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Hydrophilic Antioxydant Systems for Cardiovascular Applications : Synthesis, Characterization, In Vitro and In Vivo Studies

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Ecole Doctorale Galilée

Laboratoire de Recherche Vasculaire Translationnelle – INSERM U1148

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Thèse présentée par

Marisol ZULUAGA TAMAYO

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Soutenue le 21 septembre 2017

Devant le JURY

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Defended by

Marisol ZULUAGA TAMAYO

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Abbreviations

A

ARE: Antioxidant-response element

Acyl-CoA: Acetyl coenzyme A

ATP: Adenosine triphosphate

ADP: Adenosine diphosphate

AAPH: 2,2'-Azobis(2-amidinopropane)

ABTS:2,2'-AZINO-BIS-3-ethyl-benzothiazoline-6-sulforic acid

B

Bax: Bcl-2-associated x protein

C

CAT: Catalase

Cyt C: Cytochome oxidase

CoQ: Coenzyme Q or ubiquinone

CuZnSOD: Copper-Zinc superoxide dismutase

CYPs: Cytochrome P450 monoxygenase

CD=Hydroxypropyl-Beta-Cyclodextrin

CumOOH: Cumene hydroperoxide

CLPAA: Cellular lipid peroxidation antioxidant activity

D

DCFH-DA: 2',7'-Dichlorofluorescein diacetate

DPPH: 2,2-Diphenyl-1-picrylhydrazyl

DNA: Deoxyribonucleic acid

E

eNOS: endothelial Nitric Oxide Synthase

ER: Endoplasmic reticulum

ERO1: ER Oxidoreductin

ET: Electron transfer assay

F

FAD: Flavin dependent

FADH2: Flavin adenine dinucleotide

FFA: free fatty acids

G

Gpx: Glutathione-peroxidase

GTP: guanosine triphosphate

H

HO-1: Heme oxygenase 1

HDL: High density lipoprotein

HAT: Hydrogen transfer-based assay

I

IL-1: Interleukin-1

IMS: Mitochondrial intermembrane space

K

KEAP: Kelch-like ECH-associated protein

L

LDL: Low density lipoprotein

M

MnSOD: Manganese superoxide dismutase

MitoSOX: Mitochondrial superoxide indicator

N

Nrf2: Nuclear factor-erythroid 2-related factor 2

NADPH: nicotinamide adenine dinucleotide phosphate

NQO1: NAD(P)H dehydrogenase [quinone]1

NF-Kb: Nuclear Factor kappa B

NLC: Nanostructured lipid carrier

O

O/W: Oil in Water

OMM: Outer mitochondrial membrane

ORAC: Oxygen radical antioxidant capacity

P

PUFAs: Poly-unsaturated fatty acid

PDI: Protein disulfide isomerase

PI3K: Phosphoinositide 3-kinase

PDGFRs: Platelet derived growth factor receptors

PVA: Polyvinyl alcohol

R

ROS: Reactive Oxygen species

RNS: Reactive Nitrogen species

RAC1:Ras-related C3 botulinum toxic substrate1

S

SOD: Superoxide dismutase

SLN: Solid lipid nanoparticles

T

TNF: Tumor necrosis factor

t-BuOOH: tert-butyl hydroperoxide

TEAC: Trolox Equivalent Antioxidant Capacity

V

VDAC: Voltage Dependent Anion

General Introduction

Introduction

Le fonctionnement normal des cellules dépend des équilibres redox intra et extracellulaires. L'état appelé stress oxydatif est induit par la présence excessive de radicaux libres et peut conduire à l'oxydation des constituants cellulaires, comme les lipides, l'ADN, ainsi qu'au dysfonctionnement cellulaire. Le stress oxydatif et l'inflammation jouent un rôle très important dans les pathologies cardiovasculaires. L'équipe de Bioingénierie de l'U1148 INSERM-Université Paris 13 développe depuis plusieurs années des recherches sur des dispositifs médicaux et des biomatériaux visant à améliorer la prévention et le traitement de maladies cardiovasculaires. Cette thèse s'inscrit dans cette démarche. L'objectif de cette thèse a été la mise au point d'un système de libération locale d'un antioxydant pouvant stabiliser l'équilibre redox des cellules et des tissus soumis au stress oxydatif dans des applications cardiovasculaires. La recherche a été centrée sur l'astaxanthine, un caroténoïde xanthophylle puissant capable de piéger les radicaux libres. Des travaux précédents dans notre groupe ont porté sur l'extraction, la purification et la caractérisation de l'astaxanthine à partir d'une microalgue, *l'Haematococus pluvialis* et montré *in vitro* des propriétés protectrices sur des cellules endothéliales humaines. Par ailleurs, les propriétés antioxydantes et anti-inflammatoires de l'astaxanthine ont montré leur efficacité dans différents travaux *in vitro* et *in vivo*. L'astaxanthine est une molécule hautement insaturée qui se décompose facilement lorsqu'elle est exposée à la chaleur, à la lumière ou à l'oxygène. Sa couleur pourpre foncé et sa solubilité limitée dans l'eau ont entravé ses applications. De ce fait, des dérivés, des complexes et des nanosystèmes améliorant la solubilité ou la stabilité de l'astaxanthine ont été très étudiés. Cependant, aucun de ces systèmes n'a été conçu pour la libération locale de l'astaxanthine dans le site de stress. Cette thèse a tout d'abord cherché à développer des complexes d'inclusion d'astaxanthine dans l'hydroxypropyl-β-cyclodextrin (CD-A), afin de protéger son activité, d'assurer une meilleure biodisponibilité et de permettre une action efficace.
Une partie de cette thèse est dédiée à leur étude. Puis les CD-A ont été incorporés dans des matrices polymériques pour la fabrication des plateformes de libération d'astaxanthine et leur action a été évaluée *in vitro* et *in vivo.*

Ce manuscrit s'articule autour de six chapitres : les deux premiers sont consacrés à deux études bibliographiques, les trois suivants étant constitués des articles scientifiques portant sur les principaux résultats obtenus et le chapitre final présente une conclusion générale et perspectives. La première étude bibliographique (chapitre 1) décrit les différentes espèces réactives oxygénées (ROS) impliquées dans le stress oxydatif, ainsi que les mécanismes de

défenses antioxydantes endogènes et exogènes. Le deuxième chapitre est constitué tout d'abord (chapitre 2A) d'un article de type « review » consacrée à l'astaxanthine. Y seront décrites ses propriétés physico-chimiques, les différents systèmes permettant son encapsulation ainsi qu'un état de l'art sur les études de modèles d'ischémie/reperfusion *in vivo*. Une deuxième partie dans ce chapitre (chapitre 2B) décrit les principales méthodes chimiques et biochimiques de caractérisation de l'activité antioxydante. Les résultats des travaux réalisés pendant cette thèse s'articulent ensuite en trois chapitres, chacun étant constitué d'un article soumis ou en révision. La principale stratégie d'encapsulation que nous avons développée repose sur l'élaboration de complexes cyclodextrines-astaxanthine. La synthèse de ces complexes, leur caractérisation physico-chimique et leur activité antioxydante sont décrits dans un article qui fait l'objet du chapitre 3. Des systèmes antioxydants ont alors été mis au point, constitués de matrices polymériques de PVA dextrane (chapitre 4) ou de pullulane/dextrane (chapitre 5). La bonne tenue mécanique des patchs PVA/dextrane, et la validation des matrices pulullane/dextrane dans un modèle d'ischémie/reperfusion confirment le potentiel de ces dispositifs pour agir localement.

Finalement, ce manuscrit fournit une preuve des capacités antioxydantes puissantes des systèmes basés sur des complexes cyclodextrines-astaxanthine. En outre, les résultats présentés ici sont encourageants pour continuer à évaluer ces systèmes pour une application postérieure dans la prévention et le traitement des pathologies cardiovasculaires

Mot clés: Espèces réactives oxygénées; Stress oxydatif; Antioxydants; Astaxanthine; Cyclodextrines; Ischémie/Reperfusion ; Biomatériaux, Systèmes de délivrance des médicaments.

Introduction

Normal cell function depends on intra- and extracellular redox equilibrium. The state called oxidative stress is induced by an overconcentration of free radicals which can lead to the oxidation of cellular constituents such as lipids, DNA and cellular dysfunction. Oxidative stress and inflammation play an important role in the generation of cardiovascular pathologies. For several years, the bioengineering team of U1148 INSERM-University Paris 13 has been developing research on medical devices and biomaterials to improve the prevention and treatment of cardiovascular diseases, this thesis lies within this approach. The principal aim of this thesis has been the development of a system that allows the local release of an antioxidant molecule capable of stabilizing the redox equilibrium of cells and tissues subjected to oxidative stress for a cardiovascular application. The research focused on astaxanthin, a potent xanthophyll carotenoid able to quench free radicals. Previous work in our group centered on the extraction, purification and characterization of astaxanthin from a Haematococus pluvialis microalgae. The results showed the *in vitro* astaxanthin protective properties on human endothelial cells. In addition, the antioxidant and anti-inflammatory properties of astaxanthin have shown to be effective in different *in vitro* and *in vivo* studies. As a highly unsaturated molecule, astaxanthin is susceptible to degradation by light, oxygen and temperature. Its dark purple color and its limited water solubility have limited its applications. Hence, astaxanthin derivatives, complexes and nanosystems have been extensively conceived to improve its solubility or stability. However, none of these systems were designed for the local release of astaxanthin in the site where the oxidative stress takes place. This thesis first sought to develop an astaxanthin inclusion complexes within hydroxypropyl-β-cyclodextrin (CD-A), in order to protect its antioxidant capacities, to ensure a higher bioavailability and to allow its effective action. CD-A systems were particularly studied on this thesis. Afterwards, CD-A were incorporated into polymeric matrices for the elaboration of astaxanthin delivery platforms and their action was evaluated *in vitro* and *in vivo.*

This thesis manuscript is organized in six chapters: The first two chapters are devoted to a literature review, chapter three through five consist on scientific articles presenting the principal results obtained during this thesis. The final chapter addresses the general conclusions and perspectives of this work. The first literature review (chapter 1) describes the reactive oxygen species (ROS) involved in the generation of oxidative stress, as well as the endogenous and exogenous antioxidant defenses mechanisms in charge of their regulation. The second chapter is divided into two sections. The first section presented in the form of a review article

(chapter 2A) covers astaxanthin generalities from its physico-chimic characteristics to the systems used for its encapsulation, including a review of *in vivo* ischemic reperfusion models using astaxanthin as treatment. The second section (chapter 2B) describes several chemical and biological methods available to evaluate the antioxidant ability of antioxidants. The results of the work carried out during this thesis are then organized into three chapters, each of them presented as an article already submitted or under journal review.

The main encapsulation strategy developed in this work focus on the implementation of cyclodextrin astaxanthin complexes. The synthesis of these complexes, their physicochemical characterization and their antioxidant activity are described in chapter 3 (published article). Antioxidant systems were then developed, consisting of polymeric matrices of PVA/Dextran (chapter 4, published article) or Pullullan/Dextran (chapter 5, article under revision). The high mechanical properties of the PVA/Dextran patches and the validation of the Pullullan/Dextran scaffolds in an *in vivo* ischemic and reperfusion model, confirm the potential of these systems to act locally.

Finally, this manuscript provides evidence that fully supports the powerful antioxidant capabilities of astaxanthin cyclodextrin complexes. Moreover, the results reported here are encouraging to continue evaluating these systems for a posterior application in the prevention and treatment of cardiovascular pathologies.

Key words: Reactive oxygen species; Oxidative stress; Antioxidants, Astaxanthin, ischemia/reperfusion; Biomaterials; Drug delivery systems

A brief summary of each of the chapters that composes this thesis manuscript will be presented in the following section.

1. Oxidative stress and ROS

Oxidative stress refers to "a disruption of redox signaling and control" [1]. A cell redox state is achieved by the balance between pro-oxidant and antioxidant molecules and by spatial and temporal confinement of oxidative reactive species, ensuring the stability of cell structural integrity and physiological function [2]. Reactive oxygen species (ROS) denote a variety of highly reactive molecules and free radicals derived from molecular oxygen. ROS are formed as a normal byproducts of aerobic respiration and current cellular metabolism [3]. Moderate amounts of ROS have beneficial effects on several physiological processes by acting as signaling molecules [4,5]. In contrast, ROS overconcentration disrupts the body homeostasis inducing oxidative tissue damage and leading to oxidative stress related diseases like cardiovascular pathologies. In the cell, these reactions are counteracted by the action of enzymatic and non-enzymatic antioxidant defenses, however the generation of exogenous and endogenous radicals stand in their protective cellular mechanism, thus contributing to tissue damage [6].

Chapter one provides a general overview of redox signaling and oxidative stress, and their contribution to both physiological and pathological conditions. Also, the different reactive oxygen species (ROS), their endogenous and exogenous sources and the enzymatic and non-enzymatic antioxidant defense involved in their regulation.

2. Carotenoids action towards cardiovascular diseases: Astaxanthin, an antioxidant strategy

Cardiovascular diseases (CVD) remain a leading cause of mortality and disability around the world. The global action plan of the World Health Organization expects to reduce up to 25% of premature mortality form cardiovascular diseases and to increase at 50% the number of eligible people receiving drug therapy and counselling to prevent heart attacks and strokes before 2020 [7]. A balanced diet including vegetables and fruit as well as regular physical exercise, have been suggested as healthy living practices to reduce the risk of cardiovascular diseases. Vegetables and fruits represent important sources of carbohydrates, dietary fibers, antioxidant vitamins, and minerals, in particular, they are the main asset of carotenoids in the human diet [8]. But what makes carotenoids so special, and how they interact with our body?

Carotenoids are lipophilic pigments, not synthetized by humans, and therefore require to be ingested in the diet [9]. Different studies have shown a correlation between elevated dietary

carotenoids intake and the reduction of markers of inflammation, oxidative stress, and endothelial dysfunction [10], which are known to be associated with CVD [11]. Their action is attributed to carotenoids conjugated double bonds structure which gives their capacity to act as free radical scavengers and to enhance the oxidative redox balance [12,13].

Astaxanthin, a xanthophyll carotenoid, have shown the highest antioxidant activity toward peroxyl radical among other carotenoids [14]. Indeed, our laboratory demonstrated its powerful antioxidant capabilities on human endothelial cells subjected to exogenous induced oxidative stress [15]. Human studies have shown promising results in the improvement of antioxidants levels in patients with coronary heart disease, when administered astaxanthin both orally or intravenously [16]. Additionally, studies evaluate the effect of astaxanthin treatment on animal undergoing ischemia and reperfusion injury. However, some stability and sensibility drawbacks have been attributed to astaxanthin. So, the implementation of new biomaterials which could act as astaxanthin vectors *in vivo* is of actual interest.

Chapter 2A, presents a literature review based on the selected carotenoid-antioxidant strategy: Astaxanthin. First, the physicochemical characteristics of astaxanthin molecule will be presented. Then, a cardiovascular approach to oxidative stress related pathologies induced by ROS overconcentration will be provided. A special focus will be given to astaxanthin potential antioxidant action against I/R condition produced by ROS imbalance. Finally, different systems used for astaxanthin in vivo delivery will also be discussed Different methods used in this work for the detection of antioxidant capacities will be briefly summarized in Chapter 2B.

3. Hydrophilic astaxanthin: A powerful antioxidant

As a highly unsaturated molecule, astaxanthin has low water solubility and can be easily degraded by light, oxygen and temperature, leading to the decrease of its bioavailability and a diminution of its properties [17]. A system based on astaxanthin inclusion within hydroxypropyl-β-cyclodextrin (CD-A) was explored, avoiding any degradation of the molecule.

Chapter 3 covers the chemical, biological and molecular characterization of hydrophilic astaxanthin based system as the selected strategy to deal with cellular and mitochondrial ROS imbalance on human endothelial cells (HUVEC) subjected to chemically induced oxidative stress.

The synthesis and characterization of CD-A complexes were successfully achieved (Figure 1). Complexes antioxidant capabilities were quantified directly by *in tube* and *in vitro* generation of peroxyl, and alkoxyl radicals using two stressors: AAPH or t-BuOOH. Additionally, superoxide radicals' were generated after induction of mitochondrial depolarization by antimycin A, while lipid peroxyl radicals were initiated by addition of cumene hydroperoxide. An indirect antioxidant capacity evaluation was performed by understanding the molecular mechanisms involved in the regulation of endothelial cells gene expression by CD-A complexes. The activation of endogenous antioxidant PTEN/AKT, Nrf2/HO-1 and NQOI gene and protein expression supported the protective effect of CD-A complexes on human endothelial cells under stress conditions. Moreover, a non-toxic effect was registered after HUVEC cells were supplemented with CD-A complexes and after *in vivo* subcutaneous implantation (Figure 2).

Owing to the results presented above, CD-A complexes appear to be good candidates for the prevention of endothelial oxidative stress related diseases as cardiovascular pathologies.

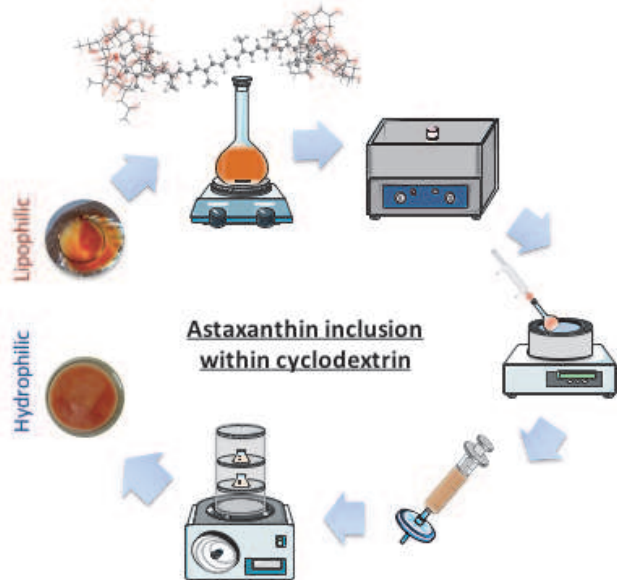

Figure 1:

***Figure 1**: Lipophilic astaxanthin inclusion within hydroxypropyl-β-cyclodextrin (CD-A).*

4. Astaxanthin targeting ischemia and reperfusion injury: two different biomaterials systems for astaxanthin delivery *in vivo*.

This study focused on a specific vascular pathology: ischemia and reperfusion (I/R). Reperfusion injury refers to the cell and tissue damage induced when blood flow is restored after an ischemic period [18]. Reperfusion while reestablishing oxygen supply, generates a production of radicals that further induce oxidative stress state and muscle damage also called 'reperfusion injury' [19]. *In vivo experimental* studies using different models of induced (I/R) confirm astaxanthin protective action when administered both orally or intravenously (Chapter 2). An *in vivo* direct release approach seems promising to evaluate the already verified antioxidant activities of CD-A complexes. Based on that, two delivery systems were explored (Figure 2).

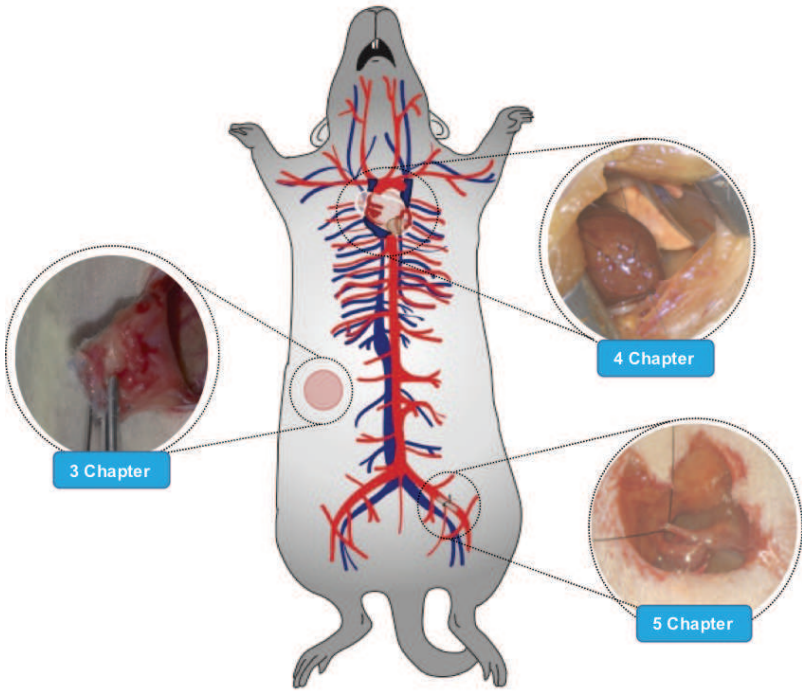

Figure 2: *Explored in vivo applications using CD-A complexes. CD-A biocompatibility was evaluated after subcutaneous implantation (Chapter 3). Antioxidant action of CD-A complexes were evaluated in two cardiovascular models: myocardial infarction (Chapter 4) and lower limb I/R (Chapter 5).*

4.1. PVA/Dextran hydrogel patches intended for the delivery of CD-A.

Myocardial infarction (MI) is caused by the infarction of the heart muscle, which is induced by the occlusion of one or more of the vessels in charge of the blood and oxygen supply. Reperfusion response carries out a burst of ROS blocking the tissue recovery and damaging the muscle wall [20]. Up to date, early reperfusion therapy and heart transplantation are available treatments to MI. However, induced secondary reactions remains a limitation.

Here, a new elastic hydrogel material obtained as hydrogel from PVA and dextran blends loaded within CD-A was evaluated. The hydrogel patches showed high *in vitro* compatibility, as well as mechanical and stability properties; in addition, CD-A capabilities were not affected by the loading process. The *ex vivo* suturability test supports the idea of using this system in the reduction of myofibril stress generated after myocardial infarction, by posterior delivery of an antioxidant molecule in the ischemic heart area to reduce the oxidative stress status produced by I/R injury, while improving the inner heart environment for repair and recover.

Chapter 4 examines a biomaterial strategy to deliver astaxanthin based on the use of synthetic polymer Poly(Vinyl Alcohol) PVA in combination with Dextran, a natural polysaccharide. A future *in situ* deliver application as myocardial patch is proposed.

4.2. Pullulan/Dextran scaffold for CD-A delivery

The lack of oxygen restricts blood supply inducing a hypoxic state and reducing the blood flow to the lower extremities leading to a ischemic condition [21]. Different strategies have been attempted to restore the ischemic tissue like surgical bypass and endovascular interventions, but reperfusion injury or even restenosis limit their action leading to the progression of the pathology [22]. Here, the feasibility of using a polysaccharide matrix based on pullulan/dextran (P/D) as astaxanthin delivery scaffold was successfully achieved. This proof of concept wanted to study the capacity of CD-A to reduce the muscular lesion produced after hind-limb femoral ischemia in a rat model. After implantation periods of 60 min, 7 and 15 days of scaffold-loaded astaxanthin, the gracilis muscle evidenced an inner body defense mechanism to foreign materials, activating an immunomodulatory response, which may disturb astaxanthin antioxidant effect. However, the reduction of transcription factors like Nrf2 revels a protective effect of CD-A in treated tissues.

