production of nitric oxide (NO) and pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) through the NF-κB signalling pathway (Kim et al., 2004). It was causing an inhibitory effect on macrophage activity, which in turn can increase susceptibility to intracellular pathogens (Nuñez et al., 2002).

In vitro studies, low concentration of DDE (≈ 10 μg mL^{-1}) enhanced the intracellular antioxidant enzyme superoxide dismutase 1 (SOD1), and pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) expression at protein and mRNA level in peripheral blood mononuclear cells (Alegría-Torres et al., 2009; Cárdenas-González et al., 2013). Gerić et al. (2012) evaluated the cytogenetic status of human lymphocytes after exposure to low DDT concentrations and its metabolites DDE *in vitro*. The results suggested that even at low concentrations, these pesticides could induce cytogenetic damage to human peripheral blood lymphocytes. Therefore, they could impact human health (Gerić et al., 2012).

Chronic environmental exposure to DDE or other organochlorines could eventually reach concentrations detrimental to human immune system function, causing an increased risk of infectious diseases (Nowak et al., 2019). In a study carried out on Inuit infants during their first year of life, a relationship among DDE exposures, immune status, and the occurrence of infectious diseases has been suggested (Dewailly et al., 2000). Moreover, workers engaged in DDT manufacture showed an alteration in the nervous system and a deterioration in the function of neutrophils, being more susceptible to infections (van Wendel de Joode et al., 2001; Nowak et al., 2019). Alterations in the immune system induced by EDCs could affect

the well-regulated immune capacity to respond to microbial and vaccine antigens, allergens, self, and tumour antigens. Therefore, possible immune system changes should be routinely investigated in ecotoxicity studies. On the other hand, it is essential to gain further insight into the cellular mechanisms by which environmental pollutants act on immune and endocrine systems. A cellular mechanism by which DDE may contribute to immunodeficiency could be related to unregulated apoptosis (Cetkovic-Cvrlje et al., 2016), such as apoptosis induction by increased reactive oxygen species (ROS) production (Pérez-Maldonado et al., 2004, 2005). Considering the pivotal role of mitochondria in ROS production, the next section will focus on DDT and DDE's impact on mitochondrial function.

3.7 DDE impact on ROS production, mitochondrial function, and cellular stress

According to evidence, xenobiotic compounds can affect mitochondria and cause mitochondrial dysfunction, ROS production, and apoptotic pathway induction, which seem to be involved in DDE action as an endocrine disruptor and metabolic regulator (Pérez-Maldonado et al., 2004, 2006; Alegría-Torres et al., 2009; Shi et al., 2010; Song et al., 2008, 2011; Bestman et al., 2015; Sehonova et al., 2018). In particular, the mechanism of action of DDE seems to be related to a ROS overproduction that led to lipid peroxidation and consequently to apoptotic pathway induction (Harada et al., 2016; Burgos-Aceves et al., 2018a). ROS is produced in biological systems as by-products of reducing molecular oxygen. These include the superoxide radical anion, hydrogen peroxide (H_2O_2), hydroxyl radical, hydroperoxyl radical, singlet oxygen, and peroxyl radical. Under physiological conditions, ROS plays a crucial role in regulating intracellular signals involved in cell division, metabolism, and programmed death (Regina et al., 2005; Hamanaka and Chandel, 2010). It

has also been demonstrated that increased ROS levels may be associated with inflammation and xenobiotics exposure (Turrens, 2003; Renaud et al., 2012; Klotz and Steinbrenner, 2017). Cells can use different mechanisms involving antioxidant enzymes and proteins with indirect antioxidant function to contain ROS accumulation and limit oxidative damage. For example, antioxidant defence stimulation represents one of the attempts to limit oxidative damage caused by environmental pollutants (Poljšak and Fink, 2014). ROS can be produced in different cellular districts. ROS endogenous sources are the mammalian CYP-dependent microsomal electron transport system and the mitochondrial electron transport chain (ETC). Mitochondria represent ROS production main sites, especially in terms of superoxide anion and hydrogen peroxide that can in part diffuse out of mitochondria by escaping from reduction by glutathione peroxidase (GPx) in the mitochondrial matrix (Han et al., 2001). Superoxide formation occurs in ETC, where the electron flux moves from the first complex of ETC through other cytochromes and proteins that compose it. Then, the electrons react with oxygen at the cytochrome c oxidase that forms water. In general, few electrons escape the ETC. At mitochondrial level, superoxide can be buffered due to the antioxidant activities: superoxide dismutases (SODs) convert superoxide anion in H_2O_2, whereas GPx converts hydrogen peroxide to H_2O using reduced glutathione. In the presence of mitochondrial impairment, many electrons can escape the passage through the cytochromes. Therefore, the levels of superoxide produced at the mitochondrial level increase. The imbalance between ROS generation and cellular antioxidant capacity represents oxidative stress generation. Which, in turn, may be associated with different metabolic disorders such as atherosclerosis, hepatic steatosis, diabetes, hypertension, ageing, Alzheimer's disease, kidney disease, and cancer (Roberts and Sindhu, 2009; Mahjoub and Masrour-Roudsari, 2012). DDE is involved in

cellular toxicity associated with increased oxidative damage and apoptosis (Liu et al., 2001), suggesting a role of this pesticide in cellular ROS overproduction. Moreover, at the mitochondrial level, DDE affects ETC by acting on mitochondrial chain enzymatic complexes (Kudin et al., 2004). DDE can partially inhibit the succinate dehydrogenase (respiratory complex II) and succinate cytochrome-c reductase (respiratory complex III), leading to a decrease in mitochondrial respiration rate, membrane potential (ΔΨm) and ATP production (Figure 3.3) (Huang et al., 2017).

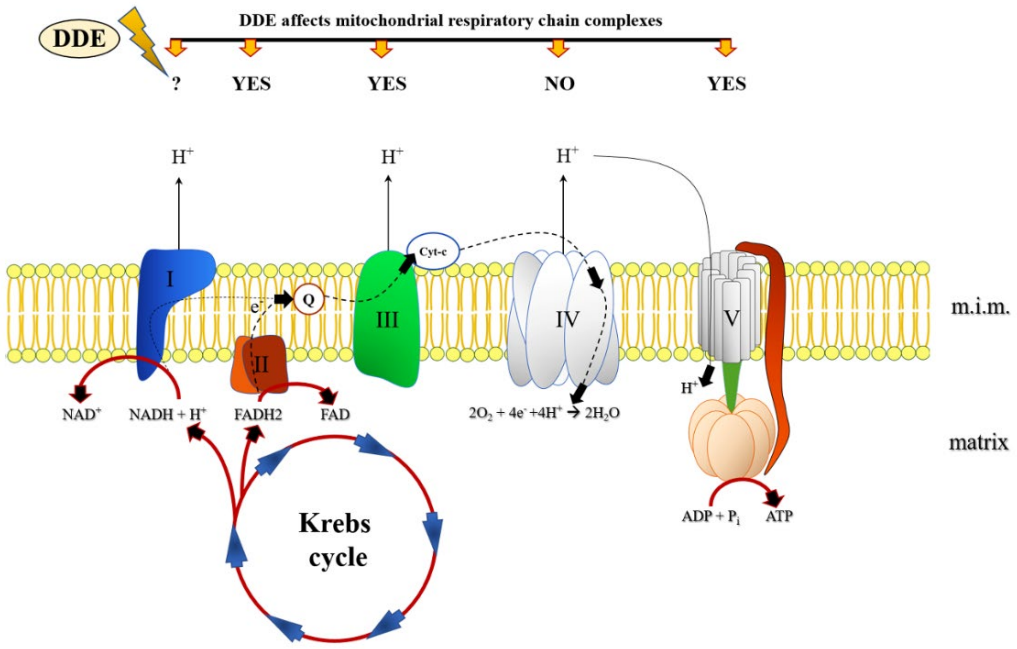

Figure 3.3. Schematic representation of the mitochondrial respiratory chain. DDE alters mitochondrial metabolism and electron flow by affecting ETC at multiple levels. It is not clear the effect of DDE on complex I, but it has been shown that DDE impairs complex II, III, and V, reducing electron flow and ATP synthesis.

A further source of ROS is represented by cytosolic enzymes or proteins that can generate ROS as secondary products of the reaction cycle in which are directly involved. For example, environmental contaminants stimulate detoxification in hepatocytes by activating proteins as cytochrome 450 (CYP450). These systems function in the presence of iron in their active site. The involvement of CYP450 enzymes in ROS production in biological systems

has been recently reviewed (Hrycay and Bandiera, 2015). CYP enzymes catalyze an organic substrate's oxygenation and the simultaneous reduction of molecular oxygen. ROS production occurs when the transfer of oxygen to a substrate is not tightly controlled. Literature evidence suggests that DDE stimulates CYP450 2B and 3A systems (Han et al., 2001; Wyde et al., 2003) and potentially induced lipid peroxidation, cell toxicity, and death (Arciello et al., 2013) or interaction with mitochondrial chain enzymatic complexes (Laing et al., 2010). DDE's putative mechanism may be involved in cellular oxidative stress, and toxicity onset was schematized in Figure 3.4.

Moreover, it has been shown that DDE-induced ROS overproduction is associated with cancer development in human hepatic cells by inhibiting the transcription factor nuclear factor erythroid 2 p45-related factor 2, both gene and protein nuclear factor erythroid 2-related factor (Nrf2) expression. This inhibition, in turn, elicited a decrement in γ-glutamyl-cysteine synthetase (γ-GCS) level (Jin et al., 2016). The γ-GCS is an important metabolic and antioxidant enzyme, and it is the rate-limiting enzyme of glutathione (GSH) biosynthesis. Both enzymes, γ-GCS and GSH, are closely related to the balance between oxidative reactions and endogenous antioxidant defence system. It has been found that the expression of these enzymes is regulated by the Nrf2/Kelch-like ECH associating protein 1(Keap1) complex (Wu et al., 2004). The Nrf2 is the master regulator of the gene expression of diverse cytoprotective protein networks, including antioxidant, anti-inflammatory, and xenobiotic metabolism enzymes (Kovac et al., 2015; Smith et al., 2016; Battino et al., 2018). It can also prevent the cell from undergoing mitochondrial apoptosis (Battino et al., 2018). An Nrf2 deficiency leads to impaired mitochondrial fatty acid oxidation, respiration, and ATP production (Sykiotis and Bohmann, 2010; Dinkova-Kostova and Abramov, 2015; Holmström et al., 2016).

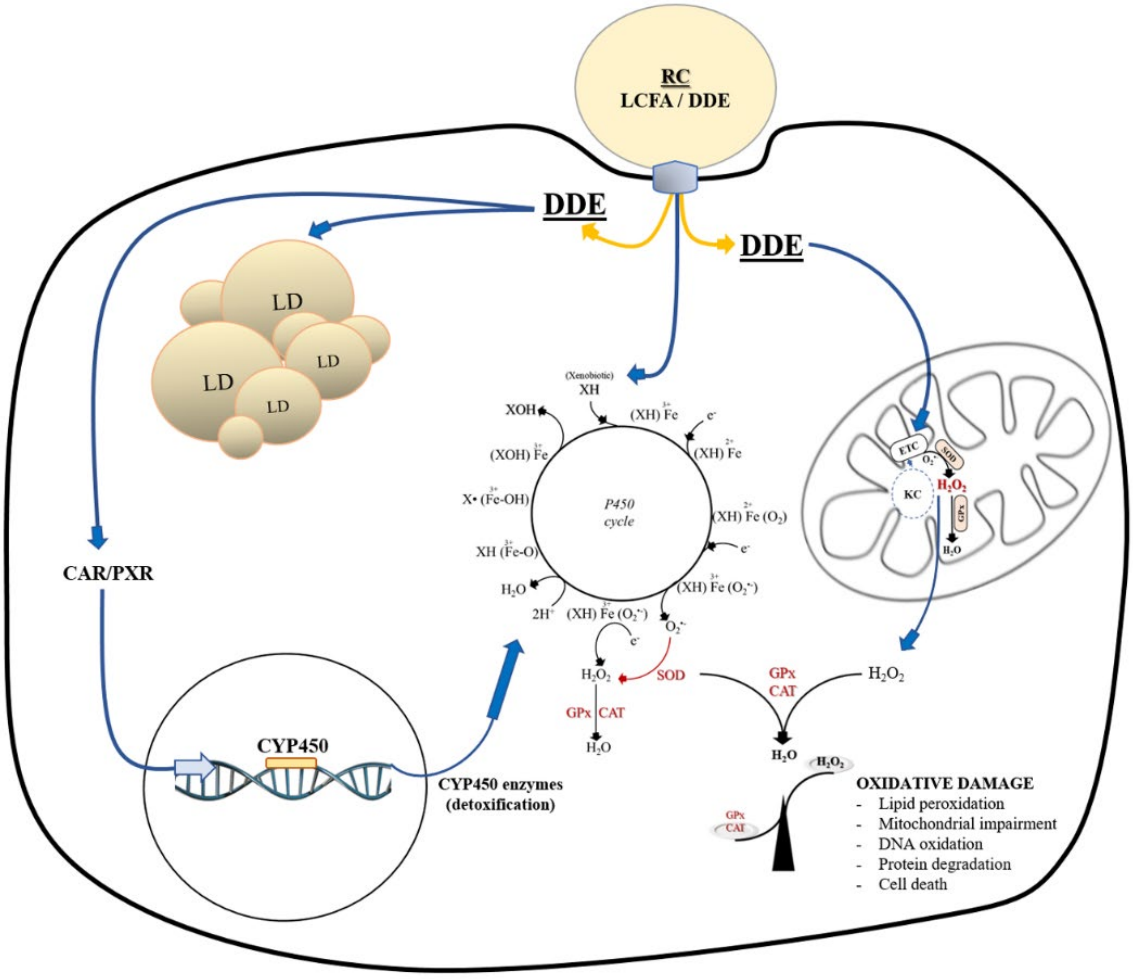

Figure 3.4. Putative mechanisms stimulated by DDE. Given its hydrophobicity, DDE can be stored in lipid droplets or act directly on mitochondria by impairing electron transport flow (figure 3.3) and enhancing mitochondrial ROS production. In addition, DDE can interact with the nuclear xenobiotic receptors, namely the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR), inducing gene expression of the P450 gene family for detoxification. However, the CYP450 reaction cycle causes an increase in ROS levels, which may generate oxidative damage and cellular death if not controlled by antioxidant defences. RC: remnant chylomicrons; LCFA: long-chain fatty acids; LD: lipid droplets; DDE: Dichlorodiphenyldichloroethylene; P450: cytochrome P450; ROS: reactive oxygen species; KC: Krebs cycle; $O_2^{\bullet-}$: superoxide anion; SOD: Superoxide dismutase; GPx: glutathione peroxidase; CAT: catalase; CAR: constitutive androstane receptor; PXR: pregnane X receptor.

DDT and DDE effects on mammalian mitochondrial oxidative phosphorylation have been recently reviewed by Elmore and La Merrill, suggesting that both xenobiotics impair the electron transport chain and oxidative phosphorylation (Elmore and La Merrill, 2019). Therefore, there are good data to suggest that DDT and DDE target specific mitochondrial protein complexes and processes causing organelle dysfunction, which could contribute to the

development of mitochondria-associated diseases related to environmental pollutants exposure. Mitochondrial dysfunction can be associated with impaired function of other cellular organelles, such as endoplasmic reticulum stress, and inducing cellular death via the mitochondrial pathway of apoptosis (Lepretti et al., 2018).

Several studies showed a relationship between DDE exposure and cellular death in different tissues associated with oxidative stress and mitochondrial impairment (Pérez-Maldonado et al., 2004, 2006; Alegría-Torres et al., 2009; Shi et al., 2010; Song et al., 2008, 2011). For example, it has been shown that DDE can trigger testicular apoptosis in pubertal male rats at a concentration greater than 20 mg Kg^{-1} body weight through the Fas/FasL pathway (Shi et al., 2010). Significant increases in both Fas and FasL mRNA levels, along with an increase in caspase-3, -8, and a decrease in antioxidant enzymes SOD and GPx activity were observed. Therefore, DDE can induce apoptosis by a mechanism involving Fas/FasL and mitochondria oxidative stress pathways (Shi et al., 2010; Kovac et al., 2015). Apoptosis induction has also been reported in peripheral blood cells with a DDE concentration of 80 μg mL^{-1} or higher, as reported *in vitro* studies (Pérez-Maldonado et al., 2004, 2006; Alegría-Torres et al., 2009). It seems that an increase in ROS production induced by DDE could be associated with apoptosis induction (Pérez-Maldonado et al., 2006). Indeed, DDE could induce mitochondria-mediated apoptosis in Sertoli cells, with an associated elevation in ROS generation and release of cytochrome c into the cytosol (Song et al., 2008, 2011; Tavares et al., 2015), confirming mitochondrial involvement.