Chapter 5 covers the evaluation of a second biomaterial strategy based on two natural polysaccharides: Pullulan and Dextran as astaxanthin delivery system to reduce muscle injury after lower hind limb I/R. An in vivo proof of the concept.

5. Conclusion

The final chapter presents a general conclusion of this thesis, and open some general perspectives for future works

Astaxanthin scavenging capabilities against reactive molecules has been conditioned by its structure sensibility to external factors. Here, astaxanthin inclusion within cyclodextrin has shown to enhance its stability, while preserving its antioxidant properties. Different compatible materials acting as astaxanthin carrier's systems were explored to allow its delivery *in situ* in a controlled manner aiming to reduce the generation and progression of cardiovascular pathologies, particularly I/R. Besides the already positive effects shown after astaxanthin dietary intake, there is still a need for futures studies to understand astaxanthin antioxidant mechanism to counteract the damaging effect induced by ROS overconcentration in oxidative stress related diseases. However, this manuscript provides evidence that supports the powerful antioxidant capabilities of astaxanthin *in vitro and* in *vivo*, when directed against oxidative stress linked to cardiovascular conditions.

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1 Chapter

Oxidative Stress Induced by ROS: An Overview

Chapter 1 aims to provide some elements in redox signaling and oxidative stress definition. A theoretical introduction about reactive oxygen species (ROS), from their endogenous and exogenous sources of production to the enzymatic and non-enzymatic antioxidant defense involved in their regulation is presented. Understanding the different concepts and reactions linked to ROS and their influence in the induction of oxidative stress and redox homeostasis will assist in a better comprehension of the present thesis.

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Résumé

Dans les conditions physiologiques, l'équilibre de réactions d'oxydo-réduction est maintenu dans les cellules et dans les tissus. Des espèces réactives oxygénées (ROS) sont produites dans le métabolisme aérobique de la cellule. Des systèmes antioxydants endogènes et exogènes assurent une vie aérobie normale par la régulation des niveaux de ROS et par leur confinement spatiale et temporelle en établissant plusieurs lignes de défense cellulaire. Cette régulation est appelée équilibre redox, ce dernier est maintenu lorsque les concentrations de ROS sont modérées. Mais une surproduction de ROS perturbe l'homéostasie du corps, la réponse anti-oxydante n'est plus suffisante et un déséquilibre apparait : c'est le stress oxydatif, dont le rôle dans de nombreuses pathologies est largement démontré. Un intérêt particulier a été accordé à la capacité des caroténoïdes à contribuer à la préservation de l'hémostase 'redox', grâce à leurs capacités potentielles de piéger les radicaux. Dans ce chapitre, un aperçu général du stress oxydatif induit par la surconcentration de ROS est fourni. Les composants cellulaires produisant des ROS et le système antioxydant responsable de leur régulation sont présentés.

Mots clés: Stress oxydatif, Espèces réactives oxygénées; Antioxydants

Abstract

Under physiological conditions, a cell redox equilibrium is maintained within the cell and tissues. Reactive oxygen species (ROS) are formed as a normal byproduct of aerobic respiration and current cellular metabolism. Endogenous and exogenous antioxidant systems ensure normal aerobic life by the regulation of ROS levels and ensuring their spatial and temporal confinement by stablishing multiple lines of cellular defenses. When ROS are overconcentrated, antioxidants systems are no longer efficient to ensure the body homeostasis inducing a cellular disruption also called oxidative stress, whose role in the generation on several pathologies has been demonstrated. A particular attention has been paid to carotenoids ability to contribute in the preservation of 'redox' hemostasis, thanks to their potential scavenging capabilities. In the following chapter, an overview of oxidative stress induced by ROS overconcentration is provided, as well as, the cellular components producing ROS and the antioxidant systems in charge of their regulation.

Key words: Oxidative stress; Reactive oxygen species; Antioxidants

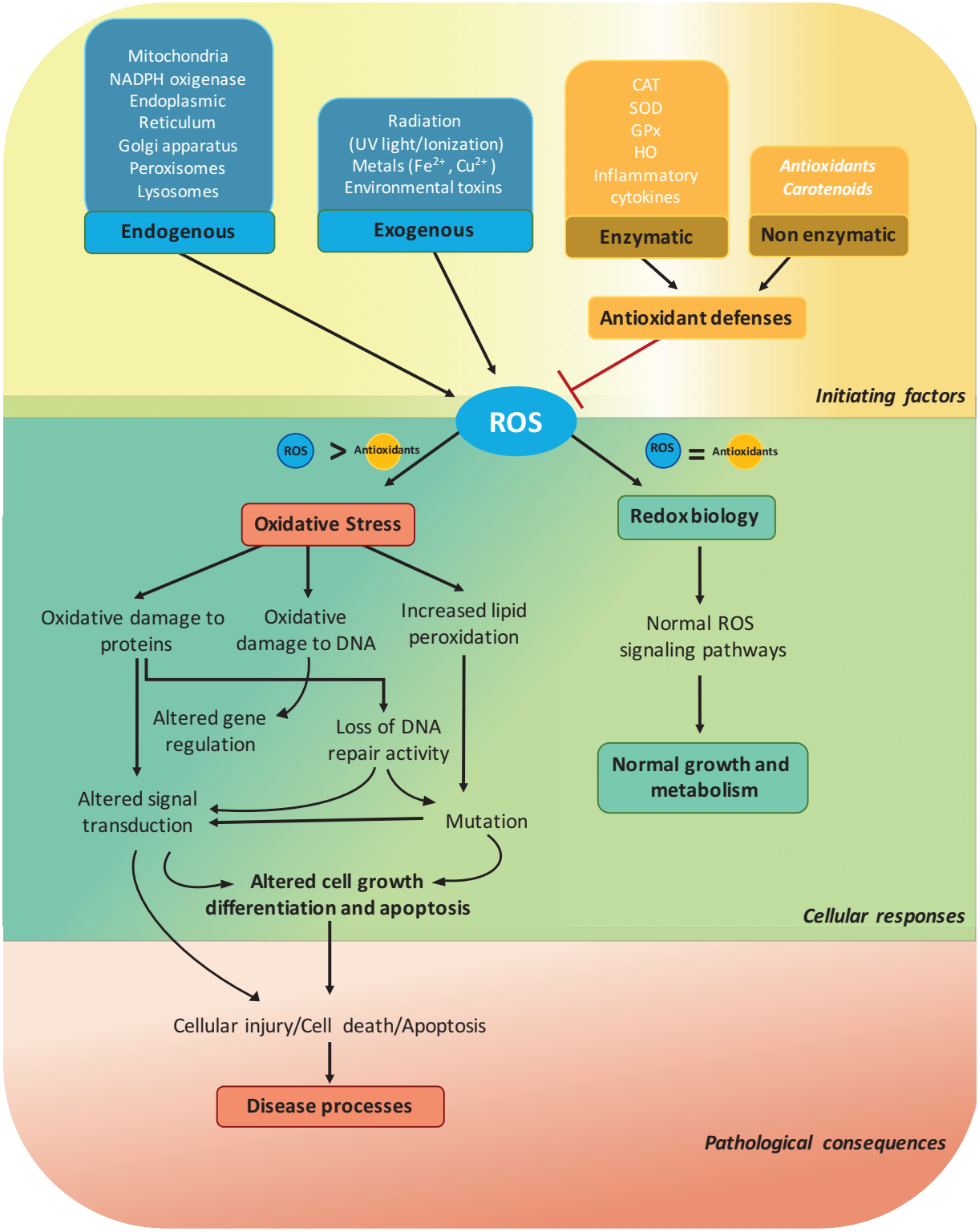

Figure 1. *The sources and cellular responses to reactive oxygen species (ROS) and their influence in either the induction of oxidative stress or redox biology. Adapted from [1–3].*

1. *Oxidative stress*

Oxidative stress was originally defined as "a disturbance in the pro-oxidant-antioxidant balance in favor of the former" [4]. This definition reveals the need to provide protection against the possible damage caused by higher concentrations of reactive oxygen (ROS) and nitrogen species (RNS) to cellular components like proteins, nucleic acids, and lipids [5,6]. However, it was necessary to consider that oxidative stress can also occur in the absence of direct structural damage by the disruption of redox homeostasis [7]. By decreasing ROS levels, signaling pathways activate, initiating different biological processes [8]. This finding led to the adoption of a more appropriate definition of oxidative stress as "a disruption of redox signaling and control" [5]. Implying that a cell redox state is achieved by the balance between pro-oxidant and antioxidant molecules and by spatial and temporal confinement of the oxidative reactive species, allowing the protection of the cell structural integrity and physiological function [9,10]. Figure 1 presents a brief description of the oxidative stress process.

2. *An overview of Reactive Oxygen and Nitrogen Species*

Reactive oxygen species (ROS) refer to a variety of highly reactive molecules and free radicals derived from molecular oxygen (Table 1). ROS are formed as normal byproducts of aerobic respiration and current cellular metabolism [11]. Moderate amounts of ROS have beneficial effects on several physiological processes like the reduction of pathogens, wound healing, and tissue repair processes by acting as signaling molecules [8,12,13]. In contrast, biological oxidation process induced either by oxidation through the removal of an electron or a hydrogen or by the addition of an oxygen [14], increase ROS levels disrupting the body homeostasis [1]. Antioxidant systems (endogenous or exogenous) initiate a regulation process to counteract this radical disturbance, however in case of ROS overconcentration, this protective mechanism fails inducing an oxidative tissue damage [2].

Free Radicals	**Sources of production**
Superoxide radical ($O_2^{\bullet-}$)	One-electron reduction of oxygen
Hydroxyl radical ($HO^{\bullet}$)	Reduction of hydrogen peroxide, that undergoes reduction to yield water. By catalysis of H_2O_2 by the action of transition metals (Cu^{+}, Fe^{2+}) (Fenton reaction)
Peroxyl ($RO_2^{\bullet}$), Alkoxyl ($RO^{\bullet}$)	Intermediate products during the breakdown of lipids in the free radical reaction of peroxidation.
Nitric oxide ($^{\bullet}NO$), Nitrogen dioxide ($^{\bullet}NO_2$)	eNOS activation by the amino acid L-arginine. $^{\bullet}NO_2$ is formed after reaction of $^{\bullet}NO$ with O_2 and it is found in polluted air and smoke.
Lipid peroxyl radical ($LOO^{\bullet}$)	Carbon-centered lipid radicals by $OH^{\bullet}$ reaction with $O_2^{\bullet-}$
Lipid radical ($L^{\bullet}$)	Oxidation product after $LOO^{\bullet}$ abstract a hydrogen from another lipid
Singlet oxygen ($^1O_2$)	Energy transfer to O_2
Non-radicals	**Sources of production**
Hydrogen peroxide (H_2O_2)	One-electron reduction of $O_2^{\bullet-}$, which is dismutated by superoxide dismutase. It can directly produce water by the action of glutathione peroxidase or catalase
Peroxinitrite ($ONOO^{-}$)	Reaction of $O_2^{\bullet-}$ with $^{\bullet}NO$
Lipid hydroxyperoxide (LOOH)	Oxidation product after $LOO^{\bullet}$ abstract a hydrogen from another lipid

***Table 1**: Description of free radicals and non-radicals: Name, chemical formula and sources of production.*

2.1. Molecular Oxygen

Atmospheric molecular oxygen is a stable diradical molecule (O_2), vital for the survival of aerobic organisms. An alteration on its concentration leads to abnormal physiological functions, making necessary a tight oxygen regulation by complex networks of signaling pathways and antioxidant defenses [15]. Oxygen stability is given by a restriction in its spin arrangements (two spin-aligned, unpaired electrons in its pi orbitals) [16]. When O_2 reacts with unpaired electrons of transition metals or organic radicals, reactive radicals "ROS" are generated [17] (Figure 2). In mitochondria, 95% of O_2 is reduced to water by the action of the protein cytochrome oxidase (Cyt C) by catalysis and by holding onto toxic partially reduced ROS until they can be fully reduced to water [18].

2.2. Superoxide Radical and Hydrogen Peroxide

The superoxide radical anion $O_2^{\bullet-}$, is formed when a single electron is supplied to O_2 (Eq.1), while two electron reduction of O_2 is required to form hydrogen peroxide H_2O_2 (Eq.2) [17].

$$O_2 + e^- \rightarrow O_2^{\bullet-} \quad \text{(Eq.1)}$$
$$O_2 + 2e^- + 2H^+ \rightarrow H_2O_2 \quad \text{(Eq.2)}$$

Additionally, $O_2^{\bullet-}$ and H_2O_2 can also be formed when molecular oxygen O_2 oxidizes redox enzymes that are designed to transfer electrons to other substrates [16]. During mitochondrial respiratory chain, electrons leak from the electron carriers and pass directly onto O_2, reducing it to $O_2^{\bullet-}$, that readily penetrates cells [19]. The concentration of $O_2^{\bullet-}$ in the mitochondrial matrix is controlled by the catalytic action of manganese superoxide dismutase (MnSOD) [20].

Hydrogen peroxide is able to leave the mitochondrion and, when produced in excessive amounts, can also diffuse across membranes reaching the extracellular space [19]. H_2O_2 is considered as an intracellular signaling molecule triggered by the activation of various cell surface receptors [21]. Indeed, H_2O_2 can also be generated as a signaling molecule by NADPH oxidase independent mechanisms [19] and by 50-lipoxygenase in the cytoplasm due to the conversion of $O_2^{\bullet-}$ to H_2O_2 by the action of cytoplasmic and mitochondrial superoxide dismutase enzymes [22,23]. H_2O_2 is more stable outside the cell due to the high oxidizing environment, where it can serve as a secondary messenger by diffusing from an already activated cell with high H_2O_2 production to another cell which initiate activation [24]. Moreover, DeYulia *et al.* [23] proposed its extracellular generation by receptor–ligand interaction, allowing it to diffuse across the plasma membrane and thus enabling the local activation of initiating kinases or inactivation of downstream phosphatases, facilitating receptor-mediated signal transduction. Antioxidant

enzymes like catalases and glutathione peroxidases are in charge of the elimination of hydrogen peroxide and organic peroxides [25].

2.3. Hydroxyl Radical

Hydroxyl radical, the most biologically and chemically active free radical is formed under conditions of oxidative stress when high concentrations of $O_2^{\bullet-}$ and/or H_2O_2 are present [11], by the influence of ferric or cuprous copper chelated Haber-Weiss reaction (Eq.3 and Eq.4) [26,27].

$$Fe^{3+}/Cu^{2+} + O_2^{\bullet-} \rightarrow Fe^{2+}/Cu^{+} + O_2 \quad \text{(Eq.3)}$$

$$Fe^{2+}/Cu^{+} + H_2O_2 \rightarrow Fe^{3+}/Cu^{2+} + OH^{-} + OH^{\bullet} \quad \text{(Eq.4)}$$

$OH^{\bullet}$ can also be generated close to phospholipids membranes where $HO^{\bullet}$ removes an hydrogen atom from one of the carbon atoms in the side chain and combines with it to form water, leaving a carbon-centered radical in the membrane able to attack the fatty acid side chains [11]. Furthermore, $HO^{\bullet}$ can react at a diffusion-controlled rate with biomolecules to form additional free radicals which can result in cellular injury [25].

2.4. Singlet Oxygen

Singlet oxygen ($^1O_2$) is generated by energy transfer when endogenous or exogenous photosensitizers absorb light at an appropriate wavelength in the presence of oxygen [28]. $^1O_2$ has been considered as a primary agent of photo-oxidative stress in microorganisms [29], due to its capacity to easily react with cellular macromolecules like proteins, lipids, and DNA, disturbing the cellular homeostasis. Mechanisms to prevent photo-oxidative stress include the use of radical quenchers, such as carotenoids [30], which interact either with photosensitizers or directly with $^1O_2$ to prevent damage of cellular molecules.

2.5. Lipid radicals

Lipids have a structural and control function in the cell membranes. Lipid peroxidation is a process under which oxidants such as free radicals or non-radical species attack lipids [31]. The first act is an initiation reaction where lipid radicals $L^{\bullet}$ are formed (Eq.5). These, in turn, can form lipid peroxyl radicals ($LOO^{\bullet}$), which abstracts a hydrogen from another lipid molecule generating two oxidation products: lipid hydroperoxide LOOH (Eq.6) and a new $L^{\bullet}$ (Eq.7) [32].

Initiation $LH + R^{\bullet} \rightarrow L^{\bullet} + RH$ (Eq.5)

Propagation $L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$ (Eq.6)

$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$ (Eq.7)

$LOOH + Fe^{3+}/ Cu^{2+} \rightarrow LOO^{\bullet} + Fe^{2+}/ Cu^{+} + H^{+}$ (Eq.8)

When transition metals are present, they decompose these peroxides into peroxyl and alkoxyl ($LO^{\bullet}$) radicals, propagating the chain reaction (Eq. 8). Under high lipid peroxidation rates, the cell repair capacity is compromised, leading to cell damage and facilitating the development of various pathological states [17,32]. Additionally, Meerson *et al*. [33] attribute to lipid peroxidation and other lipids components a contribution in the irreversible destruction of cardiomyocyte membranes during myocardial infarction.

2.6. Nitrogen Reactive Oxygen species

Nitric oxide ($^{\bullet}NO$) is a reactive molecule that can rapidly diffuse throughout the cell [34]. The formation of a non-radical product, peroxinitrite ($ONOO^{-}$) takes place when $O_2^{\bullet-}$ react with $^{\bullet}NO$ (Eq.9). At physiological pH, $ONOO^{-}$ rapidly protonates to peroxynitrous acid, ONOOH, which is a potent oxidant synthesized by the cell during normal metabolism [17].

$$^{\bullet}NO + O_2^{\bullet-} \rightarrow ONOO^{-} \quad \text{(Eq.9)}$$

In vivo, the reaction between $O_2^{\bullet-}$ and $^{\bullet}NO$ is controlled by oxyhemoglobin in red blood cells, which rapidly removes $^{\bullet}NO$ from the blood vessels [35]. The enzyme in charge of $^{\bullet}NO$ production is the endothelial synthase (eNOS), the loss of $^{\bullet}NO$ is considered to be dysfunctional, by impairing endothelium-dependent vasodilatation and promoting vasoconstriction [36], but an overconcentration can directly damage proteins, lipids, and DNA [34]. $^{\bullet}NO$ is an antihypertensive, antithrombotic and anti-atherosclerotic radical [37]. Indeed, the induction of calcific aortic valve disease has been linked to $^{\bullet}NO$ action [38].

3. *Sources of ROS*

As explained above, there are different kinds of ROS, all of them play an important role in the maintaining of redox biology or in the induction of oxidative stress. Recognizing where those radicals are produced within the cell, represents a key to understand their health impacts and to find new methods to regulate their levels. Below, we present a brief review of the major endogenous and exogenous contributors to oxidant formation within the cell (Figure 2).

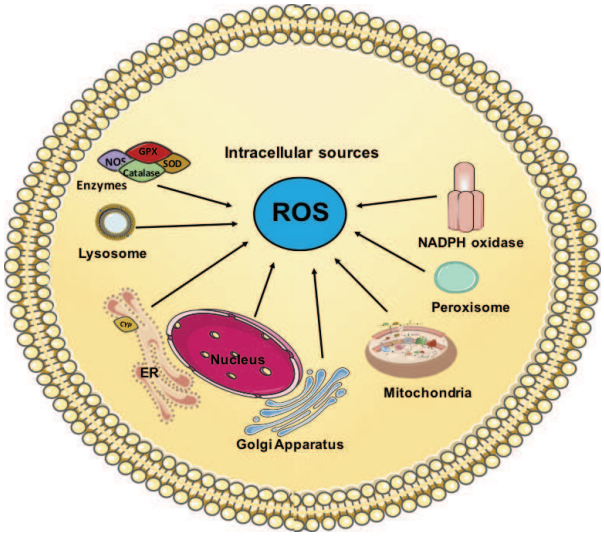

***Figure 2**: Intracellular sources of ROS: Organelles and enzymes within the cell can generate reactive oxygen species (ROS).*

3.1. ***Endogenous sources of ROS under physiological and oxidative stress conditions***

3.1.1. ROS generation by mitochondria

Although the mitochondria principal function is the generation of cellular energy (ATP) by means of the respiratory chain, these organelles are also involved in the conversion of molecular oxygen into ROS (Figure 3) [1]. The respiratory chain is localized in the inner membrane of the mitochondria and it is composed by five multi-subunit enzymatic complexes (I, II, III, IV and V) containing phospholipids, proteins and redox couples [2]. Iron is present in the respiratory chain as redox couple (Fe^{3+}/Fe^{2+}) in hemic nucleus and in Fe-S clusters of complexes located in respiratory complexes I, II, and III [39]. Each mitochondrion contains thousands of copies of the electron transport chain.

The production of mitochondrial superoxide radicals occurs primarily at Complex I (NADH-ubiquinone oxidoreductase) and Complex III (ubiquinol-cytochrome c oxidoreductase) upon one electron transfer to oxygen [2,40]. After $O_2^{\bullet-}$ release, it can be dismutated to H_2O_2 by Copper Zinc superoxide dismutase CuZnSOD in the inner membrane and cytosol (Figure 3) and by manganese superoxide dismutase (MnSOD) in the matrix. In turn, H_2O_2 is reduced to water by glutathione peroxidase (GPx) [41] and catalase (Figure 3). The movement of electrons across complex I, III and IV is coupled to protonated hydrogen (H^+) pumping, generating an electrochemical gradient that is used by complex V (ATP synthase) to generate ATP from ADP and inorganic phosphate (Pi) [42].