In line with the pro-oxidant role of DDE and its precursor DDT, other authors recently evidenced their ability to induce cellular stress at different doses and with different methods of administration (Marouani et al., 2017; Migliaccio et al., 2019c, 2019d). For example,

intraperitoneal injection of high doses of DDT (50-100 mg Kg^{-1} b.w. for ten days) seemed to act on testicular apoptosis and oxidative stress generation by inducing antioxidant impairment in adult rats (Marouani et al., 2017). Our research group showed that male Wistar rats chronically treated with a low oral dose of DDE (10 mg Kg^{-1} b.w. per day) for 28 days exhibited higher testicular lipid peroxidation levels than the control group. These were associated with a pronounced defect in antioxidant capacity, apoptosis, cellular proliferation, as well as testis tissue damage (Migliaccio et al., 2019c). Moreover, reduced metallothioneins (MTs) expression levels have been reported in different tissues in the same experimental rat model (Migliaccio et al., 2019c, 2019d). MTs are metal-binding proteins rich in cysteine (Si and Lang, 2018) that can play a role as free radical scavenging activity in cells. Chiaverini and De Ley (2010) demonstrated that MTs could reduce peroxidative damages in the liver by preventing hydroxyl radical generation and DNA degradation. Alterations in MTs gene expression and protein synthesis/localization associated with lipid peroxidation have been reported in testis, liver, and kidney in rats chronically treated with a non-toxic oral dose of DDE (Migliaccio et al., 2019d). This finding confirmed that a low amount of DDE *in vivo* could induce several metabolic changes, including alteration in cellular gene expression and protein synthesis, to adapt cellular metabolism in environmental stress conditions.

Noteworthy, DDE could alter the homeostasis of the endoplasmic reticulum and induce endoplasmic reticulum stress activating the apoptotic pathway by stimulating the unfolded protein response and Ca^{2+} signalling. Excessive or inadequate apoptosis of testicular cells induced by DDE through the involvement of mitochondria and/or endoplasmic reticulum-mediated cellular stress can lead to abnormal spermatogenesis or testicular tumorigenesis (Tavares et al., 2015). Shabbir et al. (2005) reported that DDT could also induce

an endoplasmic reticulum stress response. Other researchers pointed out that a decrement in semen quality and quantity could result from a depolarized mitochondrial membrane, elevation in ROS production, lipid peroxidation, and semen DNA fragmentation in the presence of 20 μg L^{-1} DDE concentration (Pant et al., 2014). ROS overproduction also was detected in rat testis mitochondria after hyperpolarization induced by DDE exposure, associated with Fas/FasL apoptotic pathway activation in spermatocytes (Mota et al., 2011).

Given the critical role played by both mitochondrial dysfunction and ROS production in the etiopathogenesis of obesity and insulin-resistance/diabetes onset (Sivitz, 2010; Di Meo et al., 2017; Ramalingam et al., 2017; Verma et al., 2017; Lefranc et al., 2018), it can be suggested a possible role of DDE in developing of these mitochondrial-related pathologies. It should also be noticed that increased ROS levels and cell death seem to be associated with changes not only in mitochondrial function but also in mitochondrial dynamic behaviour (Willems et al., 2015). Mitochondrial dynamics is a physiological mechanism through which mitochondria undergo fission and fusion processes to sustain the energy requirement in response to metabolic changes in the cells as well as to regulate cellular growth, differentiation, proliferation, maturation, and death (Chan et al., 2006; Khacho et al., 2016; Noguchi and Kasahara, 2018; Seo et al., 2018). Several chemical species seem to be involved in the impairment of mitochondrial dynamics associated with cellular oxidative stress, mitochondrial dysfunction, ATP depletion, and apoptosis (Chen et al., 2017). For example, the herbicide paraquat generates mitochondrial fission and oxidative stress leading to apoptosis on alveolar cell culture (Zhao et al., 2017). Increased ROS levels, apoptosis rate, and release of Cyt c from mitochondria have also been reported. Moreover, in response to cellular stress, decreased mitochondrial fusion markers dominant optic atrophy 1 (OPA1) and

Mitofusin 2 associated with increased fission markers dynamin-related protein 1 and fission protein 1 occurred (Chan et al., 2006; Willems et al., 2015). These mitochondrial dynamic balance changes induced increases in mitochondrial fragmentation and OPA1-dependent cristae remodelling (Wang et al., 2014; Varanita et al., 2015). In line with this finding, it has been suggested a correlation among oxidative stress, mitochondrial dysfunction, and mitochondrial fragmentation (Wu et al., 2011; Iqbal and Hood, 2014; Ježek et al., 2018). As far as we are aware, any research studies on the effect of DDE on mitochondrial dynamics have been published. Given the pro-oxidative role of DDE and other environmental chemicals, future studies are needed to understand the implication of mitochondrial morphology/dynamic behaviour in the adaptation/toxicity produced by these substances at the cellular level. This kind of study may also help clarify whether ROS generation by DDE is a cause or a consequence of the impairment in mitochondrial function and dynamic behaviour. These data suggested a role of mitochondria dysfunction, ROS production, cellular stress, and apoptosis in the endocrine disruptor effect of DDE.

3.8 Antioxidants for treatment and prevention of environmental pollutant toxicity

Given that DDE, like other organochlorine pesticides, can induce cellular oxidative stress through both ROS production increase and antioxidant defence decrease. It can be suggested that bioactive compounds with antioxidant activities may help treat or prevent toxicity induced by DDE or other environmental pollutants.

Antioxidants agents can be endogenous or obtained exogenously, as a part of a diet, or through dietary supplements. Several studies have demonstrated that the human body can alleviate oxidative stress using exogenous antioxidants. Vitamin C and E are among the

significant natural antioxidants derived from natural sources by dietary intake. Vitamin C is the most important water-soluble antioxidant; meanwhile, Vitamin E is a critical lipid-soluble vitamin that acts as an effective chain-breaking antioxidant within cellular membrane where it inhibits lipid membrane peroxidation. It has been shown that vitamins C and E can improve hepatic and renal function by ameliorating oxidative stress induced by the environmental pollutant abamectin in rats (Wilson Magdy et al., 2016). Vitamin C and vitamin E have also been protective against oxidative stress induced by the pesticide chlorpyrifos (Akefe, 2017). Moreover, it has been shown that both Vitamins C and E exhibit protective effects on DDT-induced cytotoxicity via the ROS-mediated mitochondrial pathway and NF-kB/FasL pathway in human normal liver cells (HL-7702) (Jin et al., 2014). This study demonstrated that DDT exposure at over 10 μM elevated ROS generation level, induced mitochondrial membrane potential, and released Cyt c into the cytosol, inducing cell apoptosis. These alterations caused by DDT treatment were prevented or reversed by the addition of vitamin C or vitamin E, and the protective effects of co-treatment with vitamin C and vitamin E were higher than the single supplement with DDT. These findings provide experimental evidence supporting that vitamin C or/and vitamin E could alleviate the oxidative stress and cytotoxicity induced by DDT in HL-7702 cells.

The protective role of antioxidants in the defence against ROS production mediated by environmental pollution has been reviewed by Poljšak and Fink (2014). The effects of the antioxidant N-acetyl-L-cysteine (NAC), a glutathione inducer, on the induction of oxidative stress and apoptosis has been analyzed in human peripheral blood mononuclear cell (PBMC) treated with DDT, DDE, or DDD. NAC prevented ROS production when normal human

PBMC were preincubated with 30 mM NAC for 30 min, before the treatment with DDT, DDE, or DDD at 80 mg mL^{-1} for 12 or 24 h (Pérez-Maldonado et al., 2005).

It has been suggested that silymarin, the dried extract of the ripe seeds of the plant *Silybum marianum*, has antioxidant property through direct and/or indirect mechanisms that provide protective effects against DDT-induced nephrotoxicity (Mohammed et al., 2012). Silymarin was orally administered twice-daily dose (500 mg Kg^{-1}) for 7-days before DDT administration (a single oral dose of DDT (100 mg Kg^{-1}) in rats. Silymarin administration caused a significant reduction in the contents of malondialdehyde (MDA), the lipid peroxidation end product, down to (61%) with the increase in the levels of GSH levels up to (82%) in renal tissue homogenate compared to DDT-treated animals (Mohammed et al., 2012). These data suggest that silymarin could be a good candidate for preventing or treating toxicity induced by organochlorine pesticides.

Recent reviews summarized the pharmacological effects and molecular mechanisms of curcumin, a lead compound in preventing and treating oxidative-associated liver diseases, such as non-alcoholic steatohepatitis and toxicant-associated steatohepatitis (Farzaei et al., 2018; Khan et al., 2019). Curcumin is the main constituent of turmeric, the rhizome of Curcuma longa, and possesses several biological activities such as antioxidant and anti-inflammatory (Farzaei et al., 2018). Curcumin can become a phenoxyl radical, so it is considered a potent lipid-soluble antioxidant, ten times more antioxidant than vitamin E (Shishodia et al., 2005). It was found to inhibit lipid peroxidation and neutralize ROS (superoxide, peroxyl, hydroxyl radicals) (Ak and Gülçin, 2008). Mechanisms involved in the protective and therapeutic effects of curcumin in oxidative associated liver disease include suppressing the proinflammatory cytokines, PI3K/Akt and hepatic stellate cells activation, as

well as ameliorating cellular responses to oxidative stress such as the expression of Nrf2, SOD, GSH and GPx (Farzaei et al., 2018). Curcumin up regulates Nrf2 genes, which leads to a rise in GSH concentration and activity of glutathione peroxidase and SOD. Thus, it protects against free radical damage (Jagetia and Rajanikant, 2015). Curcumin can protect against hepatic injury induced by lindane, a widely used organochlorine insecticide, scavenging oxygen free radicals and ameliorating the state of oxidative stress (Singh and Sharma, 2011). The antioxidant effect of curcumin also contributes to its hepatoprotective effect against heavy metals intoxication (García-Niño and Pedraza-Chaverrí, 2014). Curcumin also has anti-inflammatory properties. It is documented that suppression of tumor necrosis factor-induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation is one of the essential anti-inflammatory mechanisms of curcumin (Aggarwal et al., 2013). NF-κB can be activated in response to oxidative damage, inducing an inflammatory response. Curcumin has also proven to be a phytodrug against the damage caused by environmental exposure to ozone, a harmful tropospheric pollutant (Nery-Flores et al., 2018). Curcumin decreased NF-κB activation and serum levels of inflammatory cytokines and protein and lipid oxidation in therapeutic and preventive approaches in the hippocampus from rats treated with acute or chronic exposure to ozone. These findings suggested that curcumin has a protective effect against oxidative stress and inflammation induced by environmental pollutants in different tissue. To our knowledge, studies on the effects of curcumin on DDE-induced oxidative stress are lacking.

Another antioxidant compound with a potential protective effect against DDE is selenium (Se), an essential trace element in animal nutrition. It has been shown to have antioxidant properties that protect against some xenobiotics' toxicity (Monteiro et al., 2009;

Ravoori et al., 2010). A recent study examined diet Se-supplementation's ability to prevent damages induced by DDE in the liver of *M. spretus* mice (Morales-Prieto et al., 2018). This study showed that Se selectively acted on a specific target, restoring the redox status and functionality of some membrane proteins involved in mitochondrial functionality, protein transport, cell signalling, and protein metabolism. However, selenium supplementation was insufficient to reduce many of the health insults associated with lipid metabolisms, such as lipogenesis and hepatic steatosis.

It has also been shown the antioxidant activity and hepatoprotective effects of ethyl acetate extract of *Rhus oxyacantha* root cortex (RE) against DDT-induced liver injury in male rats (Ben Miled et al., 2017). Pre-treatment of rats with RE at a dose of 150 and 300 mg Kg^{-1} (b.w.) significantly lowered hepatic thiobarbituric reactive substances, antioxidant enzyme activities, and MTs levels DDT-treated rats. The RE may be beneficial due to some phenolic components such as gallic acid, syringic acid, and catechol (Ben Miled et al., 2017). These results strongly suggest that treatment with ethyl acetate extract protects the liver against environmental pollutant-induced oxidative damage in rats.

The use of antioxidants to prevent or treat different health problems, including environmental pollutant toxicity, is opening a new research area to improve antioxidant treatments efficiency. A recent review highlighted the promising future of nano-antioxidant therapy against environmental pollutants induced-toxicities (Eftekhari et al., 2018). Antioxidants application has met with limited success so far, given that conventional antioxidants are poorly soluble in water or show inefficient permeability. These problems can be potentially solved using an encapsulating carrier. Nanotechnology has presented novel options in this area. Antioxidants can be encapsulated with nano-materials to form nano-

antioxidants to obtain the ideal solubility and permeability profile and preserve the probable antioxidant enzymatic degradation.

As mentioned above, curcumin has antioxidant properties and protects against hepatic damage induced by lindane. However, its usage has been confined due to insufficient water solubility and bioavailability. Nanotechnology allowed overcoming these limitations. Zabihi et al. reported nano-curcumin preparation with improved aqueous dispersion and dissolution rate (Zabihi et al., 2015). Nanocurcumin has shown a robust antioxidant activity, with attenuation of cellular ROS formation, replenishment of GSH pools, and protection against H_2O_2 in environmental toxicities or diseases (Doggui et al., 2102; Flora et al., 2013). A similar increase in antioxidant activity has also been shown for silymarin, the above-mentioned polyphenolic component, with a wide range of beneficial effects against environmental pollutant oxidative injury. The synthesis of nanoscale silymarin with precipitating technique has improved its solubility and bioavailability and has exacerbated the radical scavenging activity of crude silymarin (Hsu et al., 2012).

In summary, nano-antioxidants seem to counteract environmental pollutant-induced oxidative stress more efficiently than crude antioxidants. However, it needs further investigation before their application in practice employs non-toxic, organ/ tissue specifically, on time in situ release, easy to use, and high radical scavenging function. Moreover, robust knowledge of pharmacokinetics parameters and biological processes should be taken into account in the context of designing appropriate nano-antioxidants. Meanwhile, it could help underline that correct lifestyle, including diet habits and physical activity, is essential for human health. Physical exercises can effectively upregulate antioxidant enzyme expression and activity (Bloomer, 2008; Lima et al., 2015). A positive effect of an aerobic exercise

intervention on hepatic DDT degradation and oxidative stress in rats (Li et al., 2017). Exercise-induced consistent increases in hepatic SOD activity by effectively upregulating SOD activity. Besides, aerobic exercise enhanced CAT and GPx activities and promoted MDA scavenging (Li et al., 2017). This finding may provide new insight into aerobic exercise's function in preventing environmental pollutant-induced oxidative stress and confirm that moderate aerobic exercise is an efficient strategy for public health promotion.

3.9 Endocrine-disrupting compounds' effects on mitochondria via steroid receptors. Can be the link between DDE and mitochondria?

As above discussed, DDE and other man-made EDCs can induce some alterations in the endocrine system. It can be through a variety of mechanisms, including the intracellular pathways of steroid hormone receptors, namely ER-(alpha, -beta), AR, or PR, and their responsiveness to hormones (Chen et al., 2004; Ropero et al., 2006; Shanle and Xu, 2010; Watson et al., 2014; Kiyama and Wada-Kiyama, 2015; Hanson et al., 2017). Moreover, EDCs can bind to ER and alter gene expressions by genomic pathway due to ER as transcription factors (Lee et al., 2013). On the other hand, in a non-genomic way, EDCs bind to membrane-bound ER and rapidly initiate signalling cascades influencing cellular function by post-translational modifications of various proteins and epigenome (Watson et al., 2011; Viñas et al., 2012; Casati et al., 2015). However, endocrine disruption mechanisms remain unclear for most EDCs (Zhuang et al., 2012).

Recently, nuclear steroid and thyroid receptors in mitochondria have been reported in various somatic cells (Scheller et al., 2003; Psarra et al., 2006, 2008; Milanesi et al., 2008). ER seems to function as a transcription factor for mitochondrial DNA genes (Figure 3.5)

(Chen et al., 2004; Simpkins et al., 2008) via Estrogen Response Elements in the promoter region of estrogen-response genes present in the mitochondrial genome (Yager and Chen, 2007). Notwithstanding, Yang and colleagues (2004) indicate that ER-beta is a mitochondrial protein rather than a nuclear receptor, which might explain this receptor's predominance in the mitochondria. Therefore, EDCs could act not only through nuclear ER but also directly in mitochondria through mitochondrial ER-beta (Figure 3.5). This finding suggested that ER-beta could play a vital role in the estrogen effects on mitochondria, including its ability to modulate calcium influx, ATP production, ROS production, and apoptosis. Moreover, other researchers have recently reported the ER-alpha variant, ER-alpha36, in the mitochondrial membrane. This ER variant could play a pivotal role in non-genomic signalling and mitochondrial functions in human uterine smooth muscle and leiomyoma cells (Yan et al., 2017).

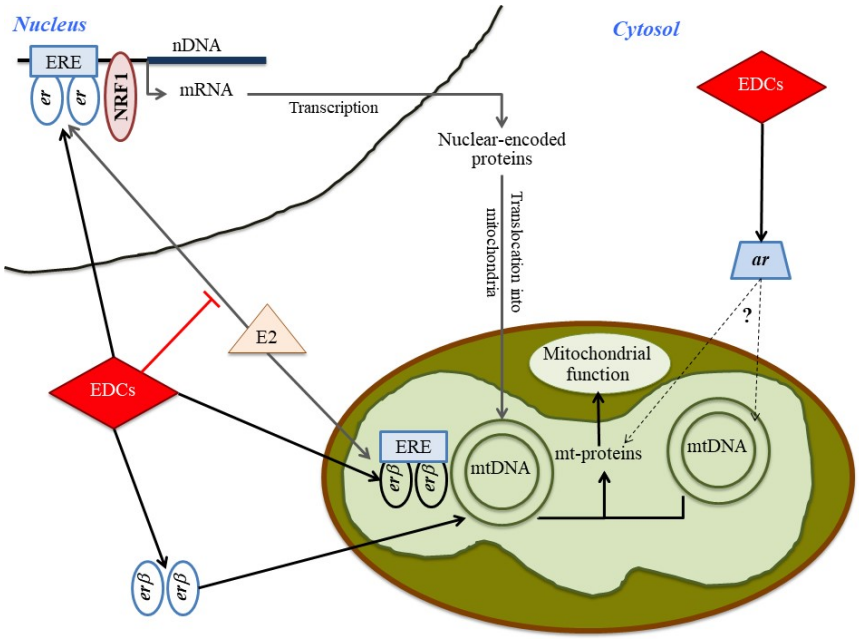

Figure 3.5. Potential effects of endocrine-disrupting chemicals (EDCs) on the expression of nuclear DNA and mitochondrial DNA proteins essential for mitochondrial function. The EDCs bind to nuclear estrogen receptors (er) and modulate mitochondrial genes' expression (mt-genes). Their proteins expressed are imported into mitochondria. The EDCs can also bind to mitochondrial er-beta (er-β) and modulate mitochondrial-encoded mitochondrial gene transcription. The direct effect of EDCs through the androgen receptor (ar) is less studied.

In conclusion, all these data regarding the role of steroid hormone receptor activation in modulating mitochondrial function suggested that DDE could affect mitochondria by altering the steroid hormone receptor responsiveness. Further studies are needed to clarify this aspect and help understand the adaptive cellular response to xenobiotics, such as DDE, and the induction of pathological pathways triggered by environmental pollutant exposure.

3.10 Conclusions and perspectives

EDCs are ubiquitous environmental pollutants and can be found in our daily lives and food. They can act as hormone receptor mediators and deregulate homeostatic mechanisms, reproduction, and immune responses. The accumulation of DDE in living tissues leads to questions regarding how such compounds accumulate and whether such cumulation has toxicity independently of the endocrine disruption effects, including cellular stress generation. Consequently, mitochondria are good predictors for acute toxicity at the metabolic level, and new investigations on mitochondrial function and morphology are necessary. Thus, evaluating mitochondrial bioenergetics can be used as a toxicological indicator, as a first-line economic model to detect the possible effects of xenobiotics (Iero et al., 2003; Fernandes et al., 2007; Pshenichnyuk and Modelli, 2013; Pant et al., 2014; Yeh et al., 2017). Moreover, mitochondrial function and ROS production can target classical or new antioxidant compounds to prevent or treat environmental pollutant-induced cellular stress. Noteworthy, the effect of EDCs at the molecular level has been gaining ground, and their potential epigenetic effects have renewed the focus of these legacy pesticides. Since EDCs not only alter the exposed individual but can also have transgenerational effects (Collotta et al., 2013; Kabasenche and Skinner, 2014; Vandegehuchte and Janssen, 2014). Therefore, assessing a potentially toxic environment's

long-term health effects is challenging. Individuals may or may not experience clinical health problems from being exposed to increasing environmental pollution. Furthermore, a matter of great concern is that unexposed progeny may also encounter the consequences of these ancestral exposures (Van Cauwenbergh et al., 2020). Currently, there is a great interest in understanding epigenetics in ecotoxicological field studies. It has a substantial potential to broaden our understanding of the molecular mechanisms of pesticide health effects, which opens up new perspectives for estimating the risk of man-made EDCs. Thus, epigenetics changes can be another promising area for further inquiry with the development/use of epigenetic biomarkers. These can be used in the future as a diagnostic tool to evaluate the susceptibility of a person to a specific disease, or exposure to environmental toxicants is necessary (Nilsson et al., 2018). Moreover, to gain further insight into the spectrum of environmental pollutants toxicities and their mechanisms of cellular adaptation or stress, future work should focus on carefully studying individual and mixture components across various concentrations to examine the cellular pathways stimulated in various tissue types.

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4. Experiment 1

Dose-dependent response to the environmental pollutant dichlorodipheniletylhene (DDE) in HepG2 cells: focus on cell viability and mitochondrial network.

4.1 Abstract

Dichlorodiphenyldichloroethylene (DDE) is the primary persistent metabolite of the pesticide dichlorodiphenyltrichloroethane (DDT). Its toxic effects on cells could be linked to mitochondrial network impairment associated with an imbalance between mitochondrial fusion and fission processes. The present work aimed to study the impact of increasing DDE doses on cell viability and mitochondrial markers in an in vitro hepatic cell model. Human hepatocarcinomatous cells (HepG2) were stimulated with a wide range of DDE doses (0.5-100 μM) for 24 h. Decreases in cell viability were observed with the highest DDE doses (50, 100 μM). Given the lipophilic proprieties of DDE, we also analyzed cell viability in the presence of fatty acids (saturated, monounsaturated, or polyunsaturated fatty acids). Cells cotreated with DDE and fatty acids showed reduced cell viability even with low DDE doses and a further cell viability reduction with the highest DDE dose, mainly saturated fatty acids. Fusion protein markers (mitofusin 2 and Optical Atrophy 1) exhibited an inverted U-shape dose-response curve, showing the highest content in the 2.5-25 μM DDE dose range. A similar trend was observed for glucose-regulated protein 75, a chaperon involved in mitochondria-endoplasmic reticulum interaction. On the other hand, the fission protein marker, dynamin-related protein1, was found significantly increased even with high DDE doses, leading to an increased DRP1/MFN2 ratio. The antioxidant enzyme mitochondrial superoxide dismutase isoform 2 showed a reduction in its content in a dose-response way, suggesting mitochondrial

impairment with the highest DDE doses. Present results indicated that DDE low doses elicited cell adaption, stimulating mitochondrial dynamic machinery to counteract the DDE effect, preserving cell viability. However, high DDE doses induced cell viability loss associated with mitochondrial fusion/fission balance and antioxidant defence impairments. Present results are helpful to clarify the mechanisms underlying the cell fate towards survival or death in response to increasing doses of environmental pollutants.

4.2 Introduction

It has been reported that mitochondria represent the primary or secondary site of action of many chemical compounds capable of producing an increase in the reactive oxygen/nitrogen species (ROS/RNS) levels in cells (Shaughnessy et al., 2010; Moreira et al., 2011; Naranmandura et al., 2011; Knecht et al., 2013; Meyer et al., 2013; Bestman et al., 2015; Brunst et al., 2015; Caito and Aschner, 2015; Kang and Hamasaki, 2002; Venkatraman et al., 2004; Migliaccio et al., 2019a, 2019b, 2019c). Among the persistent organic pollutants (POPs) produced by human activities, polychlorinated biphenyl compounds (PCBs) represent a heterogeneous group of chemical species, including some insecticides, with toxic effects on human health (Roubicek and de Sousa Pinto, 2017). In literature, it has been reported that PCBs act as endocrine-disrupting chemicals (EDCs), inducing hormonal and metabolic disorders and altering energetic mitochondrial homeostasis and the endoplasmic reticulum (EnR) (Nadal et al., 2017). Consequently, researchers identified among EDCs a lot of metabolism-disrupting chemicals (MDCs) with obesogens and diabetogens proprieties (Meyer et al., 2013; Heindel et al., 2017; Nadal et al. 2017;

Marroqui et al., 2018). The ancestral pesticide dichlorodiphenyltrichloroethane (DDT) and its metabolites (Heindel et al., 2017) are included. DDT was used mainly against several insects, vectors of human parasites. It was used primarily to control malaria cases during the second world war and post-war period. Because of its efficiency as a chemical insecticide, DDT utilization was massively extended to agriculture, producing accumulation in soil, water surface, and other environmental compartments (Harley et al., 2008; Malusá et al., 2020). Today, DDT utilization is ban in different countries due to its toxic power. However, WHO still encourages DDT to use sanitary aim in equatorial world zones where malaria cases persist and endemics (van den Berg et al., 2017). Given its hydrophobic proprieties, human exposure to DDT is mainly associated with dietary intake, consuming contaminated fatty foods like fish, milk, and fat-meat, where it could be accumulated in lipids (Schecter et al., 2010). Once inside the organism, DDT can be converted to its first major persistent metabolite, dichlorodiphenyldichloroethylene (DDE), through a dechlorination reaction (Kwong et al., 2009). Therefore, both DDT and DDE can be biomagnified in the food chain (Kidd et al., 2001) and bioaccumulated in adipose tissue and liver for many years (Mota et al., 2011; Arroyo-Salgado et al., 2016; Liu et al., 2017). It has been reported that the effect of DDE exposure can also be transmitted across generations resulting in metabolic disorders at generation F3 (Skinner et al., 2013), producing long-term toxicity (Dich et al., 1997). According to the US National Cancer Institute (NCI), the liver appears to be the principal target organ of DDE in mammalian species (USEPA, 2008), probably because hepatic cells have high sensitivity to xenobiotics exposure (Arroyo-Salgado et al. 2016). Moreover, metabolic detoxification processes occur in hepatocytes (Holloway, 1981; Angrish et al., 2016), involving EnR, mitochondria, and lysosomes (Cribb et al., 2005). Bioenergetics injury

on both humans and wildlife by DDE exposure has been reported (Elmore and La Merrill, 2019). Mitochondrial dysfunction was associated with an elevation in ROS generation, decrease in mitochondrial membrane potential, and release of cytochrome c (Cyt c) into the cytosol, which leads to apoptosis (Shi et al., 2010; Song et al., 2008, 2011; Quan et al., 2016), promoting obesity and insulin-resistance/diabetes onset (Sivitz, 2010; Kim et al., 2014; Bhatti et al., 2017; Di Meo et al., 2017; Ramalingam et al., 2017; Verma et al., 2017; Lefranc et al., 2018).

Mitochondria are essential organelles mainly involved in ATP production and cell death programming regulation by a self-destructing system, including apoptosis and cellular necrosis (Meyer et al., 2013; Wallace, 2013; Ni et al., 2015). Therefore, an adequate mitochondrial quality should be maintained in cells to avoid metabolic alterations and cellular death (McBride et al., 2006; Picard et al., 2016). Mitochondrial toxicity is a crucial determinant in the pathogenesis or biological adaptation to various chemical exposures (Wallace, 2017). Thus, the details of how different environmental species affect mitochondria and cellular redox homeostasis represent areas of rapid research growth. It has been shown that DDT and DDE can alter the mitochondrial respiratory chain and, consequently, ATP production (Elmore and La Merrill, 2019). Moreover, alteration in protein content and enzymatical activity of cytosolic and mitochondrial antioxidant enzymes were associated with DDT/DDE exposure (Song et al., 2014; Marouani et al., 2017; Migliaccio et al., 2019a). Manganese-dependent mitochondrial superoxide dismutase isoform 2 (MnSOD, SOD2) represents one of the first mitochondrial-associated antioxidant defences used by cells to quench superoxide anion produced by mitochondria (Zelko et al., 2002). It has been reported that

reduction in SOD2 protein levels or functional defects in the enzymatical activity were associated with hepatic dysfunction and oxidative stress generation (Liu et al., 2013). A condition implicated in many multifactorial chronic diseases (Sherer et al., 2002; Wallace, 2005; Koshiba, 2013; Suárez-Rivero et al., 2016; West, 2017).

Mitochondria can use several mechanisms to maintain their physiological homeostasis (Ni et al., 2015; Meyer et al., 2017), and the dynamic behaviour, i.e., the balance between fusion and fission processes, is considered cornerstones for cell survival (Westermann, 2010; Vander Bliek et al., 2013). Alteration in mitochondrial dynamics has been associated with various diseases, including nervous system impairment, endothelial dysfunction, and mitochondrial functional defects (Shenouda et al., 2011; Suárez-Rivero et al., 2016; Schmukler et al., 2020). Mitochondria are structurally constituted by two different membranes commonly defined as mitochondrial inner and outer membranes (MIM and MOM). Distinct proteins were found involved in dynamics machinery to complete both fusion and fission. Regarding the fusion process, two different mitochondrial GTPase named mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2) were involved in MOM fusion. On the other end, MIM fusion was found regulated by a GTPase named Optical Atrophy 1 (OPA1), which was also found implicated in mitochondrial cristae remodelling and regulation of apoptosis (Patten et al., 2014). Concerning the fission process, this mechanism was found essentially mediated by the dynamin-related protein1 (DRP1), a cytosolic GTPase that interacts with a mitochondrial receptor protein known as fission protein 1 (Fis1) forming a ring around mitochondria to induce fragmentation (Twig et al., 2008; Otera et al., 2010; Westermann, 2010). Both fusion and fission events in cells play a pivotal role in maintaining lifespan and mitochondrial health. In fact, through the fusion process, the exchange of materials between healthy mitochondria

can occur (Twig et al., 2008; Van der Bliek et al., 2013). On the contrary, through fission, the damaged mitochondria, or a part of them, could be segregated and eliminated. Moreover, organelles fragmentation ensures their distribution into the cell during cell division, which guarantees ATP requirement (Mishra and Chan, 2014). These two-linked sets of opposed dynamic processes are critical to mitochondrial redistribution and network and allow the cell to respond to its ever-changing physiological conditions (Westermann, 2010; Van der Bliek et al., 2013; Meyer et al., 2017). However, experimental evidence demonstrated that many factors and diseases could modulate mitochondrial dynamics, including nutrients, inflammatory events, cancer, and chemical stressors (Lionetti et al., 2014; Eisner et al., 2018; Ježek et al., 2018). As far as we know, the effect of DDE on a mitochondrial protein involved in mitochondrial dynamic processes in the frame of adaptive or toxic response to different pesticide doses has not yet been analyzed.

The present study's main aim was to analyze the dose-dependent response to DDE exposition in terms of cell viability and mitochondrial protein fusion, and fission content in human hepatocarcinoma cell line HepG2. Scientists consider this cellular model a suitable model for in vitro studies for hepatic toxicology (Ferreira et al., 1997; Knasmüller et al., 2004). Firstly, we analyzed the effect of different DDE doses on cell viability. Given DDE lipophilic properties, we also examined the impact of DDE in association with other dietary fatty acids (i.e., palmitate as a saturated fatty acid, oleate as a monounsaturated fatty acid, and EPA+DHA mixture as polyunsaturated fatty acids) on cell viability and lipid accumulation. Then, we focused our attention on the dose-dependent response to DDE exposure in terms of the content of protein involved in mitochondrial dynamic behaviour, including the fusion proteins MFN2

and OPA1 and the fission protein DRP1. We also analyzed the contents of a protein linked to mitochondrial function and dynamic, such as glucose-regulated protein 75 (GRP75), a molecular chaperon involved in mitochondria-EnR communication (Tubbs et al., 2014), and the mitochondrial SOD2 involved in the cellular response to oxidative stress (Migliaccio et al., 2019c). The present work showed a dose-dependent decrease in cell viability associated with an adaptive mitochondrial response with a pro-survival function in response to low DDE doses but with mitochondrial fragmentation and cell loss viability in response to high DDE doses.

4.3 Material and Methods

4.3.1 Cell culture and treatments

HepG2 cells 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% CO_2, 95% air-humidified atmosphere, and passaged twice a week. Cells were seeded at the density of 4.0 x $10^4/cm^2$ and cultured for 24h before DDE exposure.

A stock solution of DDE was prepared to dissolve pesticide in dimethylsulfoxide (DMSO). For each treatment, the final concentration of DMSO in the culture medium was 0.1%, which represents a non-toxic concentration for hepatocytes (Nikolaou et al., 2016). Cells were exposed to DDE for 24h using a wide range of pesticide dose (0.5 µM; 1 µM; 2.5 µM; 5 µM; 10 µM; 25 µM; 50 µM and 100 µM). A control group of cells (no-DDE treated group) was only treated with DMSO (0.1%). In the coincubation experiments with fatty acids,

cells were co-treated for 24h with the different doses of DDE and 250 µM of palmitate, oleate, or EPA+ DHA 1:1 mixture.

4.3.2 Fatty acid-BSA conjunction preparation

Oleate and Palmitate (Merck) 40 mM stock solutions were prepared to dissolve fatty acids in NaOH 0.1 M at 70ºC for 15 min under stirring. A 10% BSA fatty acid-free (Sigma-Aldrich) solution was dissolved at 55º in NaCl 0.9% and mixed to fatty acids solutions 4:1 v/v, respectively. The resulting 8mM fatty acid stock solutions were filtered using a syringe equipped to filter (0.2 nm), aliquoted, and stored at -20°C. Concerning both EPA and DHA (Merck), 40 mM stock solutions were prepared to dissolve fatty acids in methanol as indicated by the manufacturer and mixed 1:1 v/v to obtain a stock solution EPA+DHA. Then, fatty acids were co-incubated with BSA in a culture medium. The final fatty acids concentration used in the study (250 µM) was prepared, freshly, in cell culture medium at the moment of treatments.