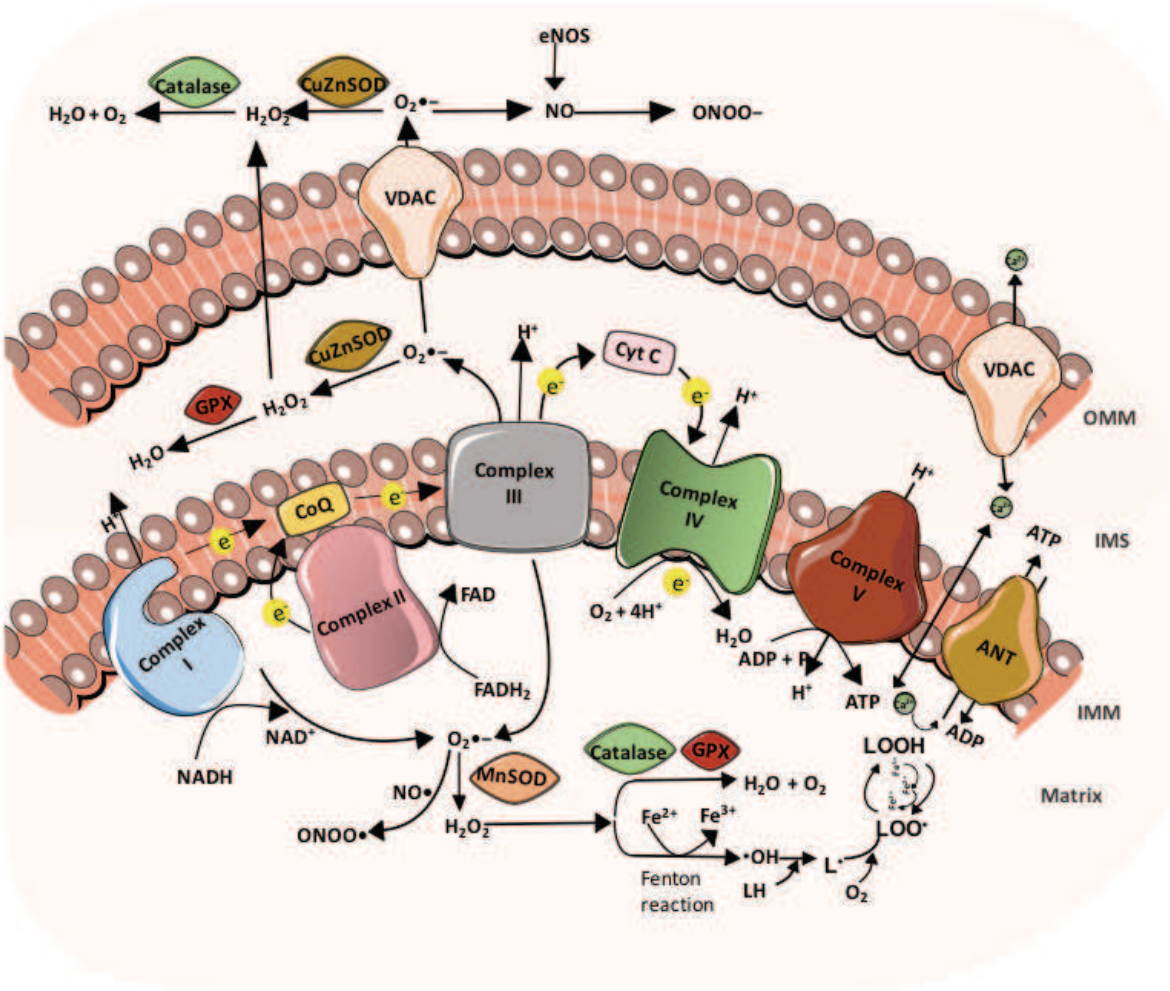

Figure 3. *ROS generation within the mitochondria. Image adapted from [42–45].*

Mitochondria dysfunction may induce an increase in electrons leak, generating highly amounts of $O_2^{\bullet-}$ [44,46], that interact with nitric oxide ($^{\bullet}NO$) to form peroxynitrite ($ONOO^{\bullet}$). Peroxynitrite may cooperate with O_2 to uncoupling the endothelial NO synthase (eNOS). Mitochondria can also lead to lipid peroxidation though the redox cycling by Fenton reaction. $HO^{\bullet}$ resulted from the interaction between free iron Fe^{2+} and H_2O_2, is capable of attacking lipid containing carbon-carbon double bonds like free fatty acids [32]. This amplification of ROS production induces endothelial dysfunction and contributes to vascular diseases [47].

Ca^{2+} and ADP production is coordinated to keep the mitochondrial balance between energy demand and supply. Under pathological conditions, the overload of Ca^{2+} and ROS levels is balanced by an increase in ADP production to maintain mitochondrial integrity and function [48]. Excess calcium may trigger mitochondrial-dependent apoptosis [49], giving to mitochondria a central role as apoptosis mediators in endothelial cells [1]. Another example of this mediation is

the activation of Bcl-2-associated X protein (Bax) which initiates caspase activation for the phagocytic recognition and engulfment of dying cells (Figure 3) [50].

3.1.2. ROS generation by NADPH Oxidases (NOX)

NADPH oxidases (NOX) are a family of enzymes formed by seven members (NOX1-5, DUOX 1/2) that catalyze the reduction of molecular oxygen to superoxide anion (Figure 4A) [1]. NOXs are major and more specific sources of ROS in the cardiovascular system. Depending on the isoform, they are expressed in different cardiovascular cell types. NOXs are involved in several process such as proliferation, migration, differentiation, apoptosis and inflammatory responses [51]. Also, they have an important role in the host defense acting as an initiator of the immune response by elimination of phagocytosed bacteria, cellular debris, and foreign material in macrophages and neutrophils [51].

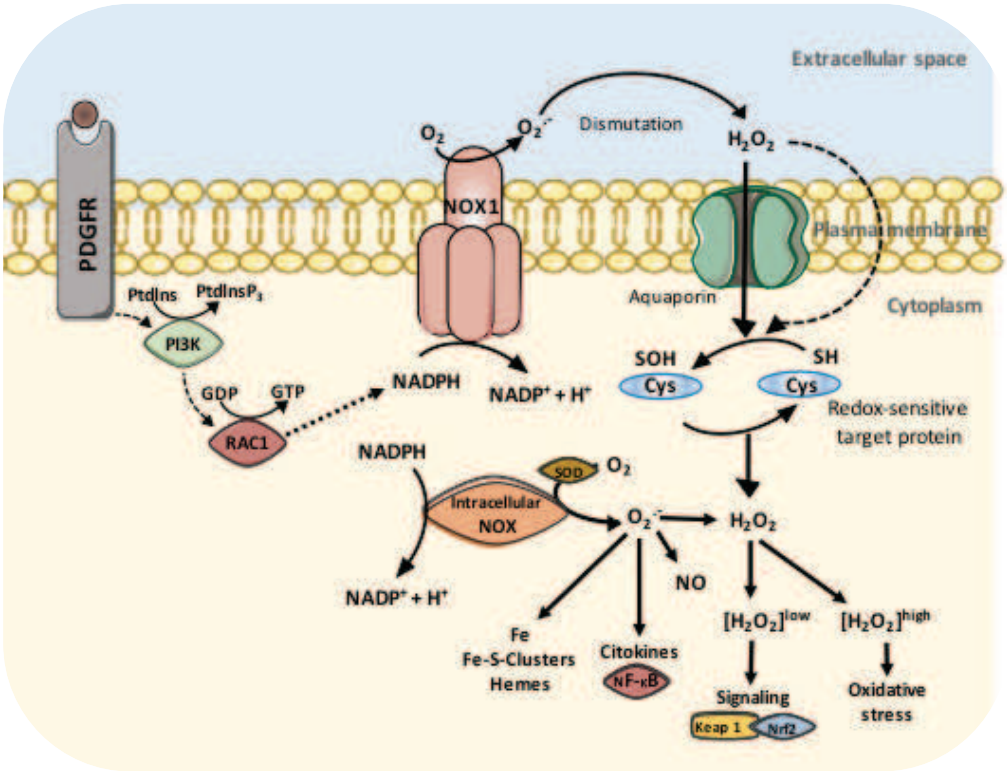

Figure 4A. *Schematic representation of NADPH Oxidase (NOX) generation of ROS. NOX constantly produce $O_2^{\cdot-}$ and H_2O_2, at controlled levels these radicals act as second messengers, while high pathological levels can induce oxidative stress and apoptosis. Image adapted from [1,10].*

Extracellular signals such as growth factors promote the initial ROS production mainly through NOXs at the cellular membrane. In fact, ROS generation in phagocytic cell takes place through the sequential activation of platelet-derived growth factor receptors (PDGFRs) and phosphoinositide 3-kinase (P13K) [51]. This interaction subsequently activates the small

GTPase protein RAC1, which mediates the propagation of the signaling response [51], leading to NOXs activation [1]. Under this condition, NOXs produce extracellular $O_2^{\bullet-}$ that can spontaneously dismutate to H_2O_2 [52]. Small amounts of H_2O_2 can diffuse back into the cell directly by aquaporin-dependent pathways. Once inside the cell, H_2O_2 alters the activity of reduction–oxidation of (redox)-sensitive targets proteins by modulating specific reactive Cys residues [53].

NOXs are important sources of vascular radical formation that allows the maintenance of normal cardiovascular health through the regulation of blood pressure [54]. The up-regulation of NOXs in vascular wall cells has been associated with the response to injury underlying atherosclerosis, diabetes, obesity, hypertension, and hypoxia [55]. Nevertheless, NOXs-mediated generation of $O_2^{\bullet-}$ had also been clinically associated with atherosclerosis [56] by the regulation of proliferation [57] and differentiation [58] of muscle cells through the activation of nuclear factor kappa B (NF-$_k$B) and inducible nitric oxide synthase (eNOS) (Figure 4B)[59].

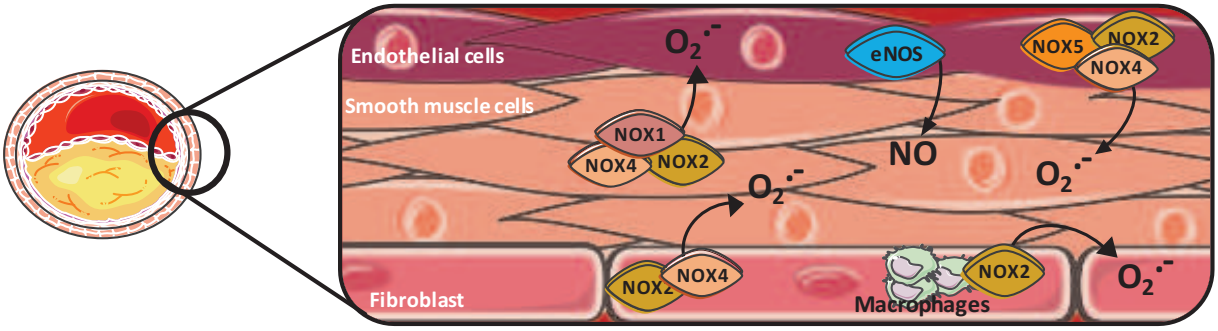

Figure 4B: *Role of NOXs signaling in atherosclerosis. Activation of specific signaling pathways by cardiovascular risk factors determine sup-regulation of NOXs and ROS production [55,60].*

3.1.3. Role of Endoplasmic reticulum in ROS generation

The endoplasmic reticulum (ER) plays an important role in ensuring cell's protein synthesis and folding. Recently, a role as oxidative stress sensor has been linked with ER function (Figure 5) [61]. The folding of proteins into the correct conformations in the ER is an energy-consuming process. Under ER physiological conditions the protein disulfide isomerase (PDI) accepts electrons from protein-folding substrates [61], inducing the oxidation of thiol (SH) groups in the protein's cysteine residues, and forming disulphide bonds, that further leads to the depletion of reduced glutathione (GSH) in oxidized glutathione (GSSG) [62]. Subsequently, the enzyme protein ERO1 by flavin-dependent (FAD) reaction, transfer electrons from PDI to molecular oxygen (O_2), forming (H_2O_2 (Figure 5) [63]. During this process Ca^{2+} ions are liberated passively

down their electrochemical gradient through the opening of release channels in the ER membrane to mitochondria [64]. RE dysfunction leads to the calcium release in the mitochondria matrix, depolarizing the mitochondrial membrane, disrupting the electron transport and increasing the ROS production. Under high mitochondrial ROS signaling, an increase of Ca^{2+} release is induced [64].

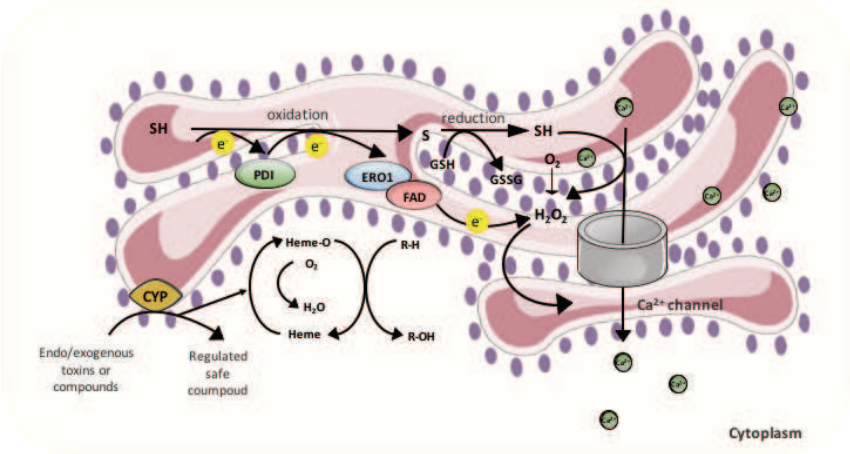

Figure 5. *Endoplasmic reticulum ROS production. Image adapted from [61,65].*

ER unfolded protein response has been linked to the accumulation of free cholesterol in macrophages [66]. This event induces apoptosis and severs ER stress promoting the progression of atherosclerotic processes [67]. ER host the hemoproteins cytochrome P450 monoxygenase (CYP), which acts as inhibitor of ROS production [65] by catalyzing H abstraction from NADPH, to reduce molecular oxygen to water [68].

3.1.4. Golgi apparatus

Golgi apparatus (GA) is the core of processing and sorting of lipids and proteins from the endoplasmic reticulum (ER) to the plasma membrane and other destinations [69]. GA also plays an important role on cell apoptosis, stress sensing, and ion homeostasis, mechanisms in which oxidative stress is involved [70]. GA ion-homeostasis function focused on the maintenance of cellular Ca^{2+} levels, particularly under stress conditions where Ca^{2+} increase rapidly [71,72]. Moreover, under severe oxidative stress, GA function is compromised by alterations and even fragmentation of its structure, carried on apoptotic events [73].

3.1.5. Peroxisomal and Lysosomal ROS production

Peroxisomes are multifunctional and ubiquitous organelles that have been identified for their role in cellular lipid metabolism. Peroxisomes play a key role in both the production and scavenging of ROS in the cell (Figure 6A). Peroxisomes oxidative pathway transform oxygen into heat

[74,75]. H_2O_2 and $O_2^{\bullet-}$ are produced by the action of peroxisomal oxidases involved in the β-oxidation of fatty acids. This process involves the decomposition of H_2O_2 into water and oxygen by catalase and glutathione-peroxidase (GPx) [76], or conversion to $OH^{\bullet}$ by transition metals [77] (Figure 6A). While $O_2^{\bullet-}$ is scavenged by manganese superoxide-dismutase (MnSOD) and by copper-zinc superoxide-dismutase (CuZnSOD) [75]. Additionally, nitric oxide synthase (NOS) catalyze the oxidation of the amino acid L-arginine (L-Arg) to nitric oxide $^{\bullet}NO$, which react to $O_2^{\bullet-}$ to form peroxynitrite $ONOO^-$ [78]. H_2O_2 and $^{\bullet}NO$ can penetrate the peroxisomal membrane and act in cellular signaling.

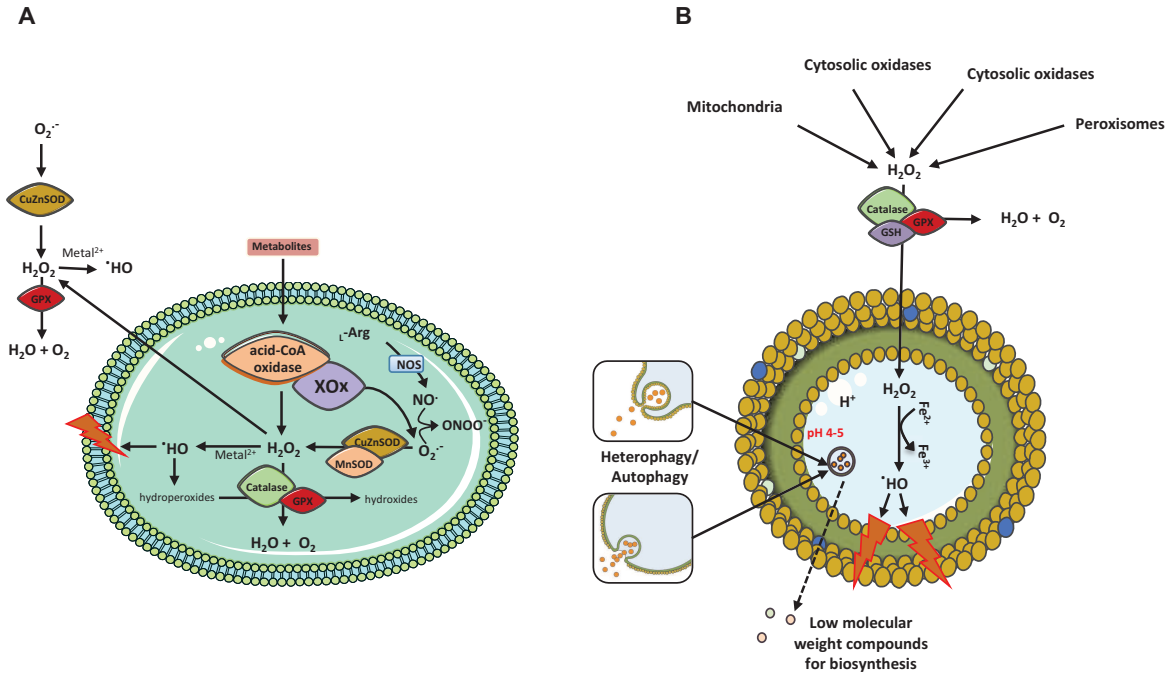

Figure 6. *Schematic overview of (A) peroxisomal and (B) lysosomal ROS production. Image adapted from [75,79].*

A close collaboration between endoplasmic reticulum, Golgi apparatus and lysosomes is placed for the degradation of ferruginous materials to avoid an excess of "free" iron. In fact, the prevalent way of iron release is protein degradation in lysosomes (Figure 6B) [80,81].

Lysosomes are acidic intracellular vesicles that provide an optimal physicochemical medium for enzymatic activities. Inside the lysosomal compartment, the degradation of endocytosed or autophagocytosed [80,82] materials takes place in an acidic environment (pH about 4 to 5), which is maintained by membrane ATP-dependent proton pumps [79]. The acidic medium and high concentration of thiols within lysosomes keep iron in a reduced (ferrous) state [83] which can easily react with endogenous or exogenous hydrogen peroxide H_2O_2 [79].

The stability of lysosomal membrane is a key factor determining cell survival-death signaling. In oxidative stress conditions, ROS compromise lysosomal integrity due to membrane lipid

peroxidation and lysosomal membrane permeation, resulting in apoptosis or necrosis [84,85]. Moreover, lysosomal enzymes promote mitochondrial permeability transition, through proteolytic activation of phospholipases, allowing cytochrome c to escape and increase levels of ROS [86].

3.2. Exogenous sources of ROS

3.2.1. Metals

Metals catalyze the oxidative deterioration of biological macromolecules inducing lipid peroxidation, DNA damage, altered calcium homeostasis, and finally leading to tissue damage [3]. For instance, iron chelates catalyze the formation of ROS and stimulate lipid peroxidation [87]. Similarly, copper metal has the ability to act as a catalyst in the peroxidation of membrane lipids [88]. While, mercury compounds act as genotoxins, altering the expression of genes that affect the human immune system [89].

3.2.2. Radiation

Ionizing radiation such as x-rays, alpha, beta and gamma rays, can cause oxidative stress by inducing ROS via secondary reactions, such as $HO^{\bullet}$ by radiolysis of water [90]. Leach *et al.* [91] demonstrated that cytoplasmic primary ionization events are amplified by a mechanism involving mitochondrial permeability transition and further production of ROS/RNS. Exposure to ionizing radiation results in complex cellular responses inducing cell death and altered proliferation states [92]. The activation or suppression of the transcriptional machinery, down-regulation of enzymes are some of the cells mechanism to respond to ionizing radiation damage and which may determine cell fate [93].

3.2.3. Photo-oxidation

Photons of relatively low energy, such as those in the visible or UV frequency range are able to interact with electrons of organic molecules to generate ROS. Reactions may involve hydrogen abstraction by an excited (photosensitized) species to form a radical ($R^{\bullet}$) which reacts with oxygen to produce a peroxyl radical ($ROO^{\bullet}$), or the absorption of the radiant energy to generate singlet oxygen that in turn, attack ground state substrates in the cell [90].

3.2.4. Environmental toxins

There is evidence suggesting that the developmental toxicological mechanisms of chemicals by oral, inhalation and/or dermal pathways and lifestyle factors involve the generation of ROS and cellular oxidative damage [94]. Additionally, air pollution generated by inorganic (sulfates, trace metals, etc) and organic carbon and biological compounds (spores, pollens) are potent oxidants, either through direct effects on lipids and proteins or indirectly through the activation of intracellular oxidant pathways [95].

4. ROS detoxification systems: Antioxidant defenses

The detoxification of ROS is one of the prerequisites of aerobic life, and one of the multiple lines of defense. Mechanisms able to counteract the potentially hazardous reactions initiated by oxygen metabolites includes all levels of protection: prevention, interception, and repair [4].

4.1. Endogenous antioxidants: Enzymes

4.1.1. Superoxide dismutases (SOD)

Superoxide dismutases (SOD) are metal ion-cofactor requiring enzymes that catalyze dismutation of $O_2^{\bullet-}$ to O_2 and H_2O_2 [17]. In Humans three isoforms are present: cytosolic and extracellular copper and zinc-containing enzyme (CuZnSOD), and manganese-requiring mitochondrial enzyme (MnSOD). SOD isoforms dismutase of $O_2^{\bullet-}$ to H_2O_2 in the mitochondrial intermembrane space and matrix [96].

4.1.2. Glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) converts glutathione (GSH), a tripeptide consisting of glutamate, cysteine, and glycine, into oxidized glutathione (GSSG), and reduces H_2O_2 to H_2O and lipid hydroperoxides (LOOH) to corresponding stable alcohols. In the other hand, glutathione reductase (GR or GSR), a flavoprotein disulfide oxidoreductase, reduces oxidized glutathione disulfide (GSSG) to reduced glutathione (GSH), which allows the protection of red blood cells, hemoglobin, and cell membranes from oxidative stress. The Isozymes of GPX are found in the cytoplasmic, mitochondrial, and extracellular compartments [97].

4.1.3. Catalase

Catalase, an heme enzyme, mainly found in peroxisomes is able to dismutate H_2O_2 to H_2O and O_2 [75], also an anti-apoptotic function has been attributed to this enzyme [98].

4.1.4. Heme oxygenase (HO)

Heme oxygenase (HO) catalyzes degradation of heme and generates carbon monoxide (CO), biliverdin, and iron [99]. HO-1 is expressed in a low level at baseline in nearly all cells, but it is strongly increased under heat shock, UV radiation, ischemia and reperfusion injury, lipopolysaccharide (LPS), cytokines, and oxidative stress [100]. Although HO does not have a direct antioxidant enzymatic function, HO and its product CO are believed to have indirect cytoprotective responses against oxidative stress [101]. Heme oxygenase-1 (HO-1), is a stress protein induced in response to a variety of oxidative challenges and pathological stimuli having cytoprotective function. HO-1 mediates the anti-inflammatory effects of some cytokines [102] and has a central role in cardiovascular protection [103].

4.2. Inflammatory cytokines

Inflammation is a protective response to various cell and tissue injuries. However, when

uncontrolled, pro-inflammatory factors are activated, a redox signal-dependent expression of genes is induce to recruit inflammatory mediators such us NF-kB, or protective antioxidants [104]. Slightly elevated ROS levels may enhance immune system function, while high levels of ROS could promote a pathological inflammatory response [8].

NF-Kb has been recognized as a major player in the regulation of inflammatory cellular responses to oxidative stress [105]. NF-kB pathway is activated by various pro-inflammatory cytokines such as the tumor necrosis factor (TNF) and interleukin-1 (IL-1), that play an important role in the regulation of the innate and adaptive immune system [106]. TNF acts in the propagation of inflammation due to the activation and recruitment of immune cells, but it can also induce oxidative stress by the activation of ROS and RNS producing enzymes [107]. Monocytes and macrophages are the major sources of TNF synthesis *in vivo* [104]. While, IL-1 is a pleiotropic cytokine classified as a dominant injury biomarker. Furthermore, ROS generation by IL-1 leads to the expression of several inflammatory genes [108]. IL-1 has been linked with markers of impaired endothelial function [8], and arterial stiffness [109], which might indicate a role of these cytokines in the early stages of development and progression of atherosclerosis.

4.3. Transcription factors

The mammalian Kelch-like ECH-associated protein 1 (KEAP1)–nuclear factor erythroid 2-related factor 2 (Nrf2) uses Cys oxidation to sense and respond to an increase in ROS levels [53]. Cellular perturbations trigger the oxidation of several reactive Cys residues in KEAP1. This redox modification releases Nrf2 from KEAP1, enabling Nrf2 levels to rise, translocate and accumulate in the nucleus. Once in the nucleus, Nrf2 binds to antioxidant response elements (ARE) [7], allowing the up-regulation of antioxidant genes, including those encoding GSH S-transferase (GST), NADPH: quinone oxidoreductase 1 (NQO1) [110], and heme oxygenase 1 (HO-1), thereby increasing the overall cell defenses against ROS [53].

4.4. Exogenous antioxidants

Current research reveals the different potential applications of antioxidant/free radical manipulations in prevention or control of certain diseases [111]. In fact, there is considerable interest in the therapeutic potential of mitochondrial-directed antioxidants to reduce ROS to physiological levels [112]. For instance, a recent work demonstrated that mitoQ treatment reduced the macrophage content and cell proliferation within plaques of atherosclerosis [113]. A similar strategy has been performed to deliver α-tocopherol or the mitochondria-targeting TEMPOL (mitoTEMPO) to mitochondria. *In vivo*, mitoTEMPO decreases vascular $O_2^{\bullet-}$, increases vascular $^{\bullet}NO$ production and improves endothelial-dependent relaxation [114].

However, some antioxidants, which may not be designed to target mitochondria, have shown potential to improve mitochondria and cell function (Figure 7) [42]. For example, vitamin D has shown capacities to prevent the loss of mitochondrial potential by inhibiting Cyt C release and caspase activation in HUVEC after undergoing oxidative stress [115]. Andrukhova *et al.* [116] attributed to vitamin D the capacity to regulate eNOS and arterial stiffness in mice. Moreover, vitamin C has shown to prevent oxidation of other compounds by electron donation. Vitamin C reduces heavy metal ions (Fe, Cu) that can generate free radicals via the Fenton reaction, revealing a pro-oxidant activity [3] although its main function is to act as an antioxidant.

Minerals such as Zinc (Zn), copper (Cu), manganese (Mn), iron (Fe), and selenium (Se) are key components of enzymes that can also act as antioxidant micronutrients at controlled concentrations [1].

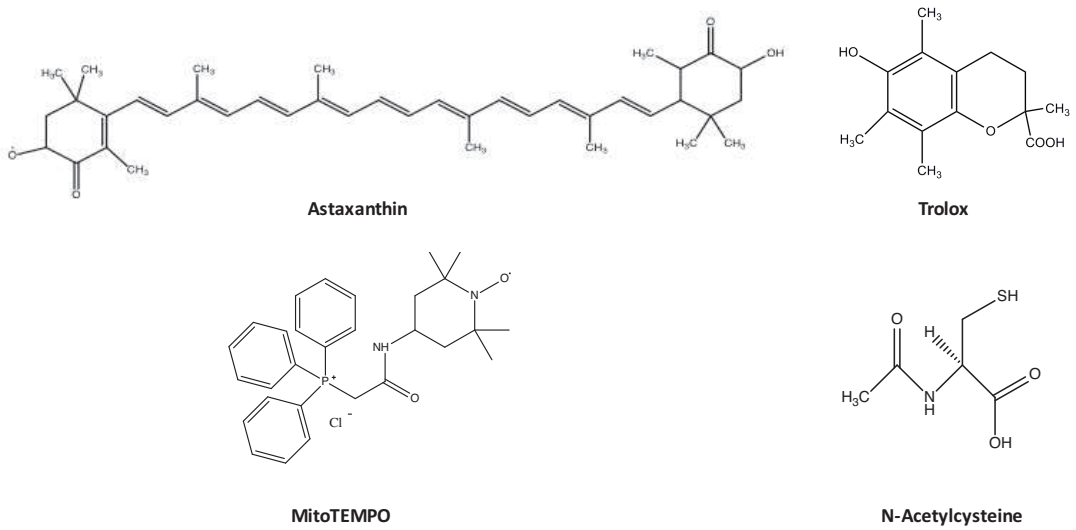

***Figure 7**. Some examples of exogenous antioxidants used in the antioxidant scavenging of ROS.*

4.4.1. Carotenoids

Carotenoids have been proposed for the prevention of chronic diseases, due to their anti-inflammatory and antioxidant properties [117,118]. Several studies suggest that carotenoids prevent the oxidation of free radical-dependent of LDL, cholesterol, proteins or DNA, by capturing free radicals and by reducing stress induced by ROS [119,120]. In this work, a special attention was given to the evaluation of carotenoids to counteract ROS production and to reestablish the cellular redox biology. **Below, a review article on *"Carotenoids from microalgae to block oxidative stress" by Marisol Zuluaga, Virginie Gueguen, Graciela Pavon-Djavid and Didier Letourneur, published on BioImPacts journal, on Febrery 2017; doi: 10.15171/bi.2017.01*** [120] ***is presented.***

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Carotenoids from microalgae to block oxidative stress

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Summary

Reactive oxygen species (ROS) are produced under normal physiological conditions and involved in several cellular biochemical processes. Their external or endogenous overproduction induces a disruption of redox signaling and control known as oxidative stress. Under oxidative stress, the cell membrane structures, enzyme functions and gene expression are compromised leading to the pathogenesis of several chronic inflammatory diseases including the cardiovascular pathologies. Attempts to find new therapeutic molecules capable of blocking the oxidative stress are of crucial importance. Owing to their anti-inflammatory and antioxidant properties, carotenoids have been proposed for the prevention and treatment of chronic diseases. In particular, microalgae carotenoids such as astaxanthin and lutein have shown promising results. Due to their protective action, these carotenoids could have a high potential to treat ROS-related pathologies. However, a better understanding of their biological mechanisms of action and the appropriate administration and uses of delivery systems are needed in the prevention and treatment of chronic pathologies.

Reactive oxygen species (ROS) are normal products of aerobic metabolism involved in cellular biochemical processes, including: signal transduction, gene expression and transcription and the activation of cell signaling cascades [121]. ROS are reactive chemical species having a single unpaired electron in an outer orbit. The increase of external ROS exposure, the decrease of antioxidant levels and the high ROS endogenous production induce excess levels of ROS, a disruption of redox signaling and control known as oxidative stress [122]. This state leads to lipid peroxidation, protein oxidation and nitration, and DNA fragmentation that ultimately affect cell membrane structures, enzyme functions and gene expression. Oxidative stress have widely been implicated in the pathogenesis of many chronic diseases such as cardiovascular pathologies, neurodegenerative pathologies [123], cancer and age related diseases [124,125].

New therapeutic molecules capable of blocking the oxidative stress are of crucial importance. Because of their anti-inflammatory and antioxidant properties carotenoids have been proposed for the prevention of chronic diseases [117,126]. Epidemiological studies suggest that carotenoids prevent the oxidation of free radical-dependent of LDL, cholesterol, proteins or DNA, by capturing free radicals and by reducing stress induced by ROS [119].

Carotenoids are fat-soluble pigments; over 750 structurally defined are present in nature, plants and microorganisms. They are directly accumulated from food or partially modified by metabolic reactions in animals. Carotenoids are divided into two major classes based on their structural elements; carotenes, constituted by carbon and hydrogen (e.g. β-carotene, α-carotene and lycopene), and xanthophylls, constituted by carbon, hydrogen, and additionally oxygen (e.g. lutein, b-cryptoxanthin, zeaxanthin, astaxanthin and fucoxanthin) [127]. The presence of conjugated double bonds allows carotenoids to accept electrons from reactive species, and then neutralize free radicals [128]. Besides, some carotenoids have been shown not only direct action but also ability to modulate gene expression of endogenous antioxidant pathways.

Some of strains of microalgae have been studied extensively for their carotenoid production activities, including astaxanthin from *Haematococcus pluvialis*, β-carotene from *Dunaliella salina* and *Spirulina*, zeaxanthin from *Synechocystis sp*., lutein from *Chlorella protothecoides*, etc. Specific advantages in the use of microalgae include a faster cultivation, processing and harvesting cycle and the ability to be cultured on waste. However, the existence of a rigid cell walls in some algal species, limits the efficiency in recovery of bioactive compounds. This is, therefore, a significant bottleneck in the overall bioprocess materials [119].

One of the better-known carotenoid is astaxanthin, a natural molecule that shows strong antioxidant activity [129]. Astaxanthin protective action involves an antioxidant mechanism based on the activation of its hydroxyl groups, which results in the formation of an ortho-dihydroxy-conjugate polyene system acting as a chain-breaking antioxidant. This molecule has shown promising results in animal and also in human experiments by the decrease of blood pressure and the increase of HDL rate. Studies show that natural astaxanthin from *H. pluvialis* is more active than synthetic one and could present therapeutic properties for the treatment of atherosclerosis and cardiovascular disease [129,130]. Moreover, growing evidences suggest that astaxanthin has potential health-promoting effect in the prevention and treatment of other disease such metabolic syndrome [131], skin disease [132], neurodegenerative pathologies [133].

Lutein is another prominent carotenoid found in several algae species, i.e. in *Scenedesmus almeriensis, Chlorella, Chlorella vulgaris, Scenedesmus obliquus, Dunaliella*

salina and *Mougeotia sp* [134]. Lutein has similar physico-chemical properties to astaxanthin and it has been used for applications related to human health, i.e. age-related macular degeneration and cardiovascular diseases. It has been stated that in a typical diet the lutein intake is lower than the daily need and it is recommended to use a supplement containing lutein [135–137]

A combination of two or more lipophilic antioxidants could also allow synergistic effects, like the elimination of reactive nitrogen species and inhibition of lipid peroxidation [117]. Natural extracts of carotenoids are a mixture of molecules; and so, their higher activities when compared with synthetics ones. Combination of phycocyanin and β-carotene contributed to antioxidant, immunomodulatory, and anti-inflammatory properties of *Spirulina* [138]. While, the ERK1/2, JNK, p38, and IκB signaling pathways are mediators in its beneficial properties [139].

Several studies confirmed the antioxidants and anti-inflammatory properties attributed to carotenoids. Plus, treatment with carotenoids showed a reduction in markers of oxidative stress and inflammation without adverse effects [130]. Nevertheless, the optimal administration and delivery systems needed to improve carotenoids bioavailability are still under investigation. Since most of the researches carried out have been based on animal and *in vitro* models, the efficiency of microalgae carotenoids in human remains to be established. Future clinical surveys should focus on large patient's cohorts. Besides, it is still needed to improve the understanding of their biological mechanisms of action to determine their efficacy in the potential prevention and treatment of chronic diseases.

***BioImpacts*, 2017, 7(1), 1-3**

This chapter aimed to show the importance of reactive oxygen species in the aerobic respiration, where ROS are produced by different organelles and external systems. Under normal rates their production and elimination is balanced thanks to powerful endogenous antioxidant mechanisms described in here, which allow ROS to act as signaling molecules protecting us against infection and further diseases. But when ROS overconcentrate, an oxidative stress state within the cells is induce, altering the redox homeostasis and thus contributing to the generation of oxidative stress related diseases such as cardiovascular pathologies. Here, the potential applications of exogenous antioxidants in the prevention and control of those illnesses was addressed. Special emphasis was given to the use of carotenoids. Current research's attribute to these pigmented components an important ability to regulate ROS levels by acting as antioxidants molecules, though their unique physicochemical properties or radical quenching.

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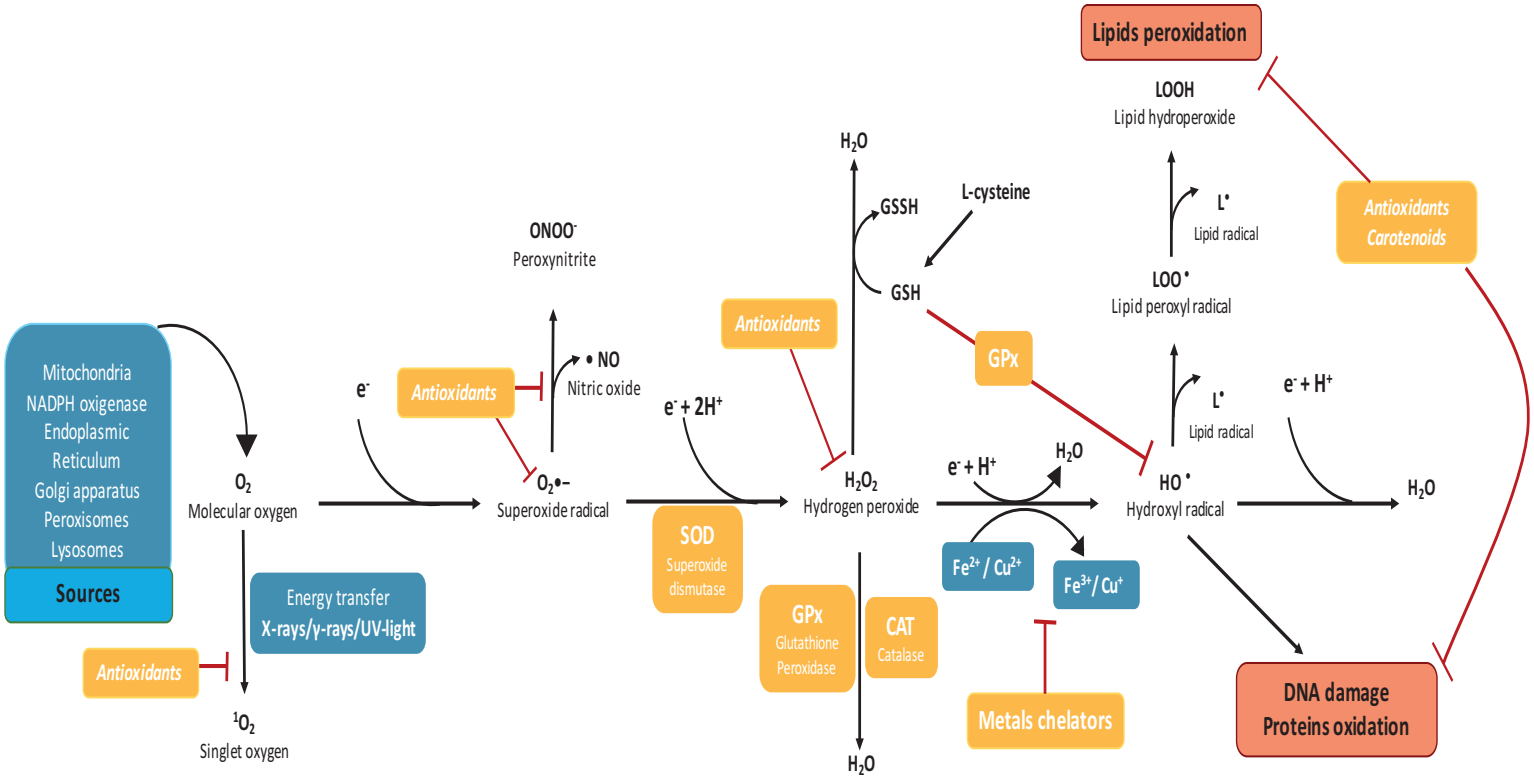

Summary: *Endogenous and exogenous sources of radicals induce the formation of reactive oxygen species. Enzymatic and non-enzymatic molecules are in charge of their regulation. Adapted from [16,91,140,141]*

2A Chapter

Review article: Astaxanthin-Antioxidant impact on reactive oxygen species induced Ischemia/reperfusion

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Chapter 2A presents a literature review focused on the selected carotenoid-based antioxidant strategy: Astaxanthin. As presented on previous chapter, ROS exert an important influence in the generation of oxidative stress related pathologies; here, a brief explanation of I/R pathology is given. Then, the physiochemical characteristics of astaxanthin molecule are presented, with special focus on its potential antioxidant action against the exposed pathology. Furthermore, an overview of encapsulation systems proposed in the literature to protect and boost antioxidant properties of astaxanthin are exposed. The format for this chapter follows the review article submitted to the Journal of Chemico-biological interactions, actually under revision.

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Résumé

Le stress oxydatif induit par les ROS est impliqué dans la pathogenèse des maladies chroniques telles que les pathologies cardiovasculaires. En particulier, le stress oxydatif semble jouer un rôle important dans la progression de la pathologie d'ischémie/reperfusion (I/R) et s'est révélé médiator du dysfonctionnement plaquettaire et de la vasodilatation dysfonctionnelle dépendante de l'endothélium, ce qui représente un facteur clé dans des conditions ischémiques. Les antioxydants naturels tels que les caroténoïdes ont été suggérés pour contribuer au traitement de la pathologie d'I/R. L'astaxanthine, un caroténoïde xanthophylle, présente des propriétés antioxydantes et anti-inflammatoires intéressantes. Des études expérimentales *in vivo* utilisant différents modèles d'I/R confirment l'action protectrice de l'astaxanthine lorsqu'elle est administrée par voie orale ou intraveineuse. Cependant, diverses études ont également montré certains inconvénients après administration orale telles que la stabilité, la biodisponibilité et la bio-efficacité. Ainsi, la mise en œuvre de nouveaux biomatériaux pour agir comme vecteurs de libération d'astaxanthine *in vivo* est particulièrement intéressante. Dans ce document est présentée une revue non exhaustive (i) des modèles *in vivo* d'I/R montrant l'effet potentiel de l'astaxanthine dans le traitement de ces pathologies et (ii) des systèmes d'encapsulation proposées dans la littérature pour surmonter les défis liés à la libération de l'astaxanthine.

Mots clés: Astaxanthine; Système de délivrance des médicaments; Stress oxydatif; Ischémie/reperfusion, ROS.

Abstract

Oxidative stress induced by ROS was shown to be involved in the pathogenesis of chronic diseases such as cardiovascular pathologies. Particularly, oxidative stress has proved to mediate abnormal platelet function and dysfunctional endothelium-dependent vasodilation, representing a key factor in the progression of ischemic injury. Antioxidants like carotenoids have been suggested to contribute in their prevention and treatment. Astaxanthin, a xanthophyll carotenoid produced naturally and synthetically, shows interesting antioxidant and anti-inflammatory properties. Experimental *in vivo* studies using different models of induced ischemia/reperfusion confirm astaxanthin protective action when administered both orally or intravenously. However, some studies have shown some drawbacks after oral administration such as stability, bioavailability and bioefficacy. So, the implementation of new biomaterials to act as astaxanthin vehicles *in vivo* is of actual interest. This document presents a brief overview of (i) *in vivo* I/R models showing astaxanthin potential effect to treat I/R injuries, and (ii) the carrier systems proposed in the literature for overcoming its delivery limitations.

Keywords: Astaxanthin; Drug delivery; oxidative stress; ischemia/reperfusion, ROS.

1. Introduction

Reactive oxygen species (ROS) refers to a variety of highly reactive molecules and free radicals derived from molecular oxygen. ROS are formed as a normal byproducts of aerobic respiration and current cellular metabolism [1]. Moderate amounts of ROS have beneficial effects on several physiological processes such as the reduction of malignant pathogens, wound healing, and tissue repair processes by acting as signaling molecules [2–4]. In contrast, ROS overconcentration disrupts the body homeostasis inducing oxidative tissue damage [5]. Indeed, high ROS levels leads to decreased bioavailability of nitric oxide, impairing endothelium-dependent vasodilatation thus promoting vasoconstriction [6]. These alterations occur early in the development of vascular diseases [7]. Moreover, overproduction of superoxide anion and hydroxyl radicals, have been considered causative agents of severe diseases, such as arteriosclerosis and ischemia and reperfusion injury [8–10], which appears to greatly increased rates of lipids peroxidation [8,11].

In the cell, these reactions are counteracted by the action of enzymatic and non-enzymatic antioxidant defenses. However, high radicals generation stand in their protective cellular mechanism contributing to tissue damage [12]. Recent studies suggest the use of exogenous antioxidant supplementation with carotenoids to enhance antioxidant defenses thanks to their potential scavenging capabilities [13–17]. Astaxanthin carotenoid is known to be a potent quencher of singlet oxygen and an efficient scavenger of superoxide anion [18] and hydroxyl radicals [19,20] by acting as an antioxidant. Moreover, within the cell, it can effectively scavenge lipid radicals and effectively destroys peroxide chain reactions to protect fatty acids and sensitive membranes [21,22] reducing the risk of atherosclerotic plaque formation [23,24]. Furthermore, astaxanthin effects in the prevention and treatment of I/R pathologies *in vivo* revels its potent action as antioxidant molecule. However, astaxanthin as a highly unsaturated molecule decomposes easily when being exposed to heat, light and oxygen. Additionally, its poor water solubility, stability and bioavailability limits its appropriate oral administration and delivery *in vivo*. The implementation of new biomaterials to act as astaxanthin vectors has been attempted through various strategies. Here, a review of *in vivo* studies reporting the effect of astaxanthin supplementation to counteract I/R injury will be presented, including a brief review of astaxanthin carrier's system successfully developed for overcoming delivery challenges.