4.3.3 Cell viability

The cytotoxicity was assessed by a 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 (Mosmann, 1983). Evaluate cell viability in the presence of DDE; after DDE treatment for 24 h, 0.5 mg mL^{-1} of MTT was added to the cell medium and incubated for 1.5 h at 37 ºC to allow MTT to be metabolized. The resulting formazan crystals were dissolved in DMSO, and the absorbance of the resulting suspension was measured at 595 and 655 nm to subtract background in each one 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% (Lepretti et al., 2015). MTT analysis was performed by using three different control groups of cells: non-treated cells (NT); DMSO-stimulated cells (used as the control group for DDE stimuli); BSA treated cells (used as the control group for fatty acids).

4.3.4 Western-Blot

Western blotting analysis was used to evaluate the number of principal markers involved in the mitochondrial dynamics (MFN2, OPA1, and DRP1) and the mitochondrial antioxidant enzyme SOD2, and the cellular chaperone GRP75 involved in the mitochondria-EnR communication/connection. After treatments, cells were washed in PBS to remove the grow medium, scraped, and lysed in RIPA buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% SDS, 1 mM, NaOVa, 1 mM PMSF, and protease inhibitors cocktail (Sigma-Aldrich, Milan, Italy). Cells were broken using an insulin syringe equipped with a thin needle. After lysis, the samples were centrifuged at 12,000 x *g* for 15 min at 4° C. Protein content was quantified by using Bio-Rad quantification method's and 30 µg of protein for each sample were loaded onto SDS-polyacrylamide electrophoretic gel (SDS-PAGE) 13% Acrylamide/Bis-Acrylamide 29:1 solution, together with a conventional protein marker. After running, proteins were transferred on PVDF membrane (Ge-Health care, P0.45) for 2h. After second run, membranes were washed in 0.1% Tween-TBS (T-TBS), incubated in blocking buffer solution (5% milk/0.1% T-TBS) for 1h at room temperature and then, incubated O.N. with the antibodies of interest: MFN2 (sc-100560, 1:1000; Santa Cruz Biotechnology); OPA1 (sc-30572, 1: 1000, Santa Cruz Biotechnology); DRP1 (sc-3298, 1:1000; Santa Cruz Biotechnology); SOD2 (PA5-30604, 1:1000, Thermo Scientific); GRP75 (sc-13967, 1:2000;

Santa Cruz Biotechnology). To normalize protein levels in samples, GAPDH (ab8245, 1:2000, Abcam) was used as a loading control guide. The second day, membranes were washed in 0.1% T-TBS, incubated for 1h at RT with the appropriate secondary antibody anti-mouse and anti-rabbit (1:10,000; Bio-Rad Laboratories) or anti-goat (1:5000, Santa Cruz Biotechnology) and then washed again in 0.1% T-TBS to remove the unbounded excess of secondary antibody. To detect the protein bands, each membrane was incubated 5 minutes with ECL solution (Millipore, reagent A + reagent B), exposed to autoradiographic filters, and developed by an automatized developer mashing. Protein bands were subjected to densitometric analyses and normalized with the relative bands of the correspondent loading control GAPDH.

4.3.5 Oil Red-O staining

To detect lipid content in the hepatocytes, cells were stained using Oil-Red O in qualitative and quantitative analyses. For qualitative experiments, cells were grown onto circular slides in a 24-multiwell plate dish. For each well, 110.000 cells were plated. After 24h, cellular treatment was started as previously described. Then, cells were washed using PBS at 37°C and fixed in 4% PFA for 45min at room temperature. After fixing, cells were washed twice using distilled water and stained as indicated by Oil-Red-O conventional protocols. Briefly, cells were firstly incubated in isopropanol 60% for 5 minutes, and then, lipid droplets were stained using oil red-O working solution composed of 3 parts of oil red-O stock solution (0.5g oil Red/100mL isopropanol 100%) and 2 parts of distilled water filtered using a syringe equipped with a 0.22 µm filter. After staining, cells were washed 3-5 times

with distilled water and photographed using a light optical microscope fitted with a TV camera.

To quantify oil-Red O accumulated in cells, HepG2 were grown in a separate 24 multiwell plate dish. As previously described, the stain was extracted by cells using 300 µl isopropanol 100% after the procedure. Finally, 100 µl of each sample in triplicate was transferred in a 96-multiwell plate. The relative oil-Red O extracted by cells was measured using a 96 well plate reader at 490 nm. Quantitative and qualitative analyses were compared to DMSO-stimulated cells.

4.3.6 Statistics

All data were expressed as mean ± standard error on the media (SEM). Each experiment was produced in biological triplicate or more. The differences between samples were analyzed using GraphPad Prism software (GraphPad Software Inc. San Diego, CA, USA), applying a one-way ANOVA test followed by Bonferroni's post hoc test. Students t-test also was used as indicated in figure captions. Differences were considered statistically significant at a P-value < 0.05.

4.4 Results

4.4.1 Dose-dependent effect of DDE on cell viability

To investigate the outcome of DDE on cell viability, we examined the dose-response effect of this chemical on cell viability by using an MMT assay. A dose-dependent decrease in cell viability was observed with no significant effect to the low DDE doses, and with the highest decreases to the highest doses (50 µM and 100 µM), where cell viability values of

64% and 46%, respectively, compared to control cells (100%) were observed (Figure 4.1 A). In the light of DDE lipophilic proprieties, we also tested the dose-dependent effect of DDE on viability in cells cotreated with different dietary fatty acids (i.e., palmitate as a saturated fatty acid, oleate as a monounsaturated fatty acid, and EPA+DHA mixture as polyunsaturated fatty acids). Cells treated only with 250 µM of palmitate or 250 µM of oleate reduced their viability values to 71% and 76%, respectively, compared to control cells. In contrast, cells treated with 250 µM EPA+DHA mixture did not show any change in viability (Figure 4.1 B, C, D). Notably, cells cotreated with fatty acids and increasing DDE doses exhibited a higher dose-dependent reduction in viability than cells only treated with DDE. Cells cotreated with 250 µM of palmitate and different doses of DDE showed a significant dose-dependent decrease in cell viability starting from low doses, showing values of 43%, 33% and 28% with 2.5 µM, 5 µM, and 100 µM DDE, respectively, compared to control cells (100%) (Figure 4.1 B). A similar trend was observed with the cotreatment with oleate but with a lower degree of toxicity. Cell viability was reduced to the values of 66% and 55% with 2.5 µM, 5 µM, and 100 µM DDE, respectively (Figure 4.1 C). Dose-dependent decreases in cell viability values were less marked mainly with high DDE doses when cells were cotreated with EPA+DHA mixture (54%, 56%, and 47% values with 2.5 µM, 5 µM, and 100 µM DDE dose, respectively) (Figure 4.1 D).

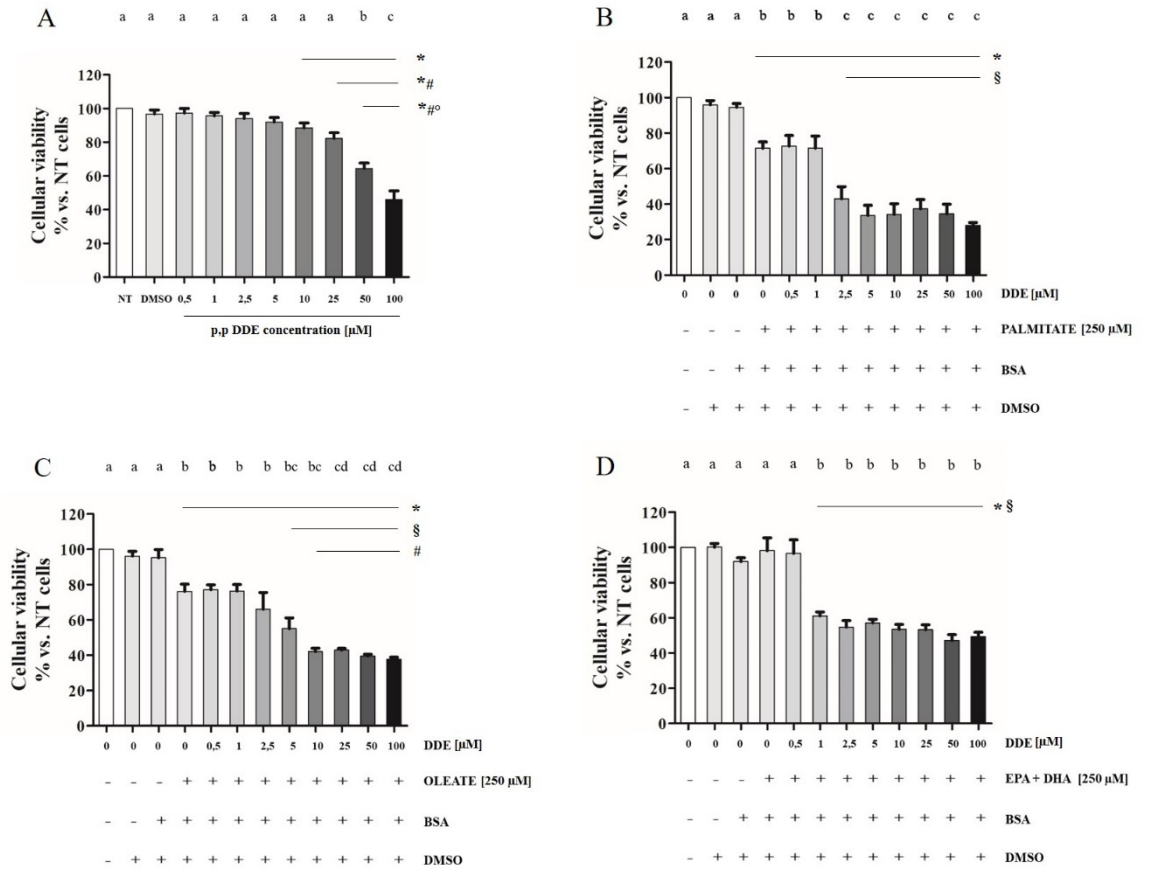

Figure 4.1. HepG2 cellular viability. Data were obtained stimulating cells with different doses of DDE (0.5 µM, 1 µM, 2.5µM, 5 µM, 10 µM,25 µM, 50 µM, and 100 µM) (A) or with DDE in association with palmitate (250 µM) (B), oleate (250 µM) (C), or EPA+ DHA (1:1) mixture (250 µM) (D). Results were graphically represented as media ± standard error of the media (SEM) of at least 3 biological replicates. Statistical analyses were performed by using GraphPad Prism software. One-way ANOVA analysis followed by Bonferroni's posthoc test: different letters indicate statistically different values. Student T-test: Graph A) * P<0.05 vs. DMSO; # P<0.05 vs. 5 µM DDE; ° P<0.05 vs. 25 µM DDE; Graph B) * P<0.05 vs. BSA; $ P<0.05 vs. 1 µM palmitate; Graph C) * P<0.05 vs. BSA; § P<0.05 vs. oleate; # P<0.05 vs. 5 µM DDE; Graph D) * P<0.05 vs. BSA; § P<0.05 vs. EPA+DHA.

4.4.2 Dose-dependent effect of DDE on lipid depots in cells cotreated with dietary fatty acids

Qualitative and quantitative tests were performed using the oil-Red O method to detect total intracellular lipid accumulation in cells treated with DDE alone or associated with fatty acids. In cells treated only with DDE, both microscopic (Figure 4.2A) and spectrophotometric

(Figure 4.2B) analyses did not show any differences in lipid accumulation between DDE-treated and DMSO-treated cells.

As expected, lipid content significantly increased in the presence of fatty acids (Figure 4.3). Cells treated with 250 µM of palmitate, oleate, or EPA+DHA mixture (1:1) increased lipid content by 52%, 72%, and 98%, respectively, compared to control cells (Figure 4.3 C, E, G). Microscopy images confirmed the increasing intracellular fat depots with the growing degree of fatty acid unsaturation, with EPA+DHA treated cells showing the highest oil red staining.

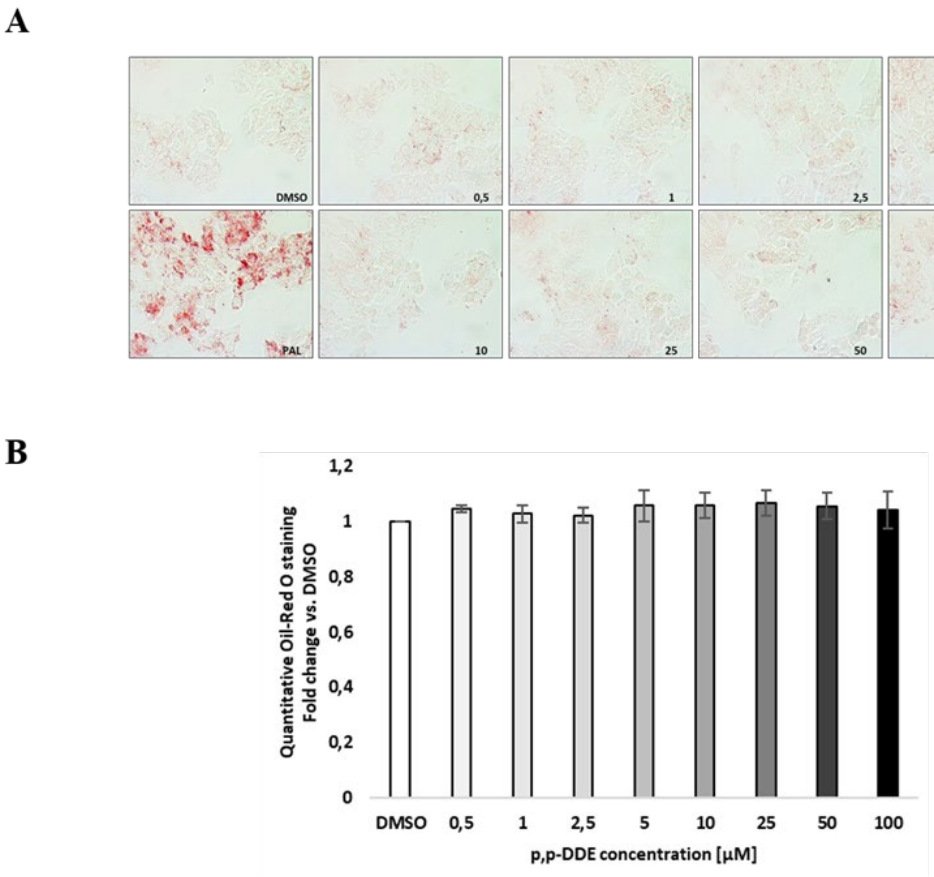

Figure 4.2. Total lipid content in response to different doses of DDE. Qualitative (A) and quantitative (B) analyses of total lipid content were performed using Oil-Red O staining. Panel A showed representative images of lipid droplets accumulated in cells after exposure to DMSO and DDE-doses. Positive control was included in the analysis to validate lipid accumulation, stimulating a group of cells with sodium palmitate (PAL), 250µM. Original image magnification used: 40X. Panel B showed Oil-Red O quantification measured spectrophotometrically (490nm). Data were graphically represented as media ± standard error on the media (SEM) for 4 biological replicates.

Notably, in cells treated with fatty acids associated with increasing DDE doses, we observed a slight progressive reduction in intracellular fat content in response to the increasing DDE dose. Cells treated with 250 μM of palmitate and increasing doses of DDE, showed a dose-dependent decrease in fat depots, with an increase in fat content of 43% and 26% with 0.5 μM and 100 μM DDE, respectively, compared to control (Figure 4.3 B, C). In contrast, palmitate alone induced a 52% increase.

A similar trend was observed with oleate, with an increase of 63% and 33% in fat content with 0.5 and 100 μM DDE, respectively, compared to control (figure 4.3 D, E), whereas oleate alone induced a 72% increase. DDE dose-dependent increase in the intracellular fat depot was lower in the presence of EPA+DHA mixture with a rise of 83% and 43% with 0.5 μM and 100 μM DDE, respectively, compared to control (Figure 4.3 F, G). In contrast, EPA/DHA mixture alone induced a 98% increase.