2. Astaxanthin: A powerful antioxidant molecule

2.1 Astaxanthin sources

The carotenoid astaxanthin is found in various microorganisms and marine animals, such us yeast, microalgae, salmon, krill, shrimp, complex plants and some birds [25–29]. As general carotenoids, astaxanthin is not synthetized by humans and therefore requires to be ingested in the diet, being seafood the main intake source [30,31].

Haematococcus pluvialis (H. pluvialis), a unicellular biflagellate green microalga, is believed to have the highest capacity to accumulate astaxanthin in nature under environmental stresses such as starvation, high salt or pH, elevated temperature, or irradiation [25,32]. Under these unfavorable conditions microalgae modifies its cellular morphology, increasing their size to become red cysts charged with ~80% of astaxanthin pigment [54] and 20% comprised a mixture of other carotenoids [33]. Due to the high astaxanthin concentration, microalgae represent the primary natural source of processed astaxanthin for human applications such as dietary supplements, cosmetics, food and beverages [34], while the synthetic [35,36], yeast (mutated *Xanthophyllomyces dendrorhous*) [37], and bacteria sources (from *Paracoccus carotinifaciens*, an aerobic bacteria) [38] are predominantly used in the aquaculture sector [35]. Moreover, dietary supplements containing *H. pluvialis* astaxanthin has proved to be safe and accepted by the American Food and Drug Administration at daily doses of 2-12 mg [39,40].

2.2 Chemical characteristics

Astaxanthin (3,3'-dihydroxy-β,β'-carotene-4,4'-dione) carotenoid is a fat-soluble orange-red color pigment with the molecular formula $C_{40}H_{52}O_4$ and molar mass of 596.84 g/mol. Astaxanthin structure consists of 40 carbon atoms which contain two oxygenated β-ionone-type ring systems linked by a chain of conjugated double bonds (polyene chain). The oxygen presence in astaxanthin ionone rings in both hydroxyl (OH) and keto (C=O) groups, makes it a member of the xanthophyll carotenoid family, and confers to astaxanthin a more polar nature than other carotenoids [41]. Additionally, conjugated double bonds allow astaxanthin to act as a strong antioxidant by electron donation and by reacting with free radicals [42] (Figure 1A).

In its free form, astaxanthin is considerably unstable and particularly susceptible to oxidation, this form is mainly produced synthetically or from yeast [43]. In nature, it is found either conjugated with proteins (e.g., salmon muscle or lobster exoskeleton) or esterified by hydroxyl reaction with one (monoester) or two (diester) fatty acids, which stabilize the molecule. Natural astaxanthin from *H. pluvialis* contains 70-90% of monoesters, about 8% of diester and 2% of free form [41,44,45] (Figure 1B-C). A protective role against high light and oxygen radical has been attributed to astaxanthin accumulation in *H. pluvialis* [33]. The stereogenic carbons in the 3

and 3' positions on the β-ionone moieties, define astaxanthin conformation as chiral [(3S, 3'S) or (3R, 3'R)] or as meso form (3R, 3'S), being the chiral conformation the most abundant in nature [27] (Figure 1D-E). Astaxanthin from microalgae *H. pluvialis* biosynthesizes the (3S, 3'S) isomer whereas yeast produces (3R, 3'R) isomer [41], and the synthetic source comprises isomers (3S, 3'S) (3R, 3'S) and (3R, 3'R) [27].

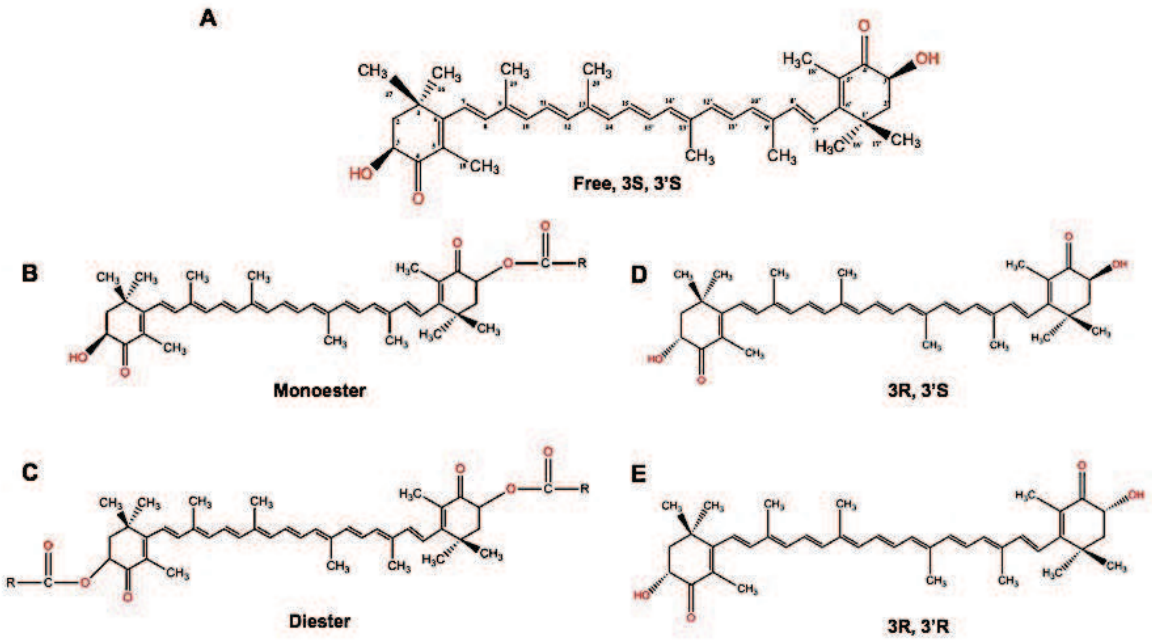

Figure 1: *(A) Structure of free astaxanthin with a numbering scheme in the stereoisomer form 3S,3'S. Astaxanthin (B) monoester and (C) diester form. (D-E) Astaxanthin stereoisomers 3R,3'S and 3R,3'R. Natural H. pluvialis produced astaxanthin 3S,3'S containing 2% free, 90% monoester and 8 % of diester, while synthetic astaxanthin exists as free form constituted by 3S,3'S, 3R,3'S and 3R,3'R in a ratio of 1:2:1, respectively [25,43,44].*

2.3 Extraction, storage, stability and bioavailability of astaxanthin

After induced stress, microalgae *H. pluvialis* becomes encysted cells and accumulate high quantities of astaxanthin [46]. This growth stage is usually done by either enclosed outdoor systems or closed indoor photo-bioreactors, which are preferred to avoid contamination by other microorganisms and to guaranty optimal and controlled growth conditions [36]. Different methods had been carried out to extract the greatest quantity of the carotenoid from *H. pluvialis* biomass by cracking the cell [47]. Some of them are based on the use of solvents [48], edible oils [49], or enzymatic digestion [50], however, supercritical fluid extraction represent the most frequently method used in the ago-alimentary industry [51–53]. Astaxanthin extraction by supramolecular solvents (SUPRAS) was performed from different samples such as salmons and foods, the extraction from *H. pluvialis* by SUPRAS is under development [54]. Once astaxanthin

is extracted from the biomass, its stability and storage must be assured to protect its biological activity from environmental factors such as temperature, pH and light [26].

The low bioavailability of astaxanthin and all xanthophyll carotenoids after oral administration has been attributed to their poor water solubility/dispersibility. Particularly, the limited solubility in digestive fluid compromise the uptake of astaxanthin by intestinal epithelial cells and their final secretion to lymph as chylomicron [30,55]. After ingestion, xanthophyll carotenoids are solubilized in the mixed micelles in the intestine tenue. These micelles are a mixture of bile acids, phospholipids, cholesterol, fatty acids and monoacylglycerols surrounded by the bile acid [56]. Then carotenoids transfers from the micelles to the epithelial cells by simple and facilitated diffusion across the phospholipid bilayers of the cytoplasmic membrane [30]. Once degraded, carotenoids are stored in the liver and re-secreted as very low-density lipoprotein (VLDL), low density lipoprotein (LDL), and high-density lipoprotein (HDL) reaching a higher level of bioavailability, to be posteriorly transported to the tissues via the circulation [57].

Due to the presence of polar ends in its structure, astaxanthin can be absorbed better than other non-polar carotenoids, like lycopene and β-carotene [58–60]. In the case of esterified astaxanthin, before LDL transport, esters need to be hydrolyzed by cholesterol esterase [21,22]. Coral-Hinostroza *et al.* [59] showed that after oral administration, astaxanthin esters are hydrolyzed selectively during absorption, suggesting that unesterified astaxanthin may be preferentially absorbed or selectively transported through circulation in human. Conversely, animal studies showed a higher uptake of astaxanthin diesters after oral administration [61]. These results could indicate that bioavailability and a half-life of astaxanthin is influenced by its esterification status [34]. Additionally, It suggest that the lipophilic properties of the molecule require the use of additives and surfactants to incorporate it into carrier systems for use in foods, beverages and pharmaceutical products [62].

3. Influence of oxidative stress in Ischemia/reperfusion: Astaxanthin antioxidant treatment

3.1 ROS production during ischemia/reperfusion injury

Reperfusion injury is induced after blood flow is restored following an ischemic period. Recent studies shown than oxidative stress could be a critical factor involved in the pathogenesis of this injury [63]. The induction of an ischemic state leads to an imbalance in the oxygen production and consumption in cells and tissues. This lack of oxygen restricts blood supply, inducing a hypoxic state which leads to microvascular dysfunction [64]. Restoration of blood flow and reoxygenation is frequently associated with an increase of tissue injury and a high inflammatory

response [65] also called 'reperfusion injury' [66]. Reperfusion injury leads to the depression of the inner body defense mechanism, inducing an imbalance between a burst of ROS production and the inability of reoxygenated cells to handle this radical load [8]. Under these conditions, cell death programs including apoptosis, autophagy-associated cell death and necrosis [67] are activated, leading to a multi-organ failure, even if only one organ underwent ischemia and reperfusion [68]. Here, extracellular ATP depletion from apoptotic cells acts as a 'find-me' signals that attracts phagocytes [69,70]. Additionally, limited oxygen availability is associated with activation of inflammatory signals which control the stability of the transcription nuclear factor NF-kB [66], through a mechanism involving hypoxia-dependent inhibition of oxygen sensors [71], and adaptive immune responses that involves the infiltration of various types of inflammatory cells (neutrophils, T lymphocytes, monocyte/macrophages) [72,73].

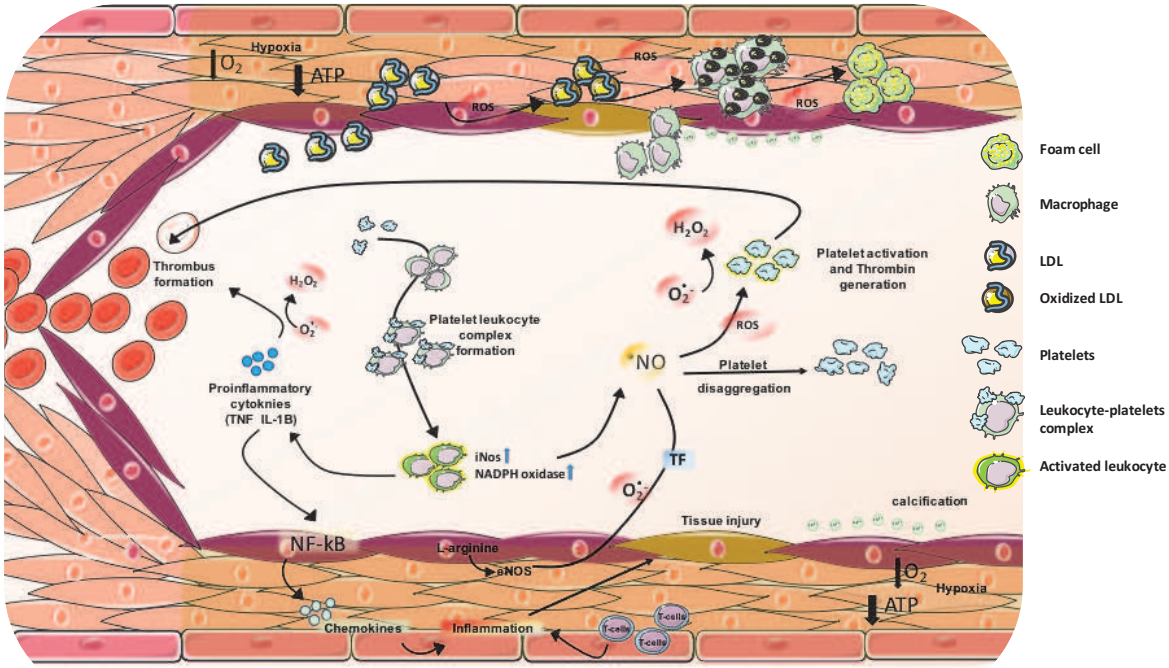

Figure 2: *I/R injury enhance ROS levels and oxidative stress conditions. Adapted from [15,64,79].*

In physiological conditions the undamaged endothelium prevents adhesion and activation of platelets and leukocytes by several mechanisms [74]. During reperfusion injury, adhesion of platelets and leukocytes to endothelial cells are enhanced leading a procoagulant state and platelet and leukocyte activation [75]. This activation results in the induction of proinflammatory cytokines and chemokines (TNF and IL-1B) [76] that are further released by activated leukocytes in the reperfused blood [77]. Under these circumstances, the endothelial cell barrier gets weak, increasing vascular permeability and leakage [78]. Endothelial damage is enhanced by the

hydroxyl radical, superoxide and peroxynitrite overproduction, that are formed following the reaction of NO with oxygen in the reperfused blood [79]. L-arginine is the main precursor of endothelial nitric oxide synthase, eNOS, in charge of NO synthesis, further enhanced by inducible NOS, iNOS [80]. Moreover, activation of NADPH oxidase in activated neutrophils, induces peroxynitrite, which generates itself more ROS by the increased availability of free iron in ischemia [81]. The excessive radical generation leads to lipid peroxidation of tissue in the subendothelial space. Accumulation of oxidized low-density lipoproteins (ox-LDL), within monocytes-derived macrophages, generates foam cells that further amplify the inflammatory cascade [82], ultimately leading to the formation of thrombus and occlusion of the vessel [83] (Figure 2).

3.2 Astaxanthin: An antioxidant ROS blocking agent

Natural carotenoids have shown particular abilities to entrap ROS and enhance the cellular capacity to block oxidative stress [84]. The effects of carotenoids vary dependent on how they interact with cell membranes [85,86]. Astaxanthin carotenoid has been shown to reduce lipid peroxidation damages by preservation of membranes structures using a polyunsaturated fatty acid enriched membrane model [87]. This action was attributed to its polar end groups which extended toward the polar regions of the membrane bilayer [88]. Additionally, astaxanthin has been currently studied in the cardiovascular field thanks to its antioxidants and anti-inflammatory properties [88]. Astaxanthin showed reduction of blood coagulation, platelet aggregation and promoted fibrinolytic activity in a high-fat diet-induced hyperlipidemic rats. These positive effects were correlated with decrease of serum lipid and lipoprotein levels, antioxidants production and protection of endothelial cells [89].

Moreover, the protective effect of astaxanthin has been studied on different *in vivo* models of I/R as myocardial, cerebral, liver and renal (Table 1). In which animals received an oral or intravenous injection of astaxanthin dose ranging between 5-500 mg/Kg/day. In these cases, astaxanthin was administered in both hydrophilic (solubilized on DI water and other organic solvents) and lipophilic (solubilized on oils) formulations before inducing the ischemic damage, thus acting as a preventive agent. A water soluble synthetic astaxanthin derivative or disodium disuccinate astaxanthin (Cardax Hawaii Biotech, Inc., USA) was studied on experimental myocardial I/R models in rat, rabbit and dogs (Table 1). After parental administration this derivative showed a potential efficacy to reduce infarct size and plasma lipid peroxidation levels attributed the direct scavenging of superoxide anion [90]. Similar results were obtained with natural astaxanthin and carotenoids mixtures (VitaePro, astaxanthin, lutein and zeaxanthin) in a rat I/R model. Oil-based astaxanthin formulations, administered mostly by oral gavage, showed

a potential reduction of inflammatory cytokines expression, decrease of organ infarct size area and reduction of arterial blood pressure lowering the risk of strokes. These studies are briefly summarized on Table 1.

Ref.	Source	Model	Pathway	Doses/Duration	Effects of Astaxanthin treatment
Gross and Lookwood 2004 [91]	Disodium Disuccinate Astaxanthin (from Dr. Samuel F. Lockwood, Hawaii Biotech, Inc.) Sterile DI Water	Rat myocardial I/R I: 30 min R: 2 h	Intravenous injection	One of 3 doses (25, 50, and 75 mg/kg) for 4 days prior to I/R	Cardax at 50 and 75 mg/kg for 4 days significantly reduces infarct size at area at risk to 35±3% (41% salvage) and 26±2% (56% salvage), respectively.
Gross and Lookwood 2005 [92]		Dog myocardial I/R I: 60 min R: 3 h	Intravenous injection	50 mg/kg 2 h or 4 days prior to I/R	Reduction in infarct size at area at risk to 11.0±1.7% (47.3% salvage) in dogs treated only once IV at 2h prior to occlusion, and 6.6±2.8% (68.4% salvage) in dogs treated for 4 days.
Lauver *et al.* 2005 [93]		Rabbit myocardial I/R I: 30 min R: 3 h	Intravenous injection (1 ml/min)	50 mg/kg/day 4 consecutives prior to I/R	Infarct size reduction expressed as a percentage of the area at risk (25.8±4.7%) in the DDA-treated. Myocardial salvage of 51%. Reduced erythrocyte hemolysis indicated by highly favorable mean myocardium/serum ratios (10.1±1.6 μM) Reduce deposition of CRP and MAC antibodies in the infarct region
Gross and Lookwood 2006 [94]		Rat myocardial I/R I: 30 min R: 2 h	Oral administration as feed supplement	0.1 and 0.4%; ~125 and 500 mg/kg/day, respectively for seven days	CardaxTM at 0.1 and 0.4% in feed for 7 days resulted in a significant mean reduction in infarct size at area at risk to 45±2.0% (26% salvage) and 39±1.5%(36% salvage), respectively. Myocardial levels of Cardax achieved after 7-day supplementation at each of the two concentrations 400 ± 65nM and 1634 ± 90 nM, respectively. Reduction of arachidonic acid and linoleic acid (lipid peroxidation products) in plasma levels
Adluri *et al.* 2013 [95]	VitaePro (2%astaxanthin, 8.1% lutein and 1.23%zeaxanthin; VitaeLab AS, Enebakkveie Oslo, Norway)	Rat myocardial I/R I: 30 min R: 2 h	Oral gavage Dissolved in safflower oil	70 mg/kg body weight for 21 days	Increased left ventricular functional recovery after I/R Decrease infarct size (27.68 ± 1.7) Decrease apoptotic cardiomyocytes (61.7 ± 10.6) measured by TUNEL assay Decrease thiobarbituric acid reactive substances levels (80 ± 3)
Wang *et al.* 2017 [89]	Astaxanthin (from Jianhe Biotech Co. Ltd. Jianhe, Hebei, China)	Mice transvers aortic constriction	Oral gavage	200 mg/kg/day for 12 days prior to TAC	Mitigation of TAC induced cardiac dysfunction, myocardial fibrosis and myocardial disorder showing that SIRT1 participates in these protective functions by attenuating R-SMAD acetylation. Reduction in the expression of protein and transcript levels of TGF-β, α-SMA and COL I.
Lu *et al.* 2010 [96]	Astaxanthin (from Sigma-Aldrich, St. Louis, MO, USA)	Rat cerebral I/R I: 2 h R: 24 h	Oral gavage	20, 50, 80 mg/kg intragastrically twice at 5h and 1h prior to ischemia	Reduction of infarct size volume 14.6±5.4% and 11.4±4.9% after doses of 50 and 80 mg/kg respectively Improved neurological deficit to 1.5±0.7 and 0.9±0.8 after doses of 50 and 80 mg/kg respectively Neurons cells protection up to 99.5±12.6.
Lee *et al.* 2010 [97]		Rat cerebral I/R I:10min R: 0 and 90 min	Intraperitoneal injection Dissolved in pure ethanol	30 mg/kg of at 0 and 90 min of cerebral reperfusion.	Protective effect of 59.5% at doses of 30 mg/kg on CA1 hippocampal neurons. Inhibition of poly (ADP-ribose) polymerase (PARP-1, apoptotic marker) cleavage at a dose of 20 mg/kg
Li *et al.* 2015 [98]		Mice renal I/R	Oral gavage	30 mg/kg or 60 mg/kg) for 14	Decrease of ALT and AST enzymes in a dose-dependent manner: Low

		I: 60 min R: 2h, 8h and 24 h	Dissolved in olive oil	days	astaxanthin doses reduce renal necrotic area, while high doses increase protection Reduction of inflammatory cytokines levels (TNF-α and IL-6) in serum and tissue. Promoted anti-apoptotic Bcl-2 and inhibited pro-apoptotic Bax levels A downward trend in protein kinases (p-P38 MAPK, p-ERK and p-JNK) expression in liver tissue
Curek *et al*. 2010 [99]	ASX (from Sigma–Aldrich Chemie, Steinheim, Germany)	Rat liver I/R I: 60 min R: 60 min	Oral gavage Dissolved in olive oil	5 mg/kg/day 14 days prior to I/R	Decreased oxidative stress by hepatic conversion of XDH to xanthine oxidase and tissue protein carbonyl levels Partial reduction of cell damage, swelling of mitochondria and disarrangement of rough endoplasmatic reticulum Reduced of protein carbonyl formation but no significant effect on GSH and nitrite/nitrate levels
Qiu *et al*. 2015 [100]	Free astaxanthin (≥97%, Sigma-Aldrich, St. Louis, MO, USA)	Mice renal I/R I: 45 min R: 12 and 24 h	Oral gavage Dissolved in olive oil	5 mg/kg/day during 14 days prior to I/R	Preservation of renal function after 12 and 24 h post I/R reflected by reduced blood serum urea nitrogen and urine creatinine levels. Decrease of apoptotic cells and α-smooth muscle actin expression assessed by TUNEL assay Decrease expressions of TNF-α, IL-1β, and IL-6 inflammatory proteins Reduce oxidative stress reflected by significantly increased of superoxide dismutase level and decreased level of malondialdehyde
Hussein *et al*. 2005 [24]	ASX-O, composed of 5.5% astaxanthin (Fuji Chemical Industry Co., Ltd., Toyama, Japan)	Mice cerebral ischemia I: 20 min	Oral gavage Dissolved in an edible oil base	ASX-O at 5, 50 and 500 mg/kg single or daily dose for 2 weeks	ASX-O showed arterial blood pressure lowering effect from the first week at the dose of 50 mg/kg. No significant change in the heart rate after ASX-O treatment ASX-O (50 mg/kg) significantly delayed the incidence of stroke

Table 1: *Summary of different in vivo studies evaluating the effect of astaxanthin treatment on induced ischemia and reperfusion injury.*

Animal studies evidenced the potential preventive effect of astaxanthin supplementation to reduce cardiovascular diseases impact. The potential astaxanthin effects in the prevention and treatment of cardiovascular disease have been extensively studied [23,25,101]. Moreover, first preclinical studies support its antioxidant abilities to prevent oxidative processes. For instance, patients who received astaxanthin supplementation showed an increased resistance to LDL oxidation when administered at doses of 1.8 to 21.6 mg/day during 14 days [102] and a slight glucose-lowering effect at doses of 4 to 20 mg/day in other studies [103]. Additionally, the amelioration of triglyceride and HDL-cholesterol in correlation with increased serum adiponectin levels after administration at doses of 12-18 mg/days during 12 weeks was also reported in other patient group [104]. In a clinical study, Park *et al.* [47] examine the action of dietary astaxanthin (2 and 8 mg/day for 8 days) in regulating immune response, oxidative damage and inflammation

in humans. Results showed an enhancement of immune markers and reduction in DNA oxidative damage biomarker and inflammation.

3.3 Encapsulation carrier's systems

Despite the positive results describe above there is still a lack in the understanding of astaxanthin therapeutic mode of action, uptake, distribution, pharmacokinetics, and metabolism. Furthermore, solve astaxanthin stability drawbacks and parenteral administration problems are of crucial interest for the development of astaxanthin-based therapies to prevent and treat oxidative stress induced cardiovascular pathologies. Consequently, the implementation of new biomaterials to act as astaxanthin vectors *in vivo* is of actual interest. Tables 2-4 summarize some carrier's systems successfully developed for overcoming astaxanthin delivery challenges. These systems were divided in three groups including polymeric systems, lipid-based carriers, and inclusion complex using cyclodextrins (Figure 3).

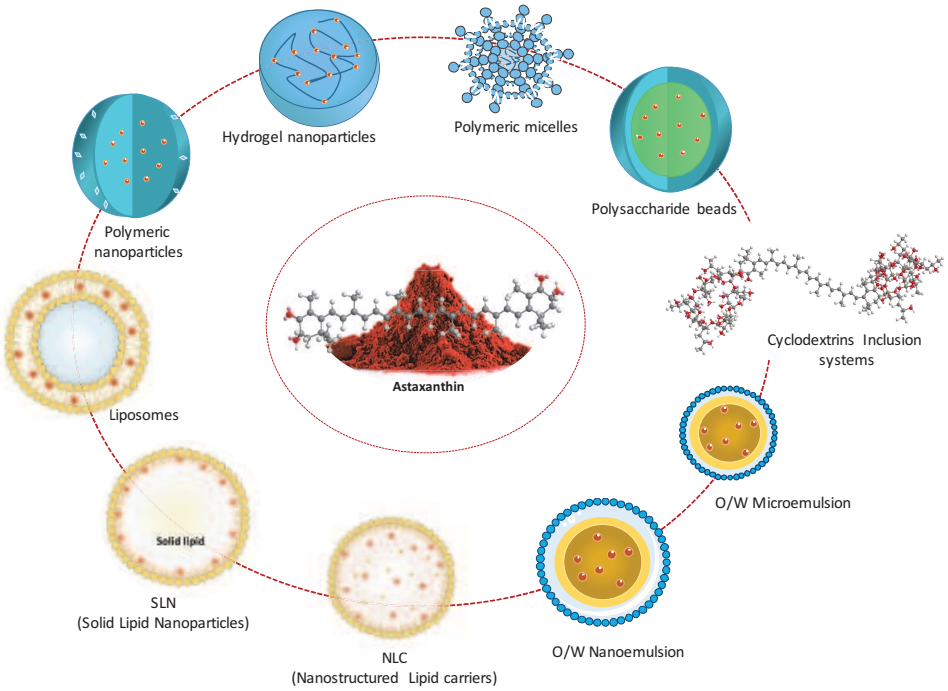

Figure 3: *Different strategies explored for astaxanthin encapsulation and posterior delivery.*

3.3.1 Polymeric systems

The microencapsulation process with polymeric systems consists in the formation of a polymeric matrix or coating layer around a particular compound to provide a physical barrier between the core material and environmental conditions. These types of systems protect the compound biological activity and enhance its physicochemical stability. Natural polymeric systems include polysaccharides like cellulose, starch, gum Arabic, alginate or chitosan [105], and the use of

proteins like albumin, gelatin or soy proteins [106] (Table 2). Microencapsulation with polymer matrices controls the molecule release, reducing the core reactivity with environmental factors and facilitating molecule handling [107,108]. In polymeric nanosytems the absorption profile of the loaded molecules is driven by the particle size, shape and surface properties of the nanoparticles [55], which could be useful for oral delivery to control the drug release until it reaches the systemic circulation [106]. Indeed, chitosan-alginate complex have shown to degraded slowly in phosphate buffer, avoiding the initial release of drugs occurring for uncoated microspheres [31]. Conversely, polymeric micelles improve their steric stabilization and ability to interact with cells due to their hydrophilic shell [106].

	Ref.	Astaxanthin source	System	Results	Antioxidant evaluation
Polymeric systems	Higuera-Ciapara *et al.* 2004 [105]	Synthetic astaxanthin (from Sigma Chemical Co. St. Louis, MO, USA)	Microencapsulation in chitosan matrix cross-linked with glutaraldehyde by multiple emulsion/solvent evaporation	Non-homogeneous size and diameter (5-50 µm) Good storage at T°<45°C for 8 weeks Yield of 92% and moisture content of 11.25% 77% of astaxanthin extraction in methanol/dichloromethane	Not performed
	Dai *et al.* 2013 [109]	Astaxanthin (from Sigma Aldrich Co.St. Louis, MO, USA). Extracted in dichloromethane	Microencapsulation using a Monodisperse Droplet Spray Dryer technique	Microparticles with relatively narrow size distribution (Mean size: 122 µm) Moisture content of 4.55 wt% Astaxanthin released immediately from the microparticles in a mixture of PBS buffer and acetone (16% released in the first 6 h).	Limited loss of antioxidant capability at 140 °C calculated by DPPH method (EC50: 1.872 x10^{-4} g/mL)
	Zhang *et al.* 2017 [110]	Astaxanthin (nutraceutical grade, obtained from Sigma Chemical Co., USA)	Microencapsulated in calcium alginate	High encapsulation efficiency (>85 %) and High storage stability at T°<50°C Uniform size distribution (1.61 mm) Sustained astaxanthin release Not cytotoxic effect on adipose-derived stem cells	Determination of lipid peroxidation inhibitory activity by the thiobarbituric acid (TBA) method
	Kittikaiwan *et al.* 2007 [111]	H. pluvialis Astaxanthin biomass (from Wako Chemicals, Japan)	Alga beads coating with multiple layer of chitosan fil	Uniform size and shape (0.431 ± 0.028 cm) Conservation of astaxanthin biomass Improved thermal stability at 80°C Optimal storage at 18°C under N_2 atmosphere in the dark	Loss of 3% of antioxidant activity after encapsulation calculated by ABTS assay
	Lee *et al.* 2011 [112]	Astaxanthin rich Xanthophyllomyces dendrorhous (Astaxanthin, nutraceutical grade IGENE Biotechnology Inc. Columbia, MD, USA	Astaxanthin-calcium alginate gel (CAG) beads prepared by ionic gelation	Beads with ovoid shape and smooth surface (average size 2.41 mm) 23-31 %t of entrapment efficiency Constant astaxanthin release rate over 12 h	Lipid peroxidation inhibition using FTC and TBA methods showed to inhibit oxidation of linoic acid
	Bustos-Garza *et al.* 2013 [113]	H. pluvialis cyst cells (fFuture Foods SA de CV,México) oleoresin Extracted in HCl and ethyl acetate	Microencapsulation using lecithin as emulsifier and whey protein and gum Arabic as wall materials.	Product yield of the astaxanthin oleoresin 61.2-70% 8.31-11.17 % of moisture content in the astaxanthin oleoresin microencapsulates particle sizes from 1 to 10 µm	Not performed

Machado et *al. 2014* [114]	H. pluvialis (Elizabeth Adair Microalgae Collection, Brazil) Extracted in dichloromethane	Co-polymer poly(hydroxybutirate-co-hydroxyvalerate) (PHBV)	Minor rigidity and crystalline Precipitation pressure influence particle size (0.128 μm) Increased encapsulation efficiency by increasing astaxanthin biomass	Not performed
Suganya et asheeba, 2015 [115]	Astaxanthin isolated from crabs (Gandhi market, Trichy, Tamilnadu, India). Solvent: hexane: isopropanol	Microencapsulation prepared by 2% sodium alginate and 3% calcium chloride using ionotropic gelation method.	Heterogeneous microspheres in size (5.65 μm to 8.98 μm) Round shaped with smooth surface. Drug content 35.25 to 42.21 mg	Not performed
Shen-Fu Lin *et al.* 2016 [107]	Astaxanthin (from Orgchem Technologies, Hsinchu, Taiwan)	Microencapsulation in calcium alginate beads Solution calcium chloride, sodium alginate, and Tween 20 as surfactant	The microencapsulation yield ranged from 97.94–25.83%, and loading efficiency ranged from 100% to 82%. Size = 685.9 to 1044.4 μm Good stability after 21 days of storage at 25°C	Not performed

Table 2*: Some polymeric systems proposed to protect and enhance astaxanthin properties.*

Table 2, presents nine different studies using polymeric matrix to improve astaxanthin solubility properties. Due to their biocompatibility and biodegradability, natural polysaccharides are currently studied for astaxanthin microencapsulation, particularly chitosan and alginate. For instance, chitosan showed an improvement in astaxanthin storage conditions, additionally, the preservation of it antioxidant scavenging ability was confirmed by ABTS chemical method. Studies using calcium-alginate reported a high astaxanthin loading efficiency, however microcapsules size distribution varied from 5.6 to 2041 μm between the studies. The preservation of lipid peroxidation inhibitory activity was confirmed by two of four studies using the TBA method and only one study evaluated the *in vitro* cytotoxicity of the system. In general, all polymeric methods improved astaxanthin solubility but lack in the full evaluation and verification of antioxidant activity preservation after encapsulation.

3.3.2 Lipid based carriers

Lipid based carriers include micelles, solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), nanoemulsions and microemulsions. These systems had been used to encapsulate, protect, and deliver lipophilic bioactive components by enhancing their long-term stability while increasing bioavailability [116]. The system stabilization is achieved by surface charge or by surface adsorption of a layer of surfactant or polymer, or by combined both methods [106].

O/W microemulsions and nanoemulsions differ by their stability. Microemulsions are thermodynamically stable colloidal dispersions consisting of small spheroid particles dispersed within an aqueous medium. While, O/W nanoemulsions refer to thermodynamically unstable colloidal dispersions consisting of two immiscible liquids, with one of the liquids being dispersed in the other liquid [116]. SLN are a mix of O/W nano/micro-emulsions in which the lipid phase is fully crystallized and has a highly-ordered crystalline structure at room/body temperature [117].

However, SLN low drug loading capacity, and drug release after polymorphic transition of the lipid core during storage represent a disadvantage [117,118]. Nanostructured lipid carriers (NLC) are modified SLN in which the lipid phase consists of a biocompatible mixture of solid and liquid lipids in a less-ordered crystalline structure [119,120]. The incorporation of oil into the core of a solid lipid leads to a higher loading capacity and controlled drug release as the drug is dissolved in the oil and simultaneously encapsulated in the solid lipid [121]. Lipids based carriers size can range from around 10 nm for micelles to hundreds of nanometers for the other systems [116].

	Ref.	Astaxanthin source	System	Results	Antioxidant evaluation
Lipid based carriers	Barros *et al.* 2001 [122]	Astaxanthin from Sigma-Aldrich Sweden AB n-hexane as solvent	Incorporation into egg-yolk phosphatidylcholine liposomes (PCL)	Astaxanthin was successfully incorporated into PCL	Reduction of lipid damage caused by addition of: Ferrous ions + H_2O_2 = 45% t-ButOOH: 45% Ascorbate=33% Reduction of H_2O_2-induced lipoperoxidation in Iron PCL by 26%.
	Peng *et al.* 2010 [123]	Astaxanthin from Sigma Chemical Co. St. Louis, MO, USA) Solvent: Chloroform/methanol (2:1 v/v)	Liposomes	Astaxanthin content 89 mg/g Improve stability after 25 h exposure in cell-free medium Homogeneous dispersion in water (size 251±23 nm) Disintegration after 8±1.8 min Better access to cell surfaces after 2 h Toxicity on Hep3B and HepG2 cell lines at concentration between 5-40 µg/mL and not toxicity on BNL CL2 line	Not performed
	Acevedo *et al.* 2014 [124]	Astaxanthin (from Sigma Chemical Co.St. Louis, MO, USA).	Microencapsulation in oil bodies carriers based on oleosomes isolated from plant seeds in a ratio (0.01–0.3).	Morphological stability (size average: 3.444±0.479 µm) Reduction of astaxanthin degradation after air and light exposure by twice. Decrease of endothelial cell viability (28% at 25–100 mg/mL of astaxanthin-microcapsules)	Intra-and extracellular ROS reduction up to 74.49% and 47.7% respectively at 1000 mg/mL
	Hama *et al.* 2012 [19,20] Kamezaki *et al.* 2016 [125]	Astaxanthin (from Sigma Aldrich Co.St. Louis, MO, USA).	Liposomes Egg phosphatidylcholine (EPC) as based lipid	Liposomes size between 151.4 to 33.6 nm Protection of mouse skin fibroblast NIH3T3 cells from hydroxyl radical induced cytotoxicity.	High hydroxyl radical scavenging at concentration <20 µM Reduction of singlet oxygen production up to 88% Inhibition of skin damage induced by UV irradiation after topical application (40 µM) Co-encapsulation of astaxanthin and tocotrienol induce a synergistic scavenging activity
	Bustamante *et al.* 2016 [126]	Astaxanthin (98% purity, from Sigma–Aldrich, Steinheim, Germany) Solvents: (1:1 v/v) diethyl ether:hexane	Microencapsulation with different fatty acid composition obtained by supercritical fluid extraction (SFE) Fatty acids: sunflower oil (SO) or high oleic sunflower oil (HOSO)	Lower degradation rate Droplet sizes of the SO+SFE and HOSO+SFE emulsions ranged from 0.31 to 0.56 µm and 0.29 to 0.76 µm, respectively	Not performed

	Odeberg *et al*. 2003 [127]	Commercial formulation of algal meal and dextrin in hard gelatin capsules (Napro Pharma, Brattvaag, Norway).	Incorporation in three lipid based formulations containing polysorbate 80 and: 1.Long-chain triglyceride (palm oil) 2. glycerol mono- and dioleate 3. glycerol mono- and dioleate, and sorbitan monooleate	Enhanced bioavailability, ranging from 1.7 to 3.7 times Highest bioavailability using formulation B after a one dose human trial. Astaxanthin doses up to 40 mg were well tolerated.	Not performed
	Ribeiro *et al*. 2005 [128]	Crystalline astaxanthin (80% purity, BASF, Ludwigshafen, Germany)	O/W Emulsions prepared by repeated premix membrane emulsification Dispersed in medium chain triglyceride oil (palm oil)	30% of Astaxanthin degradation during storage Droplets stability up to 3 weeks	Not performed
	Tachaprutin *et al*. 2009 [129]	Astaxanthin (97% (w/w) purity, Acros Organics, Geel, Belgium) Solubilized in DMF//water	Polymeric nanocarries using: poly(ethylene oxide)-4-methoxycinnamoylphthaloylchitosan (PCPLC),	Stable aqueous suspension Nanospheres diameter: 68.3±0.35 to 312±5.83 nm 40% (w/w) astaxanthin loading Improved thermal stability at 70°C	Not performed
	Anarjan et Tan 2013 [62,130,131]	Astaxanthin (>90%, from Kailu Ever Brilliance Biotechnology Co., Ltd. Beijing, China)	Nanodispersions using S1: Polysorbate 20 (PS20, 29% w/w), sodium caseinate (SC, 65% w/w) and gum Arabic (GA, 6% w/w); S2: Tween 20 with 62% w/w acetone and 38% w/w dichloromethane	Spherical-shaped with particle size of 88.9 nm for S2 and 114.6 nm using S1 Astaxanthin loss of 32.4% (w/w) S2 and 20% (w/w) at 25°C after 8 weeks of storage using S1 Higher HT-29 uptake of astaxanthin nanodispersions	Not performed
	Tamjidi *et al.* 2014 [132]	*H.Pluvialis* oleoresin (astaxanthin content 40%; from Wuhan Ereli Import & Export Co. Ldt Wuhan, China) Extracted in dichloromethane	Nanostructured lipid carriers (NCL) using tween80 and lecithin as emulsifiers and oleic acid and glycerol behenate as lipids	Good storage stability for 25 days High drug loading Particle size 85.1-138nm Decrease in astaxanthin content due to hot homogenization method but increase in long-term stability	Not performed
	Meor Mohd Affandi *et al.* 2011 [133]	Astareal 10FC grade (an oil extract containing 10% w/w of standardized astaxanthin, from Fuji Chemical Industry, Nakaniikawa, Toyama, Japan).	Nanoemulsion using Tween 80 and lecithin as emulsifiers (2.5% w/w)	Particles size at 5 cycles of homogenizing pressure 122.9±1.55 nm. Conservation of stability and storage at 25±2 °C/60%±5% relative humidity for 3 months.	Not performed
	Chun-Hung Chiu *et al.* 2016 [134] Second part of the study [123].	Astaxanthin (>99%, from the Fuji Chemical Industry Co., Ltd. Toyama Prefecture, Japan).	Liposomes	Particles size distribution of 240 ± 58 nm Astaxanthin instantaneous pharmacokinetics release from the nanoliposome particles. Efficient and stable transport allowing a higher intrahepatic uptake	Attenuation of nuclear levels of iNOS and NF-kB Hepatoprotective effects and completely alleviated the acute inflammatory status at a 10 mg/kg-day dosage.

***Table 3**: Some lipid based carriers evaluated to protect and enhance astaxanthin properties.*

Lipid based carrier systems presented in table 3 showed to enhance astaxanthin solubility properties. Five of twelve studies evaluated the *in vitro* interaction of the system after cellular

supplementation without reporting toxicity problems. The *in vitro* antioxidant capacity of the system was assessed in four studies demonstrating a reduction of ROS levels and the attenuation of cellular inflammatory markers. Moreover, one study revealed the improvement of astaxanthin bioavailability incorporated into a lipid based formulation after one dose human trial [127].

3.3.3 Inclusion complex using cyclodextrin

Cyclodextrins have been used extensively as additives to increase the solubility of poorly water-soluble organic compounds [106]. Cyclodextrins are natural macrocyclic oligosaccharides well known for having toroid-shaped structures with rigid lipophilic cavities and a hydrophilic outer surface. These systems are able to enclose highly hydrophobic molecules inside their hydrophobic cavity, constituting a true molecular encapsulation [135]. The resulting non-covalent inclusions or host–guest complexes are of current scientific and technological interest for their peculiar physical, chemical and biological properties. Such non-covalent associations can actually improve the guests water solubility, bioavailability and stability [136], while regulating the release of the guest molecules [137,138]. Cyclodextrin systems highly increased astaxanthin solubility in water and stability against heat light and oxygen. Two of the six systems presented in table 4 evaluate the chemical antioxidant activity of the inclusion system by DPPH, reduction power assay and hydroxyl radical scavenging. Moreover, one study confirmed the system bio-accessibility on HepG2 cell line.

	Reference	Astaxanthin source	System	Results	Antioxidant evaluation
Cyclodextrin inclusion complex	Dong *et al.* 2014 [139]	Astaxanthin (purity>98%, from Dr. Ehrenstorfer Co. Ltd. Germany). Chloroform and Acetone (1:1 v/v) as solvents	Inclusion with Hydroxypropyl-β-cyclodextrin	Inclusion rate 30.4% Starting decomposition temperature at 50°C Good water solubility at 50mg/m of astaxanthin concentration even after 12 days. Stable storage under dark and light conditions at 4, 25, 37, and 50°C within 260 h	Complex showed a DPPH radical activity lower than astaxanthin at same concentrations High Fe^{3+} to Fe^{2+} reduced power capabilities than pure astaxanthin under the same concentration
	Lockwood *et al.* 2003 [140]	Astaxanthin Sigma lot 71K1540	Inclusion in captisol (Sulfobutyl Ether b-Cyclodextrin)	Increased the apparent water solubility approximately 71-fold, to a concentration of 2 mg/mL	Not performed
	Kim *et al.* 2010 [141]	Astaxanthin (purchased from Sigma Chemical Co.St. Louis, MO, USA). Solvent: Dichloromethane and Acetone	Inclusion with β-cyclodextrin	Enhance water solubility up to 110-fold at pH 6.5 and 25°C. Improved stability against heat, light, and oxidation by over 7-9 folds Thermal stability even at 100°C.	Not performed

Chen *et al.* 2007 [142]	Astaxanthin prepared from small shrimps Solvent: Dichloromethane/ acetone 1:1 v/v	Inclusion with β-cyclodextrin	Improve water solubility <0.5 mg/ml thermal (57°C) and light stability after 6 days Inclusion rate of 48.96%	Not performed
Nalawade et Gajjar 2015 [143]	Astaxanthin (from Shangyu NHU Bio-Chem, China) Solubilized in Hydro-alcoholic solution (2:1 v/v)	Inclusion with methylated-β-cyclodextrin using spray drying technique	Solubility enhanced 54 times over astaxanthin alone. A dissolution rate of 85% over 45 min Increased bio-accessibility on HepG2 cell line.	Not performed

***Table 4**: Cyclodextrin inclusion complexes evaluated to protect and enhance astaxanthin properties*

Almost all reviewed studies were focused on chemical formulations and stabilization parameters. Polymeric, lipid and cyclodextrin systems showed an improvement on astaxanthin solubility and stability properties. However, very few evaluations of astaxanthin antioxidant capacities after encapsulation were found in published researches.