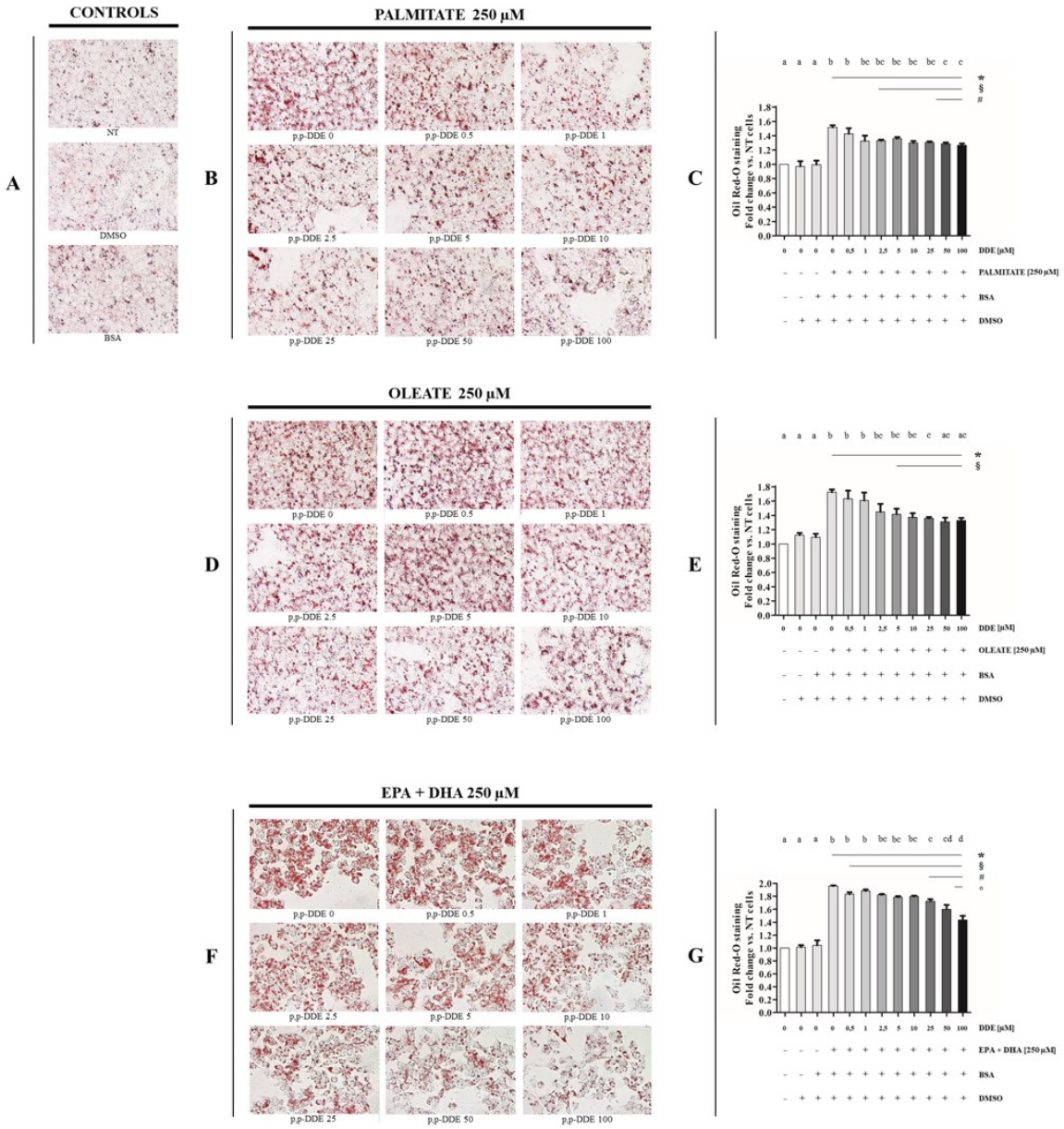

Figure 4.3. Total lipid content in response to different DDE doses coincubated with palmitate, oleate, or EPA/DHA mixture. Qualitative (A, B, D, F)) and quantitative (C, E, G) analyses of total lipid content were performed using Oil-Red O staining. Original image magnification used: 40X. Panel B showed Oil-Red O quantification measured spectrophotometrically (490nm). Data were graphically represented as media ± standard error on the media (SEM) for 4 biological replicates. Panel A showed representative images of lipid depots in control cells (NT, DMSO, BSA); panel B showed representative images of cells treated with palmitate or with palmitate and increasing DDE doses; panel D showed representative images of cells treated with oleate or with oleate and increasing DDE doses; panel F showed representative images of cells treated with EPA+DHA or with EPA+DHA and increasing DDE doses. Quantitative analyses were also obtained quantifying oil Red-O accumulation in cells. Original image magnification used: 40X. Graphs C, E, and G showed Oil-Red O quantification measured spectrophotometrically (490nm). Data were graphically represented as media ± standard error on the media (SEM) for 4 biological replicates. Three different control groups of cells were tested (non-treated cells, NT; DMSO, and BSA) to specifically evaluate the effect of DDE on fatty acid deposition. Statistical

analyses were performed by using GraphPad Prism software. One-way ANOVA analysis followed by Bonferroni's posthoc test: different letters indicate statistically different values. Student T-test: Graph C) * P<0.05 vs. control cells; § P<0.05 vs. palmitate; # P<0.05 vs. palmitate + 5 µM DDE; Graph E) * P<0.05 vs. control cells; § P<0.05 vs. oleate; Graph G) * p<0.05 vs. control cells; § P<0.05 vs. EPA+DHA; # P<0.05 vs. EPA+DHA + 5 µM DDE; ° P<0.05 vs. EPA+DHA + 25 µM DDE

4.4.3 Dose-dependent effect of DDE on mitochondrial content of proteins involved in fusion and fission processes

To evaluate the dose-dependent effect of DDE on mitochondrial dynamic machinery, western blot analysis was performed to measure cellular contents of proteins involved in mitochondrial fusion and fission processes, namely MFN2 and OPA1, as fusion markers, and DRP1, as a fission marker. Figure 4.4 showed that Mfn2 content significantly increased in response to DDE in a dose-dependent manner, reaching the highest increases (about +35%) in the 2.5-25 µM DDE dose range compared to control cells. However, Mfn2 contents decreased progressively with higher DDE doses and reached the control cells value with the highest DDE dose (100 µM). Thus, the dose-dependent response of MFN2 content to DDE showed an inverted U-shape pattern (Figure 4.4 A). In line with Mfn2 protein levels, OPA1 protein content described a similar inverted U-shape pattern (Figure 4.4 B) and reached the highest increases (about +25%) in the range 5-25 µM DDE.

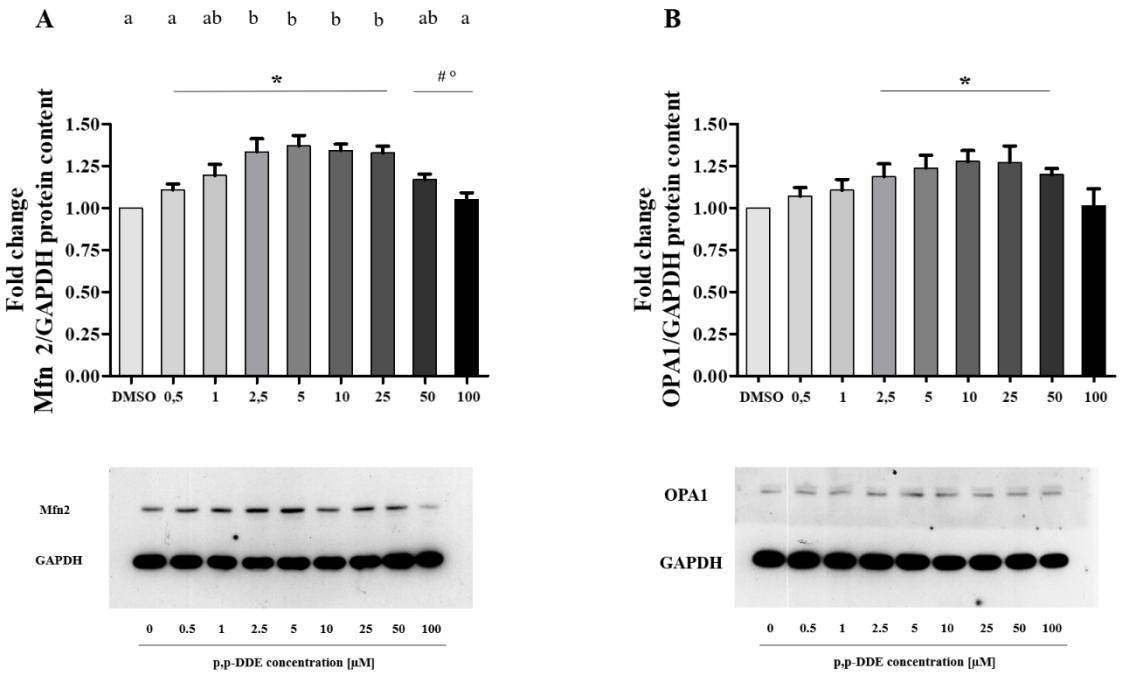

Figure 4.4. Mitochondrial fusion protein content. MFN2 (A) and OPA1 (B) protein content were analyzed by western blotting. All data were presented as mean ± standard error on the media (SEM) for 5 (MFN2) and 3 (OPA1) biological replicates. Representative images of both MFN2 (A) and OPA1 (B) (above) and the relative loading control for protein normalization GAPDH (below). Statistical analyses were performed by using GraphPad Prism software. One-way ANOVA analysis followed by Bonferroni's posthoc test: different letters indicated statistically different values. Student T-test: Graph A) * P<0.05 vs. DMSO; # P<0.05 vs. 5 µM DDE; ° P<0.05 vs. 25 µM DDE Graph B) * P<0.05 vs. DMSO.

Concerning fission marker DRP1, the relative protein content described a progressive-increase pattern in response to DDE concentrations up to the dose of 50 µM, with a significant increase (+30%) in the range 10-50 µM compared to control cells. On the other hand, the increase in DRP1 content was lower (+17%) with 100 µM DDE than the increases observed with lower DDE doses (Figure 4.5). Therefore, the dose-response curve of DRP1 to increasing amounts of DDE also showed an inverted U-shape pattern, with the highest point shifted towards higher doses compared to MFN2 and OPA1 dose-response curves.

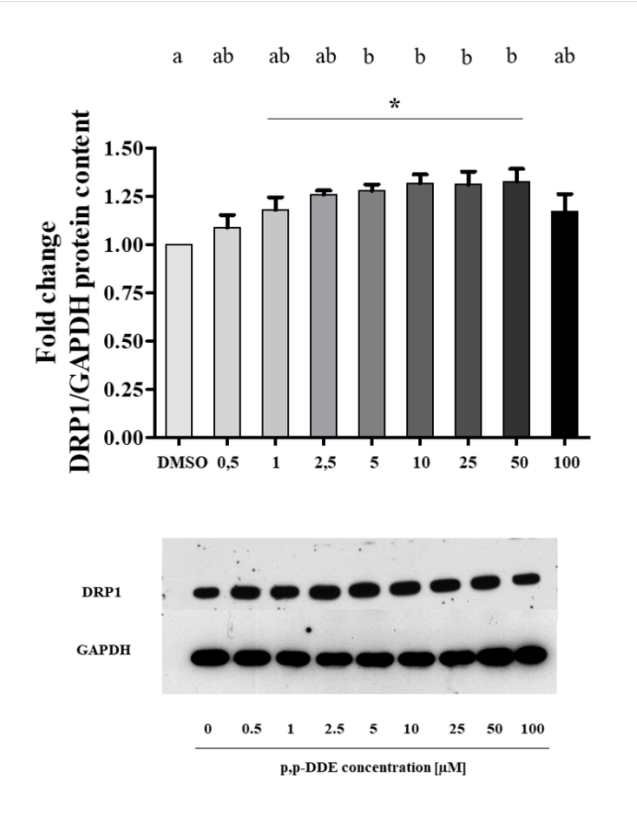

Figure 4.5. Mitochondrial fission DRP1 protein content. DRP1 protein levels were analyzed by western blotting. All data were presented as mean ± standard error on the media (SEM) for 5 biological replicates. The figure showed the representative image of DRP1 (above) and the relative loading control for protein normalization GAPDH (below). Statistical analyses were performed by using GraphPad Prism software. One-way ANOVA analysis followed by Bonferroni's posthoc test: different letters indicate statistically different values. Student T-test: * P<0.05 vs. DMSO.

4.4.4 Dose-dependent effect of DDE on mitochondrial antioxidant enzyme SOD2 and mitochondria-ER chaperon GRP75

To study the effects of DDE on the mitochondrial antioxidant system, we analyzed the levels of mitochondrial enzyme SOD2 on total cell lysates. The results showed that a progressive reduction in SOD2 protein content was observed in the range 25-100 µM DDE and reached a significant value (about -20%) with the highest dose (100 µM), compared to DMSO treated cells (Figure 4.6).

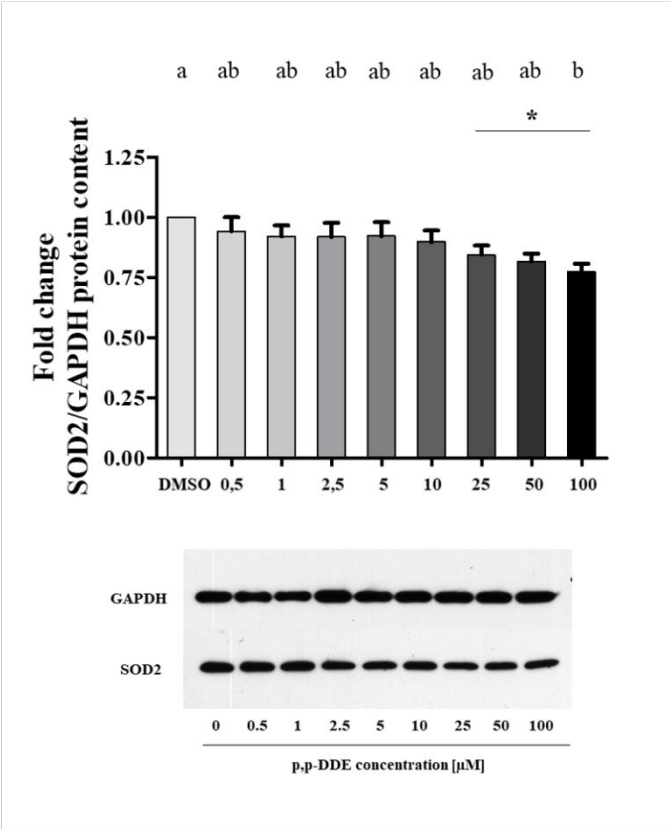

Figure 4.6. Mitochondrial antioxidant enzyme SOD2 protein levels. All data were presented as mean ± standard error on the media (SEM) for four biological replicates. The figure showed representative images of SOD2 (below) and the relative loading control for protein normalization GAPDH (above). One-way ANOVA analysis followed by Bonferroni's posthoc test: different letters indicate statistically different values. Student T-test: * P<0.05 vs. DMSO.

The analyses of GRP75 levels in the total cell lysates showed an inverted U-shape dose-response curve. A significant increase (+30%) in protein content was found in the range 5- 10 µM compared to the levels measured in DMSO-treated cells, whereas the highest doses induced a decrease in GRP75 content lower doses (Figure 4.7).

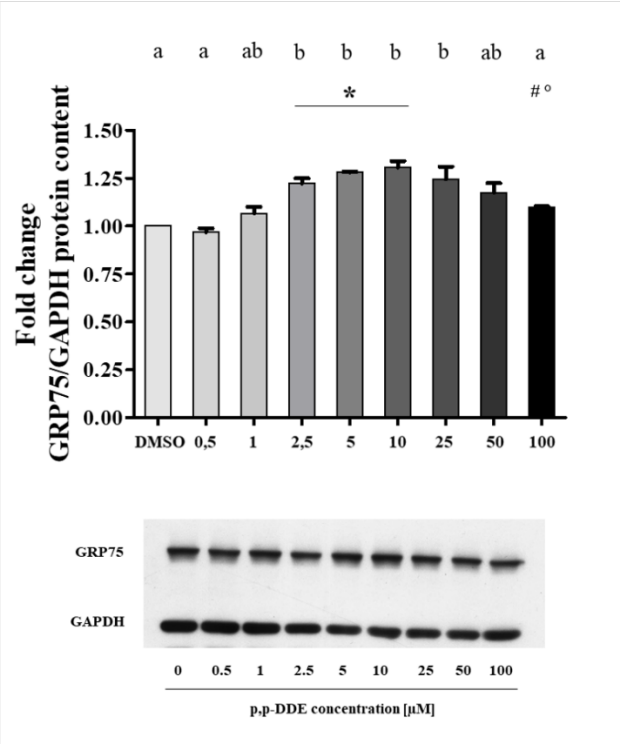

Figure 4.7. GRP75 protein levels. All data were presented as mean ± standard error on the media (SEM) for 3 biological replicates. The figure showed representative images of GRP75 (above) and the relative loading control for protein normalization GAPDH (below). Statistical analyses were performed by using GraphPad Prism software. One-way ANOVA analysis followed by Bonferroni's posthoc test: different letters indicate statistically different values. Student T-test: * P<0.05 vs. DMSO, # P<0.05 vs. 5 µM DDE; ° P<0.05 vs. 25 µM DDE.

4.5 Discussion

In vitro and *in vivo* studies showed the adverse effects of DDE in terms of cellular stress generation (Torres-Avilés et al., 2016; Migliaccio et al., 2019b), apoptosis (Shi et al., 2013), and mitochondrial impairment (Auger et al., 2015). The present work aimed to analyze other DDE adverse effects on cell viability and mitochondrial parameters in an *in vitro* hepatic cell model (HepG2 cells), focusing on the impact of increasing doses of the pesticide to shed light on the adaptive or toxic outcomes in a dose-response approach.

Concerning the effect of DDE on cell viability, our study on HepG2 cells confirmed that this environmental chemical reduced cell life in line with literature data in other cellular models (Shi et al., 2009). Our results showed a dose-dependent effect of DDE on cell viability.

Low DDE doses did not seem to have severe toxic properties, whereas high DDE doses (50 and 100 µM) elicited a significant cell viability reduction.

Given the lipophilic proprieties of DDE and its ability to accumulate in lipid depots, we found of interest to analyze cell viability as well as intracellular fat depots under conditions of cotreatment with increasing doses of DDE and different dietary fatty acids, namely palmitate (as a saturated fatty acid), oleate (as monounsaturated fatty acids) and EPA+DHA (1:1) mixture (as polyunsaturated fatty acids). Regarding the effect of fatty acids on cell viability, diverse fatty acids differently affected cell viability. Palmitate or oleate (250 µM) elicited a 25% or 20% reduction in cell viability, respectively, whereas EPA+DHA mixture did not seem to have any adverse effect on cell viability at the dose utilized in the present experimental work.

Noteworthy, fatty acids induced an increase in DDE toxicity. Cells cotreated with fatty acids, and DDE showed a significant reduction in cell viability with low DDE doses, which did not elicit any considerable cell viability decrease in the absence of fatty acids. Moreover, cells cotreated with saturated fatty acid (palmitate) exhibited higher cell viability reduction with high DDE doses than cells cotreated with oleate or EPA+DHA. In the presence of palmitate, 2.5 µM DDE dose dramatically decreased cell viability to the value of 43%, whereas a similar value (46%) was achieved with 100 µM DDE in cells treated only with DDE. Moreover, 100 µM DDE elicited a further higher decrease in cell viability (28%) when cells were cotreated with palmitate. A value of 44% cell viability was observed in oleate exposure, starting with a 10 µM DDE dose. On the other hand, the EPA+DHA mixture elicited a decrease to the value of 60% with a 1 µM DDE dose and a reduction to 47% with a 100 µM DDE dose. Considering the effect of the highest DDE dose, cell viability in the presence of

unsaturated fatty acids decreased to a value (about 46%) similar to that observed in cells treated only with DDE. In contrast, in the presence of palmitate, the reduction in cell viability was higher (28% compared to control cells). These data showed that DDE associated with fatty acids was particularly detrimental for cell viability and that mainly saturated fatty acids could exacerbate environmental pollutants toxicity. In the presence of fatty acids, DDE reduced cell viability starting with low doses (2,5 μM for palmitate or oleate, 1 μM for EPA+DHA), suggesting that fatty acids could allow an easier uptake of DDE into the cells leading to increased toxicity. Further experiments are needed to test this hypothesis.

It should also be noted that xenobiotics toxicity could be associated with lipotoxicity with an additive adverse effect on cell survival in the presence of fatty acids (Engin, 2017). Lipotoxicity is the harmful effect of lipid accumulation in non-adipose tissues. It depends on the balance between fatty acid deposition and oxidation and the production of lipid intermediate metabolites ceramides diacylglycerols. We analyzed intracellular lipid depots with a qualitative and quantitative test in the different experimental conditions. As expected, no changes in lipid depots were found in cells only treated with DDE, whereas intracellular lipid droplets increased in cells treated only with fatty acids. Quantitative analysis showed that lipid accumulation was higher in oleate than with palmitate and further increased with EPA+DHA compared to oleate or palmitate. The higher lipid accumulation was observed in cells treated with unsaturated fatty acids that were less toxic toward cell viability than saturated fatty acids. This finding was in line with recent evidence, suggesting that the total amount of triglycerides stored in the liver cells was not the primary determinant of lipotoxicity and specific lipid classes; such as ceramides diacylglycerols acted as damaging agents (Svegliati-Baroni et al., 2019). The hepatic accumulation of triglycerides in lipid droplets has

been suggested to be protective rather than harmful in steatosis progression to non-alcoholic fatty liver disease (Yamaguchi et al., 2007; Alkhouri et al., 2009).

The coincubation of HepG2 cells with fatty acids and increasing DDE doses induced a progressive decrease in lipid accumulation compared to HepG2 cells treated only with fatty acids. The reduction degree was similar for all fatty acids. A 20% decrease with low DDE doses could be observed, and a 50% decrease with the highest DDE dose. This finding was in line with our previous results in an animal model of hepatic steatosis. We showed that simultaneous treatment with DDE and a high-fat diet elicited a significant reduction in hepatic lipid content associated with increased beta-oxidation rate and detoxification, compared to high-fat diet treatment absence of DDE (Migliaccio et al., 2019c). This suggests that hepatocytes could utilize fatty acids as metabolic substrates to sustain detoxification under the condition of exposure to xenobiotics partially.

Present findings on cells cotreated with increasing DDE doses and different types of dietary fatty acids suggested that it could be of interest further to investigate the interaction between DDE as a lipophilic pollutant and dietary fatty acids, given that they could be the main route of entry of DDE in the body. These studies could shed light on the complex interactions among environmental pollutants and nutrients and suggest whether different dietary nutrients (such as saturated or unsaturated fatty acids) could potentiate the harmful effects of environmental pollutants or could be protective towards them.

The present work's main goal was to analyze the dose-dependent mitochondrial marker dose-response to increase DDE dose in HepG2 cells. Mitochondria are cellular targets susceptible to chemicals, such as DDE (Mota et al., 2011). Given that mitochondrial function is closely associated with their morphology and their cellular network (Ni et al., 2015), we

analyzed the main proteins involved in the mitochondrial dynamic machinery, namely proteins involved in fusion (MFN2 and OPA1) and fission (DRP1) mechanisms. The balance between fusion and fission processes produces a mitochondrial network reorganization in response to various mitochondrial stressors and diseases (Suárez-Riveiro et al., 2016). Our previous finding evidenced a possible role of mitochondrial dynamics in different tissues to control cellular health and function (Lionetti et al., 2014; Migliaccio et al., 2019d). Suggesting the importance of studying this physiological mechanism, mainly in the liver, plays a crucial role in lipid metabolism and detoxification processes against external toxic agents (Migliaccio et al., 2019e). As far as we know, this is the first time that mitochondrial proteins involved in mitochondrial dynamic behaviour are investigated in response to an increasing dose of DDE in HepG2 cells. Our results demonstrated that both Mfn2 and OPA1 (fusion proteins) were modulated by DDE exposure in a dose-dependent manner with a similar trend showing an inverted U-shape dose-response curve. MFN2 and OPA1 contents progressively increased with increasing DDE doses and reached the highest increase (about +30%) in a similar dose range (2.5-25 µM DDE and 5-25 µM DDE for MFN2 and DRP1, respectively). DRP1 fission protein content also increased with increasing DDE doses and reached the highest increase (+30 %) in the 10-50 µM doses range. It should be noted that both fusion and fission proteins analyzed were increased by about 30% with the dose of 25 µM, whereas with the highest doses of 50 and 100 µM, a marked increase in protein content was still observed only for DRP1 (+33% and +17%). No significant changes were observed in MFN2 and OPA1 content with the highest DDE dose (100 µM) compared to control cells. Our results showed that DDE at low doses (up to 25 µM) caused a general increase in the dynamic mitochondrial machinery. An increase in mitochondrial mass through mitochondrial biogenesis could help adapt cellular

metabolism to cope with increased energy intake. It could be suggested, require detoxification processes, and counteract DDE toxicity. In line with this suggestion, our results showed that cell viability did not significantly decrease with low doses of DDE up to 25 μM.

On the other hand, our results showed that the increase in DRP1 fission protein was still observed with the highest DDE dose, in contrast to the rise in fusion proteins that was not found with the highest dose. This finding suggested that the balance between fusion and fission processes could be shifted towards mitochondrial fission with the highest DDE doses. This suggestion was supported by the increased DRP1/MFN2 ratio (about 12%) with 50 and 100 μM DDE doses compared to control cells. It should be noted that cell viability was significant reduced in cells treated with 50 and 100 μM DDE, suggesting a link between fission processes and reduction in cell viability. Further analyses are needed to confirm mitochondrial biogenesis as an adaptive cellular response to survive low DDE doses and assess mitochondrial morphology network changes towards fission phenotype as a cellular response to high toxic DDE doses.

In the present study, we also analyzed SOD2 content as a marker of mitochondrial function and antioxidant system and GRP75 as a marker of mitochondria/endoplasmic reticulum interaction. A decreasing trend in the SOD2 range was observed in DDE-treated compared to DMSO cells, with a significant reduction with the highest DDE dose (-20% vs DMSO). It has been suggested that the SOD2 enzyme ensures mitochondrial functional capacity, so its decline could be associated with mitochondrial dysfunction (Paul et al., 2007; Flynn and Melov, 2013). Moreover, SOD2 has been suggested to improve the mitochondrial fusion process beyond its antioxidant activity (Bhaskar et al., 2020). Therefore, the decreased

content of the SOD2 enzyme observed in our results could be associated with the shift toward fission processes in cells treated with the highest dose of DDE.

We also analyzed the content of intracellular chaperone GRP75, a protein involved in mitochondria/endoplasmic reticulum interaction (Honrath et al., 2017). In association with other reticular and mitochondrial proteins, this chaperon plays a vital role in generating functional structures known as an associated mitochondrial membrane (MAMs). These contact points are essential to regulate calcium homeostasis, autophagy, lipid metabolism, mitochondrial morphology, and cell survival (Filadi et al., 2017; Lee and Min, 2018). It has been reported in the literature that GRP75 represents a critical protein involved in several physiological processes in cells. For example, chaperone overexpression prevents endoplasmic reticulum stress and apoptosis induced by glucose deprivation (Heal and McGivan, 1996; Ikesugi et al., 2006; Liu et al., 2005). Moreover, E and co-workers (2013) evidenced that GRP75 overexpression protected hepatic mitochondria from H2O2 and CCl4-induced oxidative damages, ameliorating ATP production preserving cell viability. In our study, GRP75 protein levels produced a similar inverted U-shape dose-response curve observed for Mfn2 and OPA1 levels, with the highest significant increase (about +30%) with 5 and 10 µM DDE vs DMSO-treated cells. This result suggested that an additional adaptive phenomenon involving EnR-mitochondrial contact point formation/communication could be associated with mitochondrial increases in dynamic protein in response to low DDE doses. This mechanism could be used by cells to control cellular survival and mitochondrial function in the presence of a low amount of DDE. On one side, the increase in mitochondrial dynamic machinery and GRP75 chaperone in response to low DDE doses could play a role in the mitochondrial network and mitochondria/endoplasmic reticulum interaction maintenance to

maintain cell viability. On the other hand, in cells treated with high DDE doses, decreased levels in mitochondrial fusion proteins, GRP75 and SOD2 content associated with the increase in DRP1 levels suggested mitochondrial fragmentation and dysfunction in response to high DDE doses, which could trigger the observed reduction in cell viability.

In conclusion, this study added new scientific notions in terms of adaptive/toxic phenomena induced by increasing DDE dose in hepatic cell culture. Cellular adaptation to low doses of the environmental pollutant seemed to include mitochondrial network maintenance and mitochondria-endoplasmic reticulum interaction, which could play a pro-survival role. On the other hand, a high DDE dose induced a toxic effect eliciting a reduction of the cell viability associated with a disruption of mitochondrial fusion/fission balance and antioxidant defence. Further studies are needed to shed additional light on our findings on metabolic events and highlight the different roles of cellular organelles and their adaptation to environmental pollutants.

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5. Experiment 2

Effect of two pesticides on adults of Catarina scallop *Argopecten ventricosus*: Expression study of mitochondria and endoplasmic reticulum related genes.

5.1 Abstract

The effects of pesticide contamination on Catarina scallop metabolism have been little explored, especially at the level of gene transcription. To address this question, we investigated this marine bivalve's response by analyzing the up-and-down regulation of genes associated with the mitochondrial and endoplasmic reticulum (EnR) functions after a short-time exposure to an experimental dose of two endocrine-disrupting action pesticides, endosulfan and carbofuran. RT-PCR analyzed the mitochondrial-associated gene DRP1 and the EnR-associated gene ERIS under experimental laboratory conditions. DRP1 is associated with mitochondrial dynamic, whereas ERIS is linked to immune system responses. This study provides a preliminary basis for studying marine bivalves' response to short- and long-term pesticide exposure in terms of regulated gene expression and characterizes new potential genetic markers of environmental contamination.

5.2 Introduction

Seawater receives direct input of pollutants from the atmosphere and even industrial and agricultural activities. Data indicate that the presence of these could induce a wide range of biological effects in organisms, including biochemical, immunological, physiological, and bioenergetic responses to stress (Burgos-Aceves and Faggio, 2017; Jiang et al., 2019). The risk of any contaminant impacting intertidal and nearshore benthic invertebrates is related to

numerous biological and physicochemical processes responsible for the contaminant reaching and accumulating in the nearshore region (Marsden and Cranford, 2016). Due to the lifestyle and ability to gather xenobiotics (e.g., chemicals, pathogens), scallops are a susceptible group to suffering xenobiotic-induced physiological alterations related to the bioavailability of the pollutant and its bioaccumulation potential (Marsden and Cranford, 2016). For this reason, the use of scallops as sentinel species is increasing in environmental health monitoring programs (Marsden and Cranford, 2016; Loaiza et al., 2020).

Catarina scallop *Argopecten ventricosus* is an economically important resource in Mexico's northwest; however, it has shown a negative capture trend in the last ten years (SAGARPA, 2018). For this reason, efforts have been intensified to develop sustainable mariculture of this species with the ultimate goal of recovering populations in fishing areas, thus improving the livelihoods of coastal communities (Corpuz et al., 2014; Ruiz-Verdugo et al., 2016). Factors such as overfishing and water pollution in combination with other aspects such as abrupt changes in the substrate, changes in water temperature may ultimately be related to the collapse of the Catarina scallop fishery (Corpuz et al., 2014). Then, aspects such as food, chemical composition, and abundance of particles in the water influence the scallop's physiology (Félix-Pico, 2006). However, there is currently a gap in published information on the effects of water pollutants on the species' biology. Therefore, in the present study we intended to assess the single acute effect of endosulfan and carbofuran on *A. ventricosus*, which are representative organophosphate and carbamate pesticides; commonly used in agriculture activities in México (Garciá de Llasera and M. Bernal-González, 2001; Navarrete-Rodríguez et al., 2016). Endosulfan is considered a high-risk persistent organic pollutant (POP), particularly in coastal aquatic environments (Albert, 2014), banned in many countries

under the Stockholm Convention on POPs (UNEP, 2010). Meanwhile, carbofuran is considered one of the most toxic broad-spectrum and systemic N-methyl carbamate pesticides (Mishra et al., 2020), with environmental and hazardous health impacts (Dobšíková, 2003; Rocha et al., 2018). These pesticides adversely affect mitochondrial metabolism in mammals, fish, and invertebrates (Braunbeck and Appelbaum, 1999; Kamboj et al., 2008; Otieno et al., 2010; Alonso-Trujillo et al., 2020).

Therefore, this job aimed to determine, at a subcellular level, the ability of scallop to respond to the presence of a persistent organophosphate or carbamate contaminant. Using Real-Time polymerase chain reaction (RT-PCR), we analyzed the mRNA levels of DRP1 and ERIS genes involved in the mitochondrial fission and endoplasmic reticulum stress in adult *A. ventricosus* exposed non-lethal endosulfan or carbofuran concentrations (96h-LC_{50}, 0.45 µg L^{-1}) for 24h and 48h. This type of study is essential because of the role of *A. ventricosus* in aquatic ecosystem food webs, endosulfan persistence, carbofuran pesticide, and the poor knowledge about their effects on mollusks. Determining how it can affect *A. ventricosus* could help show the new mode of actions in other invertebrates.

5.3 Material and Methods

5.3.1 Experimental design

For the assessment, we used two insecticide products Endos 35 (X1; contains active endosulfan ingredient 378 g L^{-1}, PASSA Agroservicios, Mexico), and Velfuran 350 L (X2; has carbofuran active ingredient 350 g L^{-1}, VELSIMEX SA, Mexico).

The Catarina scallops *Argopecten ventricosus* (G. B. Sowerby II, 1842) have been obtained from a mariculture facility (Non-governmental association Noroeste Sustentable,

NOS, La Paz, BCS, Mexico) and transferred to the Laboratory of experimental mollusk at Centro de Investigaciones Biológicas del Noroeste-CIBNOR. Three-hundred adult scallops (5.75 ± 0.41 cm shell length) were kept in a nursery upwelling recirculating system filled with continuously aerated filtered seawater with day light exposure 12h light:12h dark, temperature 22ºC and fed with a mix of microalgae concentration around 150,000 cel mL^{-1} (*Chaetoceros calcitrans* and *Isochrysis galbana* in proportion 1:1) for five days acclimation before the onset of any experimental procedure. After acclimation, twenty scallops were randomly selected and placed into each of 9 experimental units (three experimental groups with three replications) containing 35 L aerated seawater. One unexposed control tank, two tanks (TX1; TX2), exposed to endosulfan (X1) or carbofuran (X2) at a non-lethal dose of 0.45 μg L^{-1}. The assay lasted for 48h in a semi-closed recirculating flow system supplied with filtered seawater at 21.9 ± 0.25ºC, the salinity of 38 ± 0.5 UPS. Scallops were continuously fed in a combination of *C. calcitrans* and *I. galbana* (2:1) at 2,154,099,822 cell/organism/day during the acclimatization period.