Some studied using lipid based formulations assessed the *in vitro* potential of the system. Furthermore, cyclodextrin inclusion groups, represent a non-negligible option by rendering astaxanthin water soluble which its advantageous to approach different *in vivo* delivery pathways. Independently of the results reported by the different groups, all of them require a deeper chemical and biological characterization to confirm their potential to be used as astaxanthin carrier systems and to further considerer them for a posterior evaluation in a clinical application for the prevention and treatment of cardiovascular diseases.

4. Conclusion

Despite of ROS influence to destabilize membrane and cell homeostasis, regular production of these radicals is essential in the maintaining of redox signaling. Thus, antioxidants systems oversee ROS regulation without completely eliminate them. All antioxidants have different ways action; their biological activity may also be conditioned by the cellular structure where they act. Astaxanthin has shown potent antioxidant action to stabilize ROS influx during oxidative stress related diseases such as I/R, as presented here. Indeed, astaxanthin shows a strong ability to reduce lipid oxidation thanks to its polar end groups which extended toward the polar regions of the membrane bilayer, thus contributing to the inhibition of thrombus and atherosclerotic plaque formation. However, a drawback of astaxanthin action has also been attributed to its structure, which renders it prone to oxidation, and low its bioavailability. Protective encapsulation systems have been currently studied to solve these drawbacks. Additionally, new delivery systems may also contribute to limit potential untoward effects of *in vivo* antioxidant therapy that have been limited by antioxidant appropriate doses. Finally, studies reviewed here, show the interesting

properties and potential medical use of astaxanthin to treat oxidative stress related pathologies, particularly cardiovascular diseases such as I/R injury.

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2B Chapter

Methods to determine antioxidant capacities

Chapter 3 presents a brief description of the methods proposed in the literature for the determination of scavenging capacities of antioxidants. Particularly, this chapter focus on the biochemical and biological methods used to validate the antioxidant scavenging capacities of astaxanthin after inclusion within Hydroxyprolyl-β-cyclodextrin (CD-A) on human endothelial cells (HUVEC) under oxidative stress.

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Résumé

L'utilisation d'antioxydants dans le domaine médical et nutritionnel a connu un développement important en raison de leur forte capacité à piéger les radicaux libres. Ces derniers, lorsqu'ils sont en excès, entrainent des perturbations de l'homostase ou des équilibres redox cellulaires, favorisant le développement de processus pathologiques. La mise en évidence de l'action protectrice des antioxydants et l'identification de leurs mécanismes d'action sont nécessaires pour permettre des avancées significatives dans la recherche. Cela ne peut être obtenu sans l'utilisation d'une batterie de méthodes chimiques et biologiques très spécifiques. Les techniques existantes diffèrent entre elles par leur mécanisme de réaction, les cibles et les sondes oxydantes utilisées et leurs conditions réactionnelles. Il en résulte ainsi le besoin de les appliquer simultanément et de manière complémentaire pour évaluer la capacité antioxydante réelle d'un échantillon de principes actif sélectionné. Cela permettra également d'obtenir un véritable aperçu de l'état oxydatif général du système analysé. Dans cette partie, une brève description des méthodes d'analyse des capacités antioxydantes proposées dans la littérature sera présentée en mettant l'accent sur les méthodes utilisées dans cette thèse.

Mots Clés: Espèces réactives oxygénées; Méthodes biochimiques; Méthodes biologiques; Capacités antioxydantes; Antioxydants.

Abstract

The use of antioxidants in the medical and nutritional field has seen a substantial growth due to their scavenging abilities against the potentially harmful effect of processes or reactions that alters the cellular redox homeostasis, leading to diseases. Efficient probes able to determine the effective protection as well as the mechanism of action of antioxidants will provide a key for search appropriate tools to assure the stability of the systems. Available, chemical and biological methods differ from each other in terms of reaction mechanisms, oxidant and target/probe species and reaction conditions; hence the need to applied them simultaneously to evaluate the real antioxidant capacity of the selected sample and to obtain a real valuable overview of the general oxidative status of the analyzed system. Here, we present a brief overview of the methods described in the literature for the determination of scavenging capacities of antioxidants, focusing on the methods used in this study.

Key words: Reactive oxygen species; Biochemical assays; Biological assays; Scavenging activity; Antioxidants

1. Introduction

Antioxidants are synthetic or natural substances capable to either delay or inhibit the oxidation processes which occur under the influence of atmospheric oxygen or reactive oxygen species. Antioxidants are currently used in food supplements as bioactive compounds, and for the stabilization of products such as polymeric, cosmetic and pharmaceuticals. Antioxidant can scavenge reaction oxygen/nitrogen species (ROS/RNS) which play an important role in both physiological and pathological processes, by stopping radical chain reactions, or by acting as preventive substances through the inhibition of reactive oxidants formation. Biological antioxidants include enzymatic antioxidants (e.g., superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic antioxidants such as oxidative enzyme inhibitors, antioxidant enzyme cofactors (Coenzyme Q10), ROS/RNS scavengers (Vitamin E and carotenoids), and transition metal chelators [1-4].

Efficient probes able to quantify antioxidant scavenging capabilities as well as ROS levels provide a key to understand antioxidants action mechanisms and to regulate the redox balance in the body (Figure 1). However, reactive species present some characteristics that make difficult their detection, such as very short lifetime and the endogenous antioxidant mechanism which regulate their levels *in vivo* [5]. Furthermore, the response of antioxidants to different radicals or oxidant sources vary widely [6]. The ideal chemical or biological probe would be highly reactive at low concentrations, specific, nontoxic, easy to use and to load into organelles, cells, or tissues without subsequent leakage, readily available and inexpensive [7,8].

Up to know, there is not a unique probe filling all these criteria, therefore the simultaneous use of chemical and biological methods is advised to obtain a better screening of the tested molecule properties. Chemical methods "*in tube"* provide a first approach to validate the antioxidant capacities of a specific component. On the other hand, biological methods allow measuring the antioxidant ability of a component to regulate the redox environment of an organelle and provide information about oxidative markers, intracellular antioxidant capacities and endogenous antioxidant pathways. Probes generating either a fluorescent product or inducing chemiluminescence offer high sensitivity, and can be applied using a variety of analytical techniques, from "plate readers" with fluorescence detection, through high performance liquid chromatography [5].

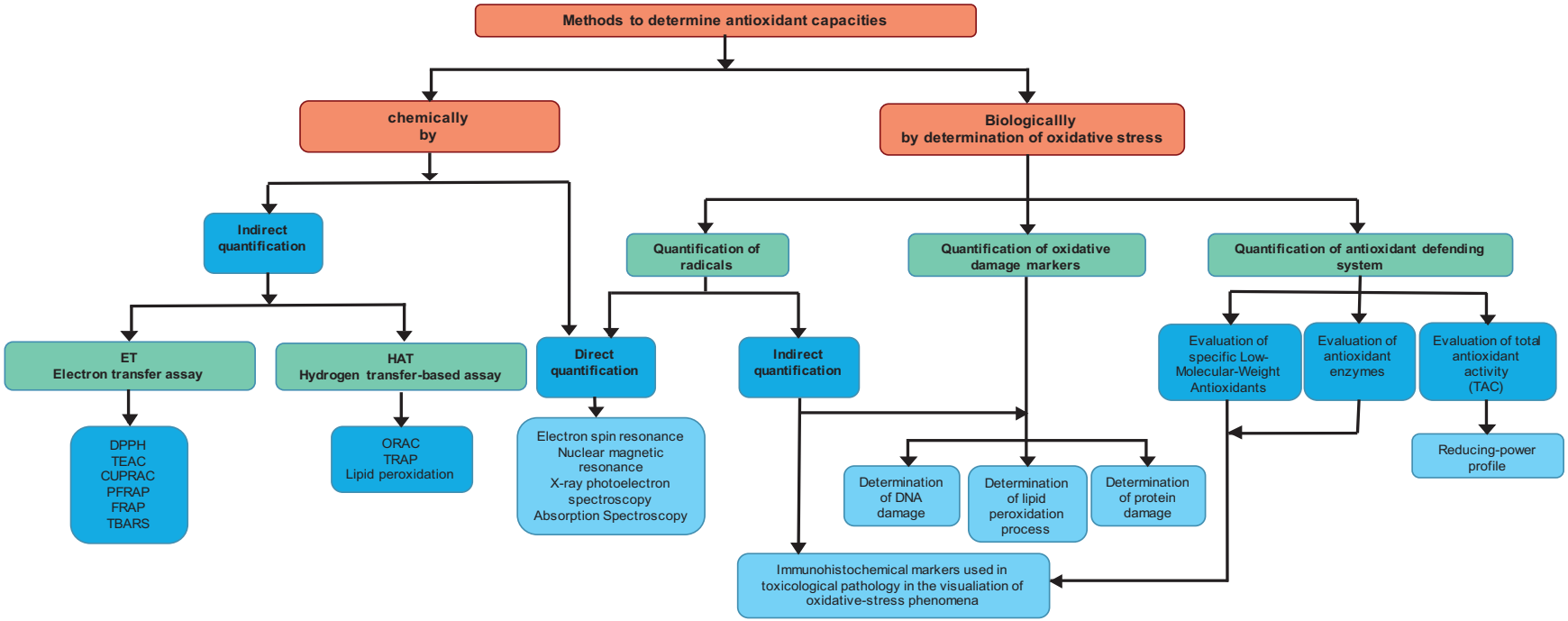


Figure 1: *Methods for the determination of antioxidant capacities, presented as biological thought the different methodologies for the determination of oxidative stress and Biochemical. Adapted from [4].*

2. Antioxidants generalities

Antioxidants can be divided as primary or secondary antioxidants. Additionally, in regards on the chemical reactions they involve, antioxidant assays can be classified as electron transfer (ET) or hydrogen transfer-based (HAT) assays (Table 1) [1,9]. As follows:

- Primary are considered as chain-breaking antioxidants or free radical scavengers, where the radical initiation (by reacting with a lipid radical, Eq.1) or propagation (by reacting with alkoxyl ($LO^{\cdot}$) or peroxyl ($LOO^{\cdot}$) radicals) steps are inhibited (Eq.2) [1,10].

$$L^{\cdot} + AH \rightarrow LH + A^{\cdot} \quad \text{(Eq.1)}$$

$$LO^{\cdot} / LOO^{\cdot} + AH \rightarrow LOH/LOOH + A^{\cdot} \quad \text{(Eq.2)}$$

- Secondary are described as preventative antioxidants which retard the rate of oxidation of new chains reaction, like in the case of Fenton reaction (Eq. 3). Endogenous Antioxidants are member of this group [1,10].

$$Fe^{2+}/Cu^{+} + H_2O_2 \rightarrow Fe^{3+}/Cu^{2+} + {}^{\bullet}OH + OH^{-} \quad \text{(Eq.3)}$$

- ET–based assays, measure the capacity of an antioxidant in the reduction of an oxidant probe, which changes of color when reduced (Eq. 4). The reduced dyes are often colorless and non-fluorescent, while the oxidized dyes are highly colored and fluorescent [7]. The degree of color change is correlated to the concentration of antioxidants in the sample [9].

$$X^{\cdot} + AH \rightarrow X^{-} + AH^{\cdot +} \quad \text{(Eq.4)}$$

- HAT-based assays: measure the capability of an antioxidant to quench free radicals by H-atom donation (Eq. 5) [1]. The antioxidant activity of the analyzed molecule can be determined from competition kinetics by measuring the fluoresce decay of the probe in the absence and presence of antioxidants, integrating the area under these curves, and finding the difference between them [11].

$$X^{\cdot} + AH \rightarrow XH + A^{\cdot} \quad \text{(Eq.5)}$$

3. Antioxidant detection methods

Methods for antioxidant capacities detection can be classified as direct and indirect. Direct methods are based on the chemical-physical properties of antioxidant compounds, without considering reactive species action (Eq.6) [12]. Direct determination use electron paramagnetic resonance methods for the measurement, which requires the use of very low temperatures for detection, which limits it use [13].

$$\text{Antioxidant} - e^{-} \rightarrow \text{Oxidant} \quad \text{(Eq. 6)}$$

$$\text{Oxidant} + e^{-}\text{(from antioxidant)} \leftrightarrow \text{Reduced oxidant} + \text{oxidized antioxidant} \quad \text{(Eq. 7)}$$

Indirect methods involve the reaction of a particular ROS with a probe molecule to yield a more stable, long-lived analyte (Eq. 7) [14]. Available indirect probes provide valuable information on changes on the redox environment of the cell, but many of these methods are not specific, do not allow subcellular localization, and their response is affected by different chemical interactions [8]. Despite that, these methods represent a valuable tool to obtain an overview of the antioxidant ability of several molecules such as antioxidants.

3.1. Chemical Vs. Biological antioxidant detection methods

Chemical "in tube" assays while being simple and reproducible, lack in selectivity and may require a catalyst for reaction [7]; despite these drawbacks these methods still provides useful information about antioxidants scavenging activities [15]. In contrast, biological methods take into account the bioavailability and metabolism of the tested compound providing information of ROS downstream effect in living cells [16]. Table 1 presents some methods currently used to chemically determine the scavenging capacities of antioxidant. The results obtained using either ET or HAT assays are hardly comparable due to the different used mechanisms, redox potentials, pH and kind of used solvent, etc. [9]. Indeed, some methods are considered to be a mix of both classifications like the chemical methods TEAC/ABTS and DPPH [9].

Method	Radical or oxidant	Wavelength	Measurement principle	HAT/ET based
ORAC (oxygen radical absorption capacity)	$AAPH^{\cdot}$ (Fluorescein)	λex=485 nm λem=528 nm	Fluorescence decay by termal degradation	HAT
TRAP (Radical-trapping antioxidant parameter)	$AAPH^{\cdot}$ (R-phycoerethrin/Luminol)	λex=495 nm λem=575 nm	Chemiluminescence quenching	HAT
Lipid Peroxidation Inhibition Assay	N-methyl-2-phenylindole	586 nm	Colorimetry/Absorbance	HAT
FRAP (Ferric ion reducing antioxidant power assay)	Chelated Fe^{3+} ions	595 nm	Colorimetry/Absorbance	ET
DPPH (2,2-diphenyl-1-Picrylhydrazyl)	$DPPH^{\cdot}$	517 nm	Colorimetry/Absorbance	ET/HAT
TEAC/ABTS (Trolox equivalent antioxidant capacity)	$ABTS^{\cdot +}$	734 nm	Colorimetry/Absorbance	ET/HAT
CUPRAC (cupric reducing antioxidant power)	$Cu^{2+} \rightarrow Cu^{+}$	450 nm	Colorimetry/Absorbance	ET
PFRAP (Potassium Ferricyanide Reducing Power)	$Fe^{3+} \rightarrow Fe^{2+}$	700 nm	Colorimetry/Absorbance	ET
TBARS (Thiobarbituric acid reactive substances)	MDA-TBA Adduct	532 nm	Colorimetry/Absorbance	ET

***Table 1**: Different biochemical methods to quantified antioxidants scavenging abilities. Adapted from [1].*

Table 2 shows some of the different biological detection probes used for ROS detection. Including some probes that are frequently used to detect a specific radical (Table 2), due to the affinity of the interaction [5,8,14,17]. Recently, new approaches to detect ROS focused on using specific components to directly target organelles involve in ROS production. For instance,

mitoSOX a red fluoresce probe is able to directly target complex III within the mitochondria to sense $O_2^{\bullet-}$ production, to form 2-hydroxy-mito-ethidium, while DHE, another probe used for $O_2^{\bullet-}$sensing recognize cytoplasmic but not mitochondrial $O_2^{\bullet-}$[18]. Also, an indirect measure of mitochondrial ROS production in living cells assess the extent of lipid peroxidation using C11-bodipy probe [19]. Another approach consists in the use of hydroxylamine spin probes to directly detect ROS intracellular, extracellular, or both, such us cyclic hydroxylamines which detect intracellular $O_2^{\bullet-}$ production [20], while the positively charged hydroxylamine or CAT1H detects only extracellular $O_2^{\bullet-}$ [18]. Additionally, probes inducing the production of cells chimeric proteins capable to detect ROS or changes in the redox status, have been currently proposed such us H_2O_2 redox-sensitive green fluorescent protein sensor (roGFP) [21], cpYFP genetically encoded bioindicator [22], or Amplex red, which used SOD to convert $O_2^{\bullet-}$ into H_2O_2 [23].

ROS Detected (Radicals and non-radicals)		**Detection Methods** (Chemiluminescent, Fluorescent and Spin probes)
Superoxide radical	$O_2^{\bullet-}$	Hydroethidine (HE) MitoSOX 1,3-Diphenylisobenzofuran (DPBF) Ferricytochrome c reduction 1- hydroxy-2,2,6,6-tetramethylpiperidin-4-yl-trimethylammonium (CAT1H) Lucigenin Luminol Coelenterazine or MCLA
Hydroxyl radical	$HO^{\bullet}$	4-(9-Anthroyloxy)-2,2,6,6-tetramethylpiperidine-1-oxyl 1,3-Cyclohexanedione (CHD) Sodium terephthalate Coumarin, coumarin-3-carboxylic acid (3-CCA) N-succinimidyl ester of coumarin-3-carboxylic acid (SECCA) 2-[6-(4V-Hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (HPF)
Peroxyl **Alkoxyl** **Lipid peroxyl radical**	$RO_2^{\bullet}$ $RO^{\bullet}$ $LOO^{\bullet}$	C11-BODIPY581/591 Lipophilic fluorescein derivatives Dipyridamole. Diphenyl-1-pyrenylphosphine (DPPP) β-Phycoerythrin/Fluorescein/6-Carboxyfluorescein
Singlet oxygen	$^1O_2$	9,10-Dimethylanthracene (DMA) 9-[2-(3-Carboxy-9,10-diphenyl)anthryl]-6-hydroxy-3H-xanthen-3-ones (DPAXs) 9-[2-(3-Carboxy-9,10-dimethyl)anthryl]-6-hydroxy-3H-xanthen-3-one (DMAX)
Hydrogen peroxide	H_2O_2	5-(and 6)-chloromethyl-20,70-dichlorohydrofluorescein diacetate (CM-H2DCFDA) Scopoletin (7-hydroxy-6-methoxy-coumarin N-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red) Homovanillic acid (4-hydroxy-3-methoxy-phenylacetic acid; HVA) Dihydrorhodamine 123 (DHR)
Peroxinitrite	ONO_2^-	Dihydrorhodamine (DHR) Boronate-containing Fluorophores

***Table 2**: Some of the different probes used for ROS detection. For a deep explanation of the action mechanism of each method and more available probes, please refer to [5,8,14,17].*

This thesis focused on the use of chemical antioxidant methods specifically TEAC, ORAC and DPPH *in tube* assays to evaluate the effect of ET and HAT based antioxidants (highlighted on Table 1). For *in vitro* analysis, DCFH-DA, MitoSOX, and C11-Bodipy, *in vitro* biological methods were used to provide information about the cellular redox environment, mitochondrial ROS, and lipid peroxidation, respectively (highlighted on Table 2). Below, these methods are explained in more detail.

3.2. Chemical methods

3.2.1. Trolox equivalent antioxidant capacity (TEAC) assay

TEAC assay is based on the ABTS radical cation ABTS•+ obtained by the oxidation of ABTS (7 mM) with potassium persulfate ($K_2S_2O_8$, 2.45 mM), by electron donation (Figure 3) [24]. ABTS and $K_2S_8O_2$ react stoichiometrically, at the ratio of 2:1. Oxidation of ABTS starts immediately, but it takes some time for absorbance to reach its maximum plateau (0.70±0.02 at 734 nm), however, incomplete oxidation of ABTS may occur [25].

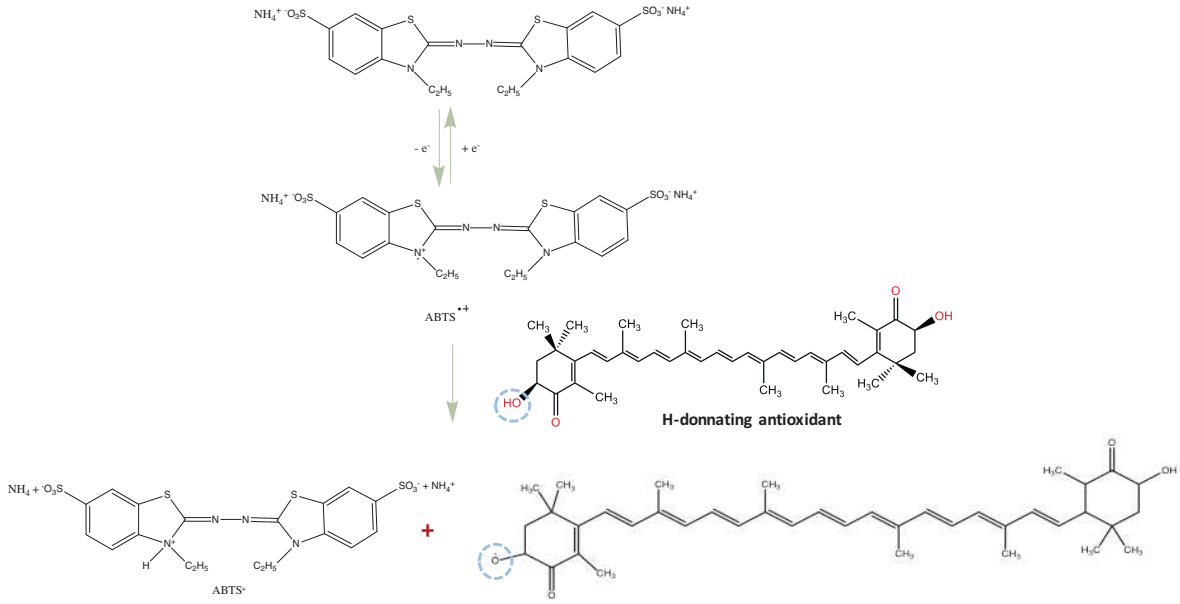


***Figure 2**. ABTS radical assays using astaxanthin as H-donating antioxidant [26]*

Different antioxidants are used to inhibit the oxidation of ABTS to ABTS•+ by electron transfer radical scavenging. The antioxidant scavenging activity of the tested components is calculated as the component capacity to decrease the ABTS maximal absorption and it is normalized to equivalent Trolox units. TEAC values are defined as the millimolar concentration of Trolox with the same antioxidant activity as 1 mM concentration of the samples (Eq.8) [26].

$$Scavenging\ activity\ (\%) = \frac{[ABTS^{\cdot+}]_{initial} - [ABTS^{\cdot+}]_{final}}{[ABTS^{\cdot+}]_{initial}} * 100 \quad \text{(Eq. 8)}$$

3.2.2. DPPH assay

The DPPH assay measures compounds that are radical scavengers. Figure 5 shows the mechanism by which DPPH• accepts one H from an antioxidant. Antioxidant effect is evaluated by following the decrease of UV absorption of DPPH• at 517 nm (purple). The color turns from purple to yellow followed by the formation of DPPH upon hydrogen absorption from the antioxidant. This reaction is stoichiometric with respect to the number of hydrogen atoms absorbed [32]. The radical scavenging activity is calculated using Eq.9; where A_c is DPPH solution absorption, A_i is the DPPH solution absorption after sample addition and A_J is the initial absorption of the experimental samples[33].

$$Scavenging\ rate\ (\%) = \frac{A_c - (A_i - A_j)}{A_c} * 100 \quad \text{(Eq.9)}$$

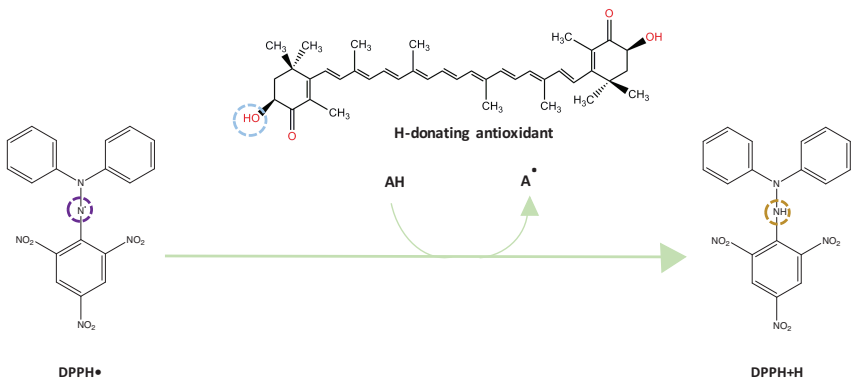


***Figure 3**. DPPH• free radical conversion to DPPH by anti-oxidant compound.*

3.2.3. The oxygen radical antioxidant capacity (ORAC) assay

The ORAC assay measures the fluoresce decay of fluorescein after exposition to a radical scavenger like 2,2'-Azobis(2-amidinopropane) dihydrochloride AAPH, that induces the formation of a peroxyl radical (ROO•) [27]. Antioxidants suppress this reaction by a H atom transfer mechanism, inhibiting the oxidative degradation of the fluorescein signal (Figure 4). The fluorescence signal is measured over 30 minutes by excitation at 485 nm, and emission at 538 nm. The concentration of antioxidant in the test sample is proportional to the fluorescence intensity through the course of the assay and is assessed by comparing the net area under the curve to that of a reference antioxidant such as Trolox [28,29]. This is the only method that

combines both the percentage of inhibition and the length of inhibition of free radical formation by antioxidants into a single quantity [30].