5.3.2 Sample collection, processing, and RNA isolation

Tissues portion, including gonad, gills, and mantle (≈ 100 mg each), were collected from a total of three scallops from each treatment at 24h, and 48h of xenobiotic exposure and preserved at -80ºC until RNA extraction. Total RNA was isolated and cleaned by DNAse from each gonad, gills, and mantle homogenate using the Ribozol™ RNA Extraction Reagent according to the protocol described by Morelos et al. (2015). The RNA concentration of each sample was calculated using a NanoDrop OneC spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the RNA quality through electrophoresis visualization.

5.3.3 Primers design for DRP1 gen

Primers were designed using the software Primer3 (Untergasser et al., 2012) and then analyzed with Oligo Analyzer Tool (https://www.idtdna.com/pages/tools/oligoanalyzer), looking for highly conserved regions of the dynamin 1 like (DRP1) gene in the yesso scallop *Mizuhopecten yessoensis* (OWF42330.1). The PCR amplification was performed in the 15 µl reaction volume containing 1 x concentrated GoTaq® Flexi DNA Polymerase reaction buffer (Promega); 2.5 mM $MgCl_2$ (Promega); 0.25 mM dNTPs (Promega); 0.3 µM of DRP1 and ERIS forward and reverse designed primers (Table 4.1); 0.125 µl of the GoTaq® Flexi DNA Polymerase (Promega); nuclease-free water and about 30-60 ng of the bisulphite-treated Catarina scallop DNA. The amplification conditions were as follows: I/95°C, 5 min; II/35 cycles: 95°C, 30 sec; 60°C, 30 sec; 72°C, 30 sec; and III/72 °C, 5 min. The PCR amplification was run on the thermocycler BioRad. In each PCR reaction, the NTC (non-template control) with 1 µl of water instead of Catarina scallop DNA was included. The amplification products were identified in the 1% agarose gel with the GelRed® Nucleic Acid Stain (Biotium). The BenchTop 100 bp DNA ladder (Promega) was used as the molecular weight standard, and the gels were photographed using the VWR GenoView (Major Science).

5.3.4 Gene expression analysis by qPCR

Total RNA was reverse transcribed into cDNA using PrimeScript RT Reagent Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions and kept at -20°C. Reactions of RT-qPCR were performed with the equipment using 2X Eva-Green® (ThermoFisher, Scientific, Waltham, MA, USA) PCR mix. Reaction mixtures were made in 15 µL, including 10 µL mix (0.45 U of GoTaq Flexi DNA polymerase (Promega,

Madison, WI, USA), 2.5 mM $MgCl_2$, 1x Go Taq Flexi Buffer, 0.2 mM dNTP Mix (Promega, Madison, WI, USA), 2x EvaGreen fluorescent dye (Biotium, Inc. Fremont, CA, USA), 0.15-0.45 µM each primer) and 5µL cDNA (diluted to 50 ng µL-1). Each sample was analyzed in triplicate using a 96-well microplate and optical adhesive films (Bio-Rad, Hercules, CA, USA) using a CFX96 Real-Time PCR Detection System (Bio-Rad). Real-time amplification conditions were: denaturation at 95°C for 5 min followed by 35 cycles at 95°C for 30 sec and 30 sec at 60°C, and 30 s at 72˚C. A melting curve of all PCR products (dissociation step from 60°C to 72°C, 0.5°C/sec) was performed at the end of the amplification to ensure the absence of artefacts and verify the specificity of the PCR product. Data were analyzed using Ct values and the $2^{-\Delta\Delta Ct}$ methods (Livak et al., 2001), taking into account the efficiency value (Morelos et al., 2015). The relative expression level of DRP1 and (Endoplasmic reticulum interferon stimulator) ERIS** genes was normalized to the abundance of sodium/potassium-transporting ATPase subunit alpha-like (ATP1A1) gene, and cAMP-dependent protein kinase catalytic subunit-like isoform X3 (PRKACA) gene for gonad, gills, and mantel tissue analyzed. The specific primer sequence was shown in Table 5.1.

According to the $2^{-\Delta\Delta Ct}$ values, data for each tissue and condition selected were converted to natural logarithm and analyzed using a student T-test (independent). Statistical comparison between the treatment and its respective control was performed, and statistical significances were set at $p < 0.05$. The log_2FC was assessed using treatment vs control data to validate selected genes expression compared to the transcriptome results and linear regression. Statistica 10.0 software (StatSoft, Tulsa, OK, USA) was used to perform statistical analysis.

Table 5.1. Polymerase chain reaction (PCR) primers sequence designed for the expression analysis of DRP1, ERIS, and constitutive genes in *Argopecten ventricous*.

Gene name	Nucleotide sequences (5' to 3')	Tm (°C)	Product size (bp)
Target genes			
DRP1*	Forward AAAGTGCCAGTGGGTGACCAGC Reverse ACGACGACCATCTGCGTCCA	60	174
ERIS**	Forward ATACCCTGCCCCATTACGGACCCT Reverse AGCTGTCGTTGGAGGTCGCCAT	60	204
Constitutive genes			
ATP1A1**	Forward AGGAGCTGGAGATGGACGAGCACA Reverse TGGTGTTGTAGGGGGAGGGGTCAA	60	152
PRKACA**	Forward AAGCCGTAGACTGGTGGGCGTT Reverse AGCGTTCTTCAGGTTGCCGTAGCG	60	212

*Sequences and 5' nucleotide designation are from GenBank. The conservative fragments were generated from alignments among the bivalve database.
**Sequences designed by López-Carvallo et al. (2020).

5.3.5 Ethics statements

All the scallops used in this work were handled according to the Official Mexican Standard protocol (NOM-062-ZOO-1999). Adults of *Argopecten ventricosus* were provided by the non-governmental association Sustainable Northwest (NOS). The specimens' transportation to the CIBNOR experimental laboratory was carried out following the standards and regulations established by the federal agency CONAPESCA. The Aquaculture Robles hatchery produced the animals used in this study in captivity for experimental purposes. The organisms were kept in optimal culture conditions to avoid stress conditions.

5.3.6 Statistical

At each tissue sampling time, the statistical analyses of results were performed using one-way ANOVA after transforming the data to natural logarithms to correct

heteroscedasticity, non-normality, and non-additivity (Zar, 1999), followed by the Tukey's test for pairwise comparisons among experimental conditions. The results are presented back-transformed.

5.4 Results

5.4.1 Effects of endosulfan and carbofuran on survival

Survival of the Catarina scallop was not affected by pesticide exposure on its own at the experimental dose. The weight of experimental animals also did not undergo significant variations throughout the time (Table 5.2).

Table 5.2. Effect on survival of Catarina scallop exposed to 0.45 µg/L of endosulfan or carbofuran.

	Weight			**Survival**		
	0 h	*24 h*	*48 h*	*0 h*	*24 h*	*48 h*
Control		47.24 ± 3.2	45.70 ± 2.2			
Endosulfan	49.99 ± 1.7	44.17 ± 3.0	42. 54 ± 3.4	100%	100%	100%
Carbofuran		48.12 ± 2.6	45.69 ± 3.4			

5.4.2 Gene expression profiling in pesticide-exposed scallops

Here, we investigated the effects of endosulfan and carbofuran on DRP1 and ERIS gene expression in the gonad, gills, and mantle of Catarina scallop. According to the results, an up-regulation of the DRP1 gene has been observed 24h and 48h endosulfan exposure than control. While the scallops exposed to carbofuran, an up-regulation of DRP1 has been reported at 48h (Figure 5.1).

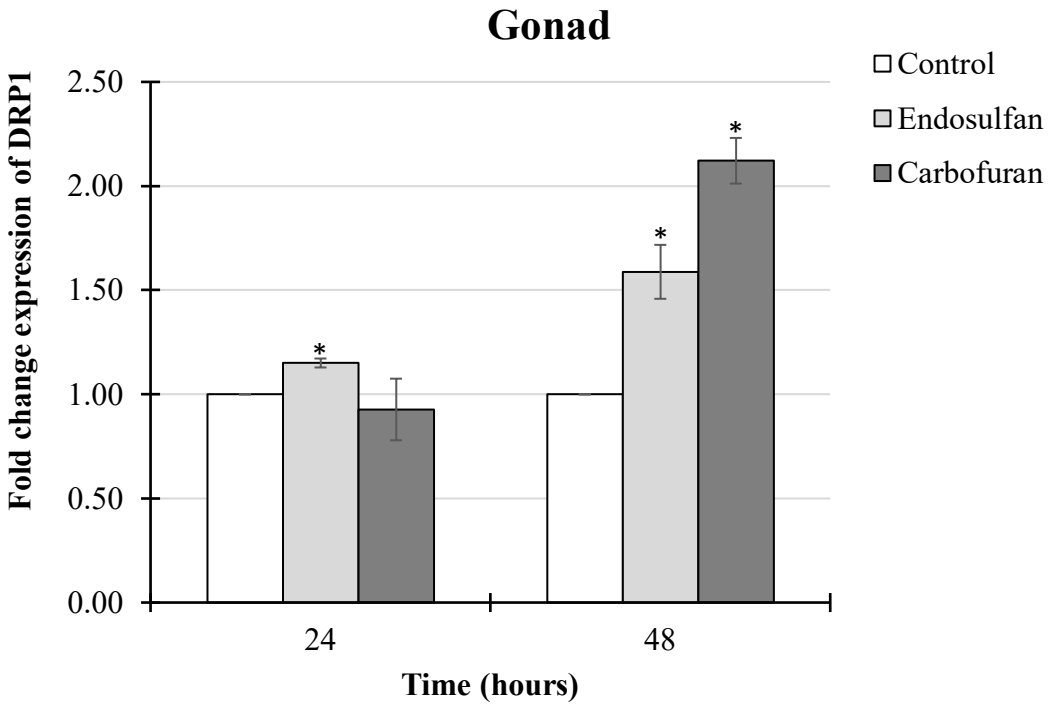

Figure 5.1. Expression of DRP1 transcripts in *Argopecten ventricosus* gonad and effects of exposure to endosulfan and carbofuran. The results are represented as gene expression fold changes of DRP1 and calculated concerning controls. Data are the mean ± S.E. from three experiments in triplicate. *P≤0.05, Tukey's test.

In gills, no changes in the expression of the DRP1 gene were observed at 24h of exposure to endosulfan or carbofuran compared to the control. An up-regulation of the DRP1 gene has been observed at 48h of exposure to endosulfan. Scallops exposed to carbofuran 48h showed up-regulation compared to the control but not with those exposed to carbofuran 24h (Figure 5.2).

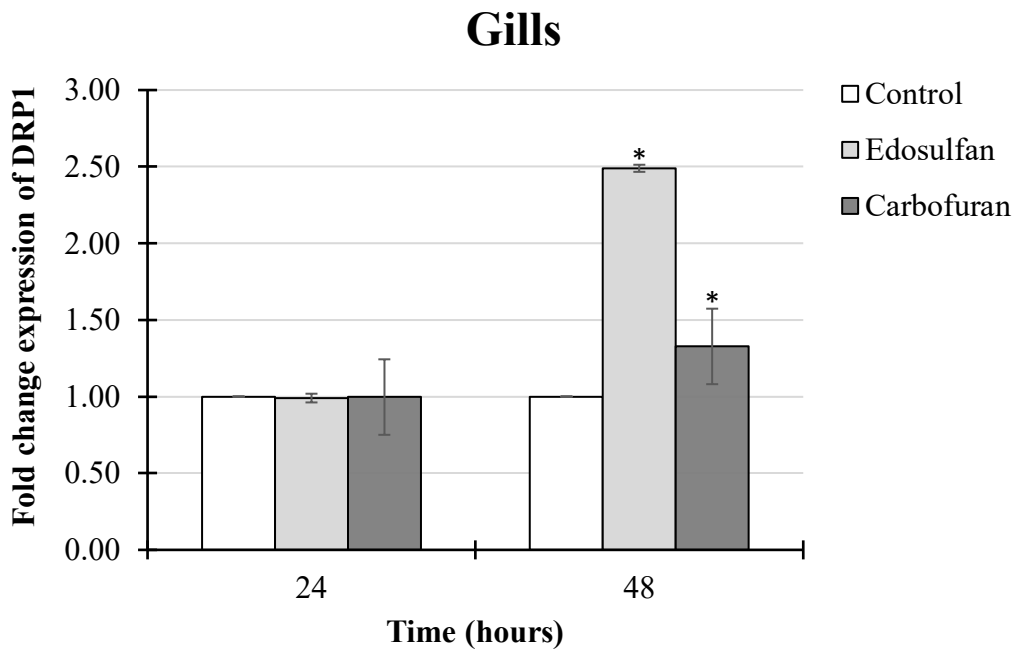

Figure 5.2. Expression of DRP1 transcripts in *Argopecten ventricosus* gills and effects of exposure to endosulfan and carbofuran. The results are represented as gene expression fold changes of DRP1 and calculated concerning controls. Data are the mean ± S.E. from three experiments in triplicate. *P≤0.05, Tukey's test.

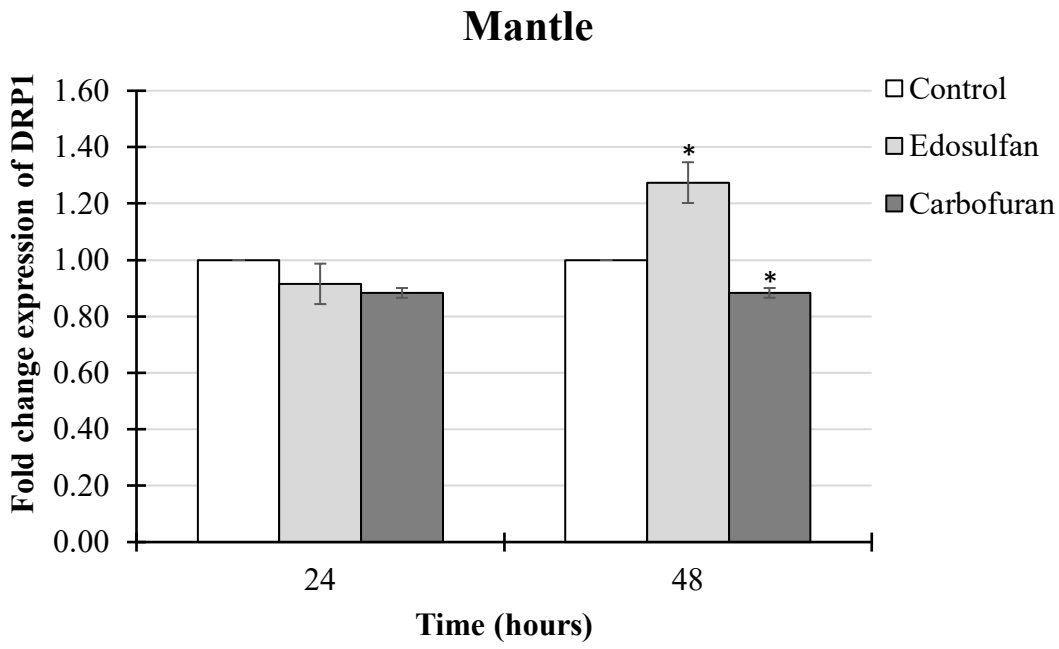

Figure 5.3. Expression of DRP1 transcripts in *Argopecten ventricosus* mantle and effects of exposure to endosulfan and carbofuran. The results are represented as gene expression fold changes of DRP1 and calculated concerning controls. Data are the mean ± S.E. from three experiments in triplicate. *P≤0.05, Tukey's test.

No changes were observed in the DRP1 gene expression at 24h of endosulfan or carbofuran exposure in the mantle. An up-regulation has been observed in the scallops exposed to endosulfan for 48h. In contrast, a down-regulation of ERIS has been observed in the scallops exposed to carbofuran compared to control (Figure 5.3).

The effect of endosulfan on ERIS gene expression in the gonad registered an up-regulation at 24h compared to the control. In contrast, a down-regulation of ERIS expression at 48h was reported similar to the control. Whereas the scallops exposed to carbofuran, the ERIS gene expression was up-regulated at 24h and 48h compared to the control but unchanged over time (Figure 5.4).

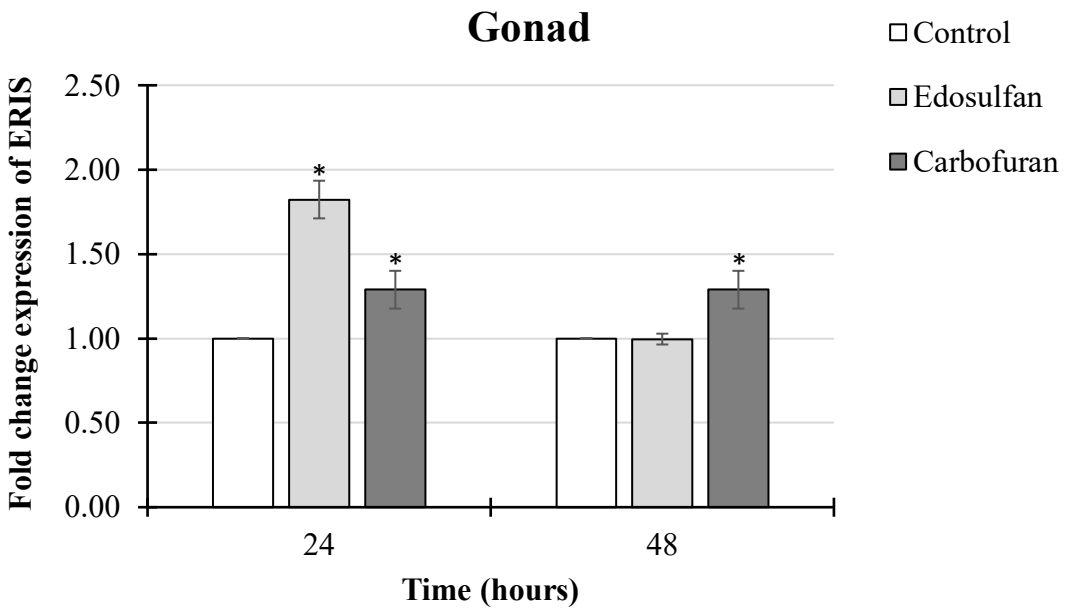

Figure 5.4. Expression of ERIS transcripts in *Argopecten ventricosus* gonad and effects of exposure to endosulfan and carbofuran. The results are represented as gene expression fold changes of ERIS and calculated concerning controls. Data are the mean ± S.E. from three experiments in triplicate. *P≤0.05, Tukey's test.

In gills, no significant changes of the ERIS gene have been observed at 24h and 48h compared to the control in scallops exposed to endosulfan or carbofuran (Figure 5.5).

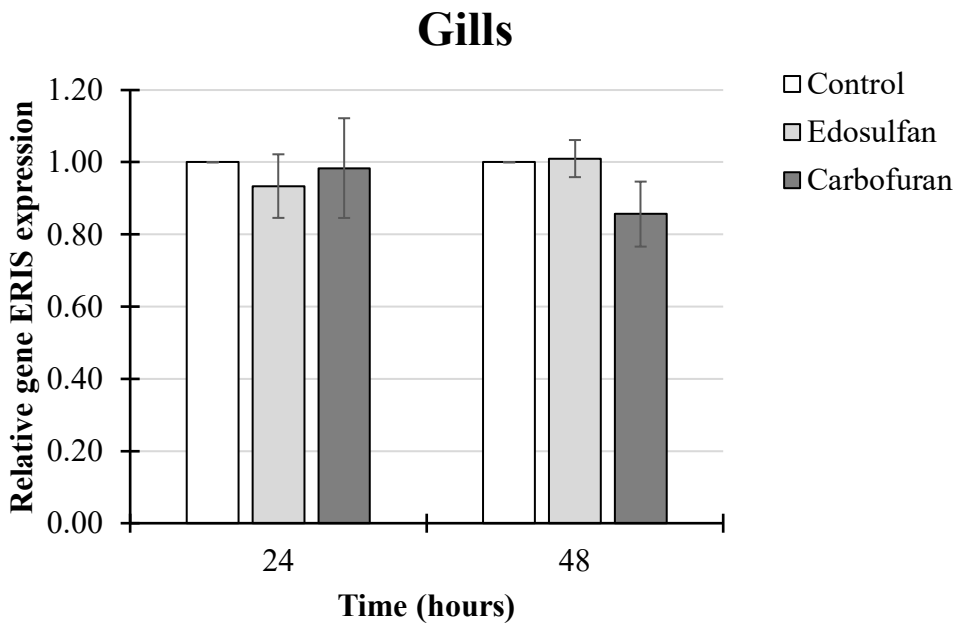

Figure 5.5. Expression of ERIS transcripts in *Argopecten ventricosus* gills and effects of exposure to endosulfan and carbofuran. The results are represented as gene expression fold changes of ERIS and calculated concerning controls. Data are the mean ± S.E. from three experiments in triplicate. *P≤0.05, Tukey's test.

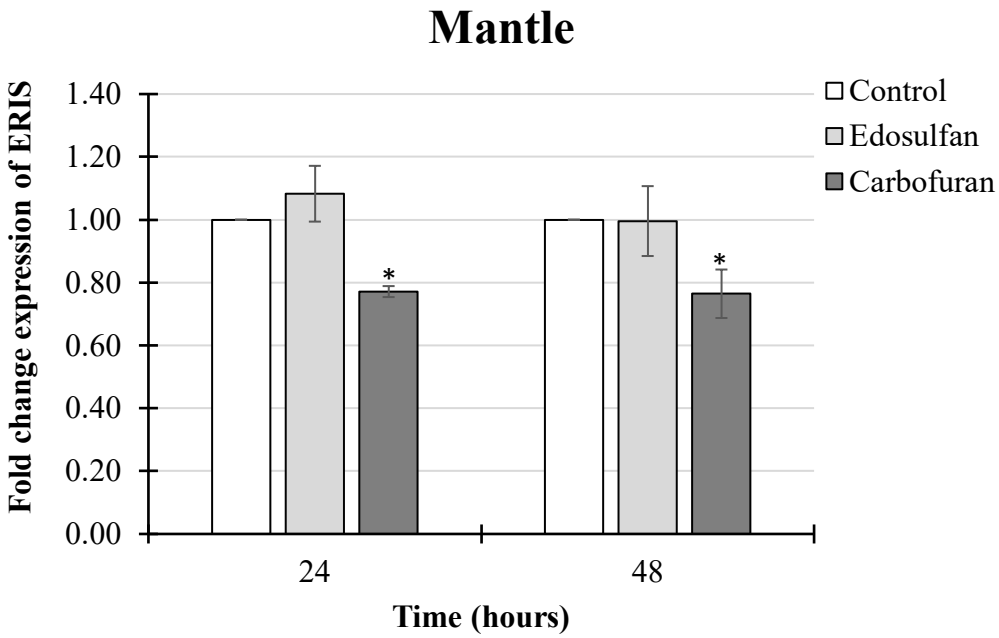

Figure 5.6. Expression of ERIS transcripts in *Argopecten ventricosus* mantle and effects of exposure to endosulfan and carbofuran. The results are represented as gene expression fold changes of ERIS and calculated concerning controls. Data are the mean ± S.E. from three experiments in triplicate. *P≤0.05, Tukey's test.

In the mantle, the ERIS gene expression in the scallops exposed to endosulfan showed no significant changes according to exposure time compared to the control. Meanwhile, a down-regulation in the ERIS gene expression was reported in the scallops exposed to carbofuran at 24h and 48h compared to the control (Figure 5.6).

5.5 Discussion

The exposure of marine bivalves to different chemical substances determines the biological responses. It may be possible to infer the organism potential state of health according to their gene expression responses. This study analyzed the Catarina scallop response to short-time non-lethal pesticide exposure under experimental conditions. We observed that some mitochondria and endoplasmic reticulum stress responses could be activated. Mitochondrial dynamics, the balance between mitochondrial fission and fusion, regulates mitochondrial quality control by segregating unwell functioning mitochondria for degradation while mixing the contents of healthy mitochondria (Twig et al., 2008). The principal executor of fission is the cytosolic dynamin-related protein 1 (DRP1), which is translocated to the mitochondrial surface to mediate the organelle fission (Serasinghe and Chipuk, 2017). Our results showed an up-regulation in the expression of the DRP1 gene in the three tissues endosulfan-exposed analyzed. Appearance may be associated with mitochondrial dysfunction and concomitant apoptosis (Hu et al., 2017). This result suggests that endosulfan can modulate the mitochondrial dynamic. A recent study reported that the different endosulfan forms impair redox status and membrane integrity of mitochondria and induce apoptosis (Lakroun et al., 2017; Rainey et al., 2017; Wang et al., 2017). Carbofuran is toxic to the animal and human nervous system and associates with reproductive toxicity

(Yenchum et al., 2011). Our results indicate that exposure to carbofuran caused up-regulation of the mitochondrial fission DRP1 gene only in the gonad of scallops exposed for 48h. Therefore, due to its endocrine disruptive behaviour (Ibrahim et al., 2014), an alteration in reproduction could be inferred, encouraging the cells to apoptosis. Studies have demonstrated that carbofuran leads to oxidative stress affecting the mitochondrial functions (Lee et al., 2004; Akbar et al., 2012; Fu et al., 2019).

The EnR is a cell essential and susceptible organelle to stressors that perturb cellular energy levels (Tebourbi et al., 2011), so its homeostasis is critical for controlling various intracellular physiological functions (Li et al., 2020). The EnR stress has been linked with activation and regulation of detoxification and immune responses (Cribb et al., 2005; So, 2018). When organisms contact foreign agents, the immune machinery kicks in (Burgos-Aceves and Faggio, 2017). The immune functions are bio-energetically expensive and mediated by several cytokines/chemokines that, in turn, are influenced by other factors like bacterial or viral infections, drugs, or exposure to environmental pollutants (Mandarapu et al., 2014). Recent data indicate the EnR-IFN stimulator (ERIS, also known as STING) is an essential innate immune mediator. ERIS is a signalling protein that is embedded exclusively on the endoplasmic reticulum membrane, which stimulates the transcription of host defence genes such as type I interferon and pro-inflammatory cytokines (Sun et al., 2009; Chima et al., 2018; Srikanth et al., 2019). An ERIS gene up-regulation was reported here only in gonad at 24h and 48h of endosulfan and carbofuran exposure. Overexpression of ERIS can be associated with an up-regulation of type I IFNs, triggering some ERIS-dependent inflammatory processes (Kumar, 2019; Hopfner and Hornung, 2020; Wan et al., 2020). Some evidence suggests that POPs exposure can potentially influence inflammatory cellular

responses (Litteljohn et al., 2011; Peinado et al., 2020). An increase in ERIS signalling can cause autoinflammation and autoimmunity diseases (Kumar, 2019). In contrast, we observed a down-regulation expression of the ERIS gene in the mantle of scallops exposed to carbofuran. This reduction in gene expression could be associated with an increase in apoptotic caspases synthesis due to an oxidative stress stage caused by the pesticide (Rai and Sharma, 2007; Luqman et a., 2019). According to White and collaborators, caspases can suppress ERIS (STING) signalling and promote cellular apoptosis (White et al., 2014). A down-regulation/suppression in ERIS expression can also be associated with a deficiency in innate immune response (Khan et al., 2020), abnormally high levels of production of some cytokines (Sharma et al., 2015), and cancer development (Konno et al., 2018).

5.6 Conclusion and Perspectives

The use of synthetic chemical pesticides carries the risk of harmful consequences for human and wildlife health. The presence of these pollutants in water, soil, air or food that, when absorbed by the body, can produce in the short term, directly or indirectly, acute, sub-chronic, chronic poisonings, diseases, and even death (Damalas and Koutroubas, 2016). Chemical pollutants can cause physiological responses, including cascade reactions, into the organisms to excrete them or mitigate their harmful effects (Wong and Candolin, 2015). Exposure to pesticides can affect organelles and cellular components such as the cell membrane, mitochondria, lysosomes, endoplasmic reticulum, nucleus, and enzymes involved in metabolism and detoxification and repair of the DNA damage. The regulation of gene expression is one of the cell's fundamental mechanisms to maintain its function and integrity in the face of changes in the environment (Singh et al., 2018). Some of these genes respond

specifically to a specific stress, while other genes are generally activated or repressed under various stress types. When the molecular control mechanisms are not sufficient, mitochondrial fission and fusion mechanisms can be activated in a balanced way. Before the appearance of damage to mtDNA or proteins of the mitochondria, the fusion can be triggered to generate a new functional mitochondrion. Instead, fission has been described as an essential component to separate those parts of the mitochondria when there is very severe damage or an accumulation of damaged components (Westermann, 2010). Therefore, mitochondrial function maintenance is necessary for mitochondrial function since the loss of balance between fission and fusion processes is associated with alterations in mitochondrial function and certain pathological conditions (Knott and Bossy-Wetzel, 2008; Suárez-Rivero et al., 2017; Liu et al., 2020)

To date, there are no reports on responses to organochlorine and carbamates pesticides exposure at the genomic level in the Catarina scallop. To answer this, we have made a first attempt to analyze the expression of genes associated with mitochondrial dynamics and endoplasmic reticulum stress in response to endocrine-disrupting agro-pesticides, endosulfan, and carbofuran. Our results reported a modification in the expression of the DRP1 gene, associated with the mitochondrial fission process, depending on pesticide exposure time. A primer was designed to analyze the mitofusin (MFN2) gene expression associated with the fusion process (data not shown). Regrettably, the primer did not show the minimum specificity required. Thus, we can analyze both sides of mitochondrial dynamics and evaluate pesticides' effects. Other primers design is proposed, optic atrophy 1 (OPA1) gene for fusion while fission protein 1 (Fis1) gene for fission process.

It has been recently shown that the endoplasmic reticulum (EnR) is the crucial site where metabolic signals are processed, integrated, and transmitted in the form of signs of overload or stress, which give rise to the activation of inflammatory immune mechanisms (Bastarrachea et al., 2006; Pen and Cox, 2016). The inflammatory process is then produced and maintained by inflammatory cell activity that synthesizes and secrete pro- and anti-inflammatory mediators caused by pathogens, physical or chemical agents (Casanova, 2006). Our results showed a variation in the ERIS gene expression response to endosulfan and carbofuran. ERIS is an EnR IFN stimulator that activates innate and inflammatory immune responses (Liu and Cao, 2016), and its expression is linked to viral infections (Sun et al., 2009). Therefore, we can deduce that, for the first time and according to the gene expression results observed here, both pesticides may cause a modulating effect on the immune and inflammatory responses through the ERIS gene in the Catarina scallop. Other studies are needed to explore the metabolic events associated with our findings. Such as evaluating the expression of Tumour Necrosis Factor-alpha (TNF alpha) gene, binding immunoglobulin protein (BiP, GRP-78) related to early EnR stress, CCAATenhancer-binding protein homologous protein (CHOP) associated with late EnR stress and mediates apoptosis as well as proteins and genes associated to antioxidant responses.

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6. Final conclusions

Today, many pesticides are available such as organophosphates, organochlorines, and carbamates. Most of them are considered dangerous to humans and wildlife. Among organochlorines is endosulfan and the well-known DDT. They are chemically stable and persistent in the environment with unchanging half-lives, highly lipophilic, and taken up by fatty tissues, tending to store and accumulate. Organochlorines can act as neurotoxins opening sodium channels in neurons, activating action potential, reducing K^+ permeability, and inhibiting calmodulin, Na^-, and K-Ca-ATPase. Carbamates, such as carbofuran, can inhibit the enzyme acetylcholinesterase (AChE), similar to organophosphate. However, the intoxication tends to be shorter because the inhibition of tissue AChE can be reversible. After all, carbamates are rapidly metabolizable. In addition to their main toxic effects, these pesticides have in common their ability to disrupt the endocrine system by interacting with several nuclear receptors. Its endocrine-disrupting actions have been investigated to a limited extent, and many long-term effects remain unknown.

Mitochondria have been established as targets for organochlorines, organophosphates, and carbamates (Karami-Mohajeri and Abdollahi, 2010; Leung and Meyer, 2019). Exposure to these pesticides can result in mitochondrial dysfunction via direct interaction with mitochondrial proteins, for example, those with a role in the electron transport chain or via oxidative stress and redox cycling. Mitochondria are highly dynamic organelles capable of responding to various external stimuli playing a pivotal role in stress adaptation. Mitochondrial morphology is regulated by a balance between fusion and fission events. Such morphology influences mitochondrial function, so keep this mitochondrial dynamic is essential for mitochondrial function, and its dysfunction is related to the endoplasmic

reticulum stress that can trigger multiple pathologies. In the present work, we observed that the pesticides analyzed here were shown to have a dose-dependent effect on both mitochondrial dynamics and endoplasmic reticulum stress, mainly via oxidative stress.

In conclusion, the mitochondrion is a dynamic organelle that can change its morphology and function in response to different physiological stimuli. For this reason, mitochondrial dynamics have begun to be studied as one of the central regulators of cell survival. All these processes are highly regulated and are aimed at optimal mitochondrial functionality and cellular homeostasis. Moreover, since pesticides can be mitochondrial toxicants, continued study is warranted to determine the scope of adverse effects that can manifest following exposures. It is critical as mitochondrial dysfunction is one of the significant events underlying human degenerative and wildlife pathogenesis. Thus, environmental exposure to mitochondrial toxins has caused growing concern limiting livestock and human health productivity. Therefore, in the last decade, there has been an increase in the number of reports that indicate the toxic effects of drugs and pollutants on mitochondria and a large number of possible gene-environment interactions in mitochondrial toxicity, including the role of mitochondrial reactive oxygen species in signalling. However, it is still necessary to generate more information that allows a better understanding of the fundamental biological processes involved in mitochondrial homeostasis. The mitochondria-endoplasmic reticulum interaction in immune functions could be considered critical for mitochondrial toxicology. Also, greater integration in clinical, experimental laboratory, and epidemiological studies (human and wildlife). It allows a better understanding of the biomarkers used in the human population and incorporates other factors that can affect mitochondria; diet, exercise, age, and non-chemical stressors.

7. References (Introduction, Final Conclusions)

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8. Published articles

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Review

Modulation of mitochondrial functions by xenobiotic-induced microRNA: From environmental sentinel organisms to mammals

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Review

Omega-3 Fatty Acids and Insulin Resistance: Focus on the Regulation of Mitochondria and Endoplasmic Reticulum Stress

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Review

Multidisciplinary haematology as prognostic device in environmental and xenobiotic stress-induced response in fish

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Environmental Pollutants Effect on Brown Adipose Tissue

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