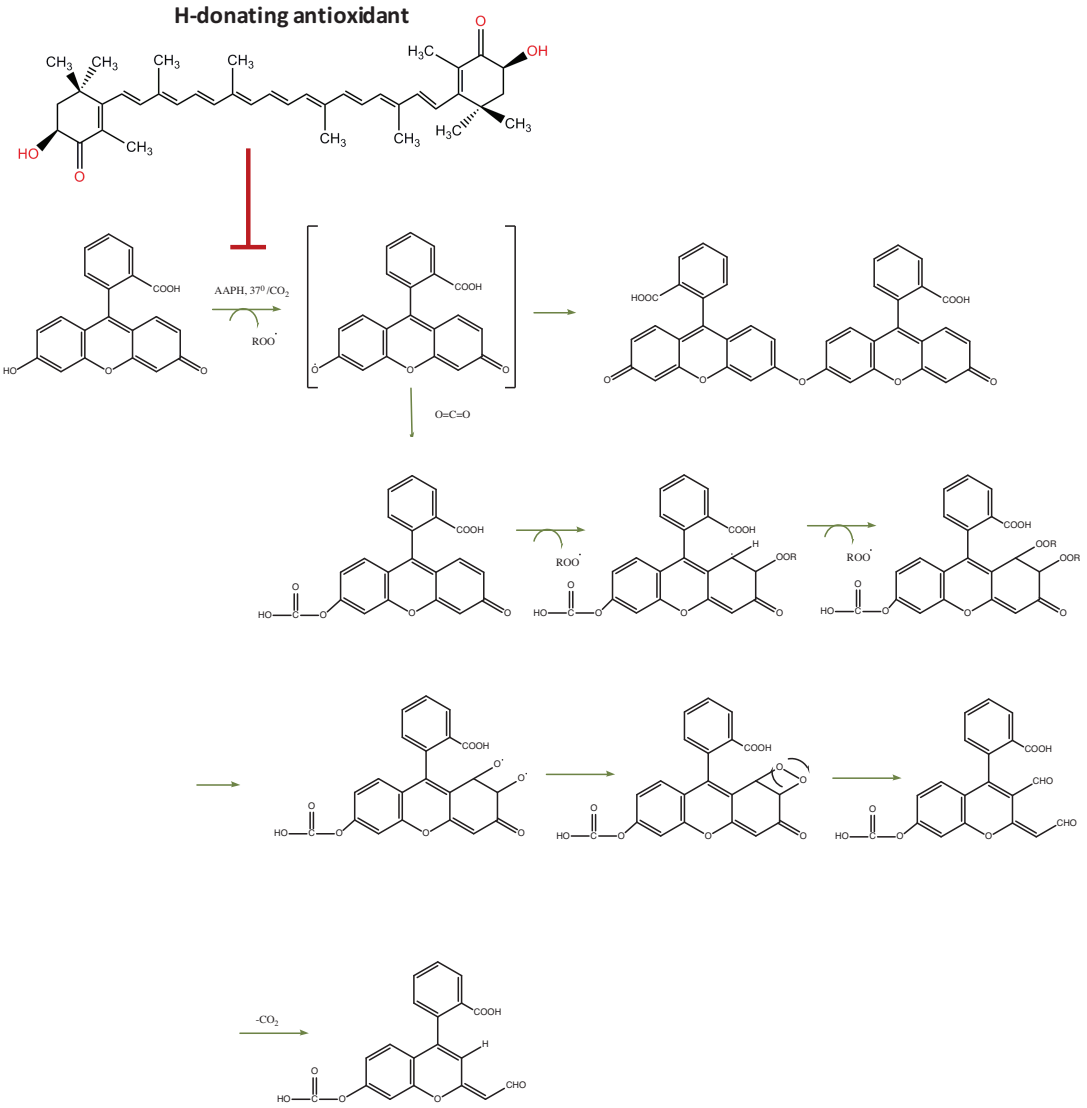


Figure 4. *The proposed reaction mechanisms for fluorescein reduction in the presence of AAPH measured by the ORAC assay. The reaction is initiated by the abstraction of a hydrogen atom from the phenol group being abstracted by a peroxyl radical, forming a stable fluorescein phenoxyl radical (FLO•) that readily undergoes dimerization. Alternatively, FLO• can attack trace amounts of CO_2 in the buffer solution. Besides hydrogen abstraction, ROO• can also add the reactive conjugated C-C double bond to form a stable delocalized radical that further reacts with ROO• to form the endoperoxide intermediate. Finally, a major oxidative product derived due to the further oxidation yield a last fluorescein product that not possess fluorescent emission [11,31].*

3.3. Biological methods

Several methods used to assess biological relevance and cellular availability of antioxidant to block oxidative stress will be presented. The CM-H_2DCFDA assay provided information concerning general disturbance in the redox state of cells [8]; MitoSOX Red allowed the measurement of the superoxide production in the mitochondrial matrix [34], and C11-BODIPY$_{581/591}$ measures free radical species formed from hydroxyl, alkoxyl, and peroxyl radicals in the lipid membrane [19]. Although assays using redox dyes are rapid and simple, they are generally not specific to a particular oxidant and are affected by numerous chemical interactions. There is therefore a need to carry out more than one assay, before attributing an effect to a particular oxidant [8]. Oxidative stress can be chemically induced by stressors such us AAPH or t-BuOOH, that induced either peroxyl (ROO•), or alkoxyl (RO•) radical, or by antimycin A and cumene peroxide (CumOOH) which are frequently used to induce superoxide radicals and lipid peroxyl radicals, respectively (Figure 6).

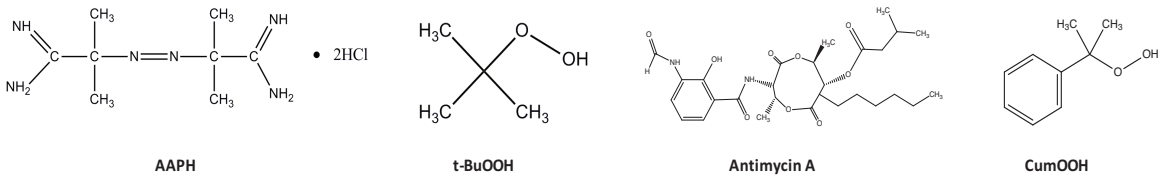


***Figure 5**. Chemical ROS stressors: 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), Tert-Butyl hydroperoxide (t-BuOOH), Antimycin A, and Cumene hydroperoxide (CumOOH).*

3.3.1. *CM-H2DCFDA probe*

Cell permeable probe CM-H_2DCFDA (*5-(and 6)-chloromethyl-20,70-dichlorohydrofluorescein diacetate)* is one of the more used probes to asses' reactive oxygen species. Particularly, it is currently used to identify H_2O_2 production in cell, however, one of the inconvenient of using this method, is that H_2O_2, itself does not react with CM-H_2DCFDA. It requires a peroxidase or other low molecular weight or protein-bound transition metal catalyst. A negative response could therefore be misinterpreted if no catalyst were present. Conversely, this method is very useful to detect a disturbance in the redox state of a cell [8].

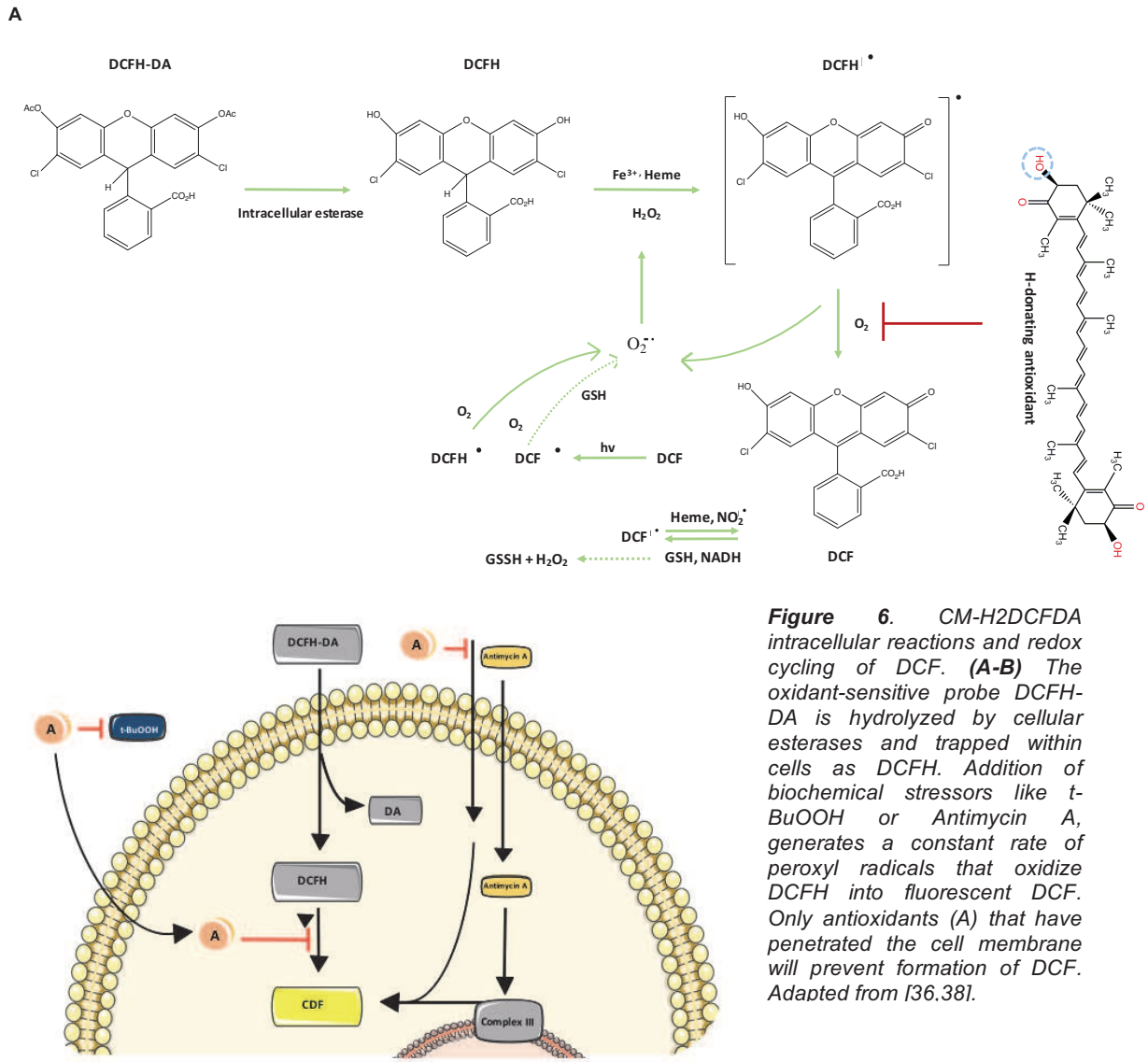


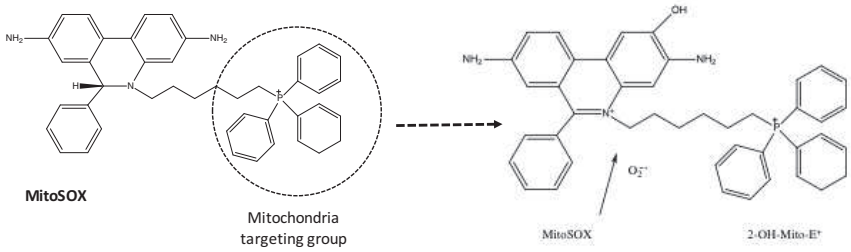
***Figure 6**. CM-H2DCFDA intracellular reactions and redox cycling of DCF. **(A-B)** The oxidant-sensitive probe DCFH-DA is hydrolyzed by cellular esterases and trapped within cells as DCFH. Addition of biochemical stressors like t-BuOOH or Antimycin A, generates a constant rate of peroxyl radicals that oxidize DCFH into fluorescent DCF. Only antioxidants (A) that have penetrated the cell membrane will prevent formation of DCF. Adapted from [36.38].*

This probe emits no fluorescence in its original state. After crossing the cell membrane, DCFH-DA is hydrolyzed by intracellular esterases into DCFH, which becomes fluorescent once oxidized to DCF in presence of ROS [7] (Figure 7). The accumulation of DCF inside the cells is measured by the fluorescence increase at 530 nm using an excitation wavelength of 485 nm in a kinetic mode [35]. Antioxidant capacities to avoid the probe oxidation and thus to reduce the oxidative cell environment is evaluated as expressed in Eq.6: Where I_{c_n} and I_{s_n} represent the intensity of stressed cells without and with antioxidant treatment at time n, respectively.

$$CAA\ (\%) = \frac{I_{c_n} - I_{s_n}}{I_{c_n}} * 100 \qquad \text{(Eq.6)}$$

3.3.2. Mitochondrial ROS detection

MitoSOX Red fluorescent probe allows the measurement of superoxide production in the mitochondrial matrix. MitoSOX Red is rapidly target to the mitochondria due to its positive charge, once target, it is oxidized by superoxide to form 2-hydroxymitoethidium (*2-OH-Mito-E*$^+$), which excites and emits at 510 and 595 nm, respectively [18] (Figure 8). Moreover, studies have shown that blockade of mitochondrial complex 3 with antimycin A, increases the conversion of mitoSOX to 2-OH-Mito-E$^+$ [36].



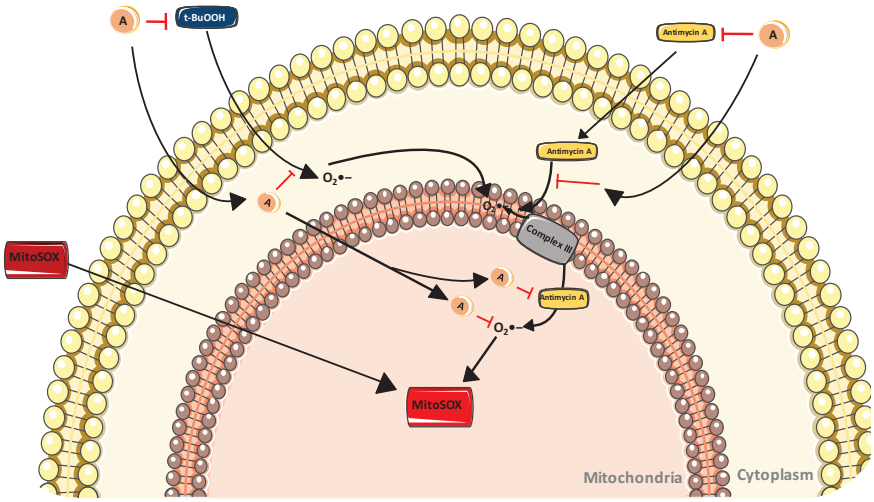
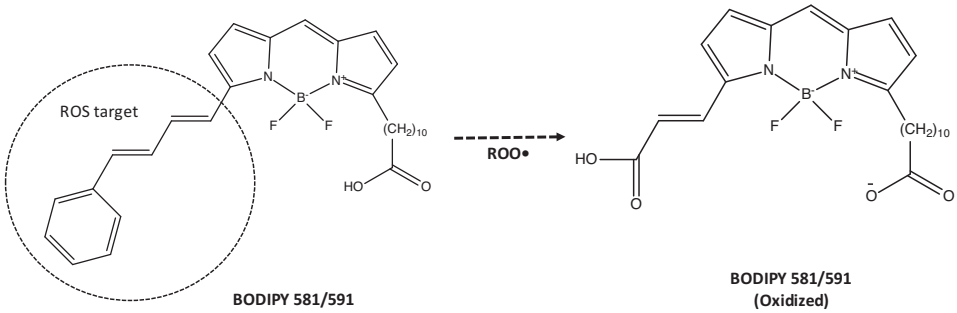


***Figure 7**. Mitochondrion-targeted accumulation of MitoSOX and detection of mitochondrial $O_2^{\bullet-}$. Antimycin A and t-BuOOH are two mitochondrial and cytoplasmic biochemical ROS stressors. MitoSOX reacts with $O_2^{\bullet-}$ to form 2-OH-Mito-E$^+$ that can be detected either in the mitochondria or in the cytoplasm. Adapted from [36].*

Antioxidants effects in decreasing mitochondrial ROS generation are compared to the percentage of control (not stressor or antioxidant treatment). Interestingly, it has been shown that the superoxide-specific product of MitoSOX Red oxidation has a specific excitation peak at ≈400 nm [34]. Moreover, probe concentrations need to be controlled in order to avoid a potential modification of mitochondrial morphology, and the redistribution of fluorescence to the nuclei [37]. Dikalov *et al.* [36] suggest to use a working concentration not greater than 2 μM or less to avoid complications.

3.3.3. Cellular lipid peroxidation

C11-BODIPY$_{581/591}$ is a fluorescent fatty acid analogue which allows the indirect quantification of intracellular membrane lipid peroxidation and antioxidant efficacy [19]. The capacities of C11-BODIPY$_{581/591}$ in the detection of lipid peroxidation have been confirmed, but its capacities to discriminate between different ROS and RNS *in vivo* has shown to be limited [16]. Lipid peroxidation is initiated by addition of stressor like cumene hydroperoxide (CumOOH) [38] (Figure 8). BODIPY C11 is a specific sensor for intracellular lipid peroxidation, which undergoes a shift in fluorescence emission from red to green upon oxidization [19] (Figure 9).



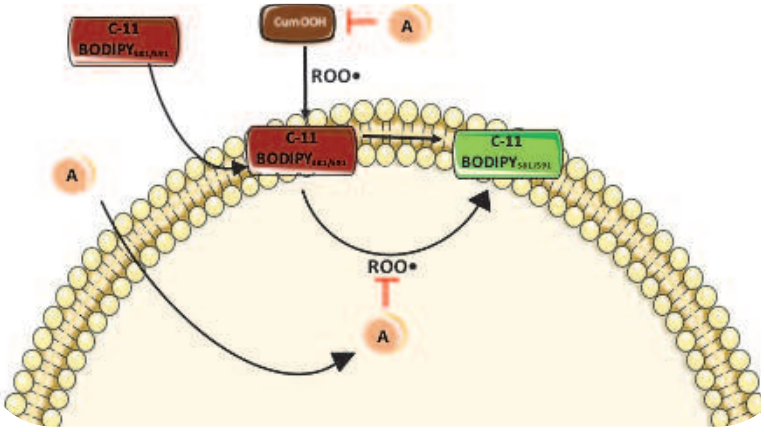


***Figure 8**. Detection of lipid peroxidation by C11-BODIPY. C11-BODIPY attach to cellular membrane, and it is gradually quenched in the presence of cumene hydroperoxide, an initiator of lipid peroxidation. This fluorescence quenching became slower in presence of antioxidants such as astaxanthin or Trolox which are well known ROO$^{\bullet}$ quencher [37].*

To evaluate lipid peroxidation, fluoresce is monitored at red λex/λem = 590/7, 632/45 nm and green λex/λem = 485/14, 520/10 nm. Drummen *et al.* showed that diene interconnection is the target for ROS, which leads to the shift in fluorescence and the formation of multiple oxidation products of the C11-BODIPY$_{581/591}$ molecule [39].

The capacity of an antioxidant to inhibit cellular lipid peroxide activity (CLPAA) is calculated as follows (Eq. 10):

$$CLPAA\ (\%) = \frac{Fluorescence\ of\ sample\ treated\ whit\ CumOOH\ and\ antioxidant}{Fluorescence\ of\ sample\ treated\ with\ CumOOH} * 100 \qquad \text{(Eq.10)}$$

4. Conclusion

Chemical and biological methods represent a valuable tool to the understanding of antioxidant action and protective mechanism. Particularly, biological methods provide information about the redox environment of the cell, allowing the evaluation of adequate antioxidant substances able to ensure the maintaining of a balanced state. However, it is important to recall that before selecting an appropiate antioxidant assay, it is necessary to understand the basic chemistry in the assays as the biochemistry of the antioxidants to be used [9]. It is also advisable to perform different test analyzing different antioxidants abilities to target divers radicals, because one assay does not accurately reflect the mechanism of action of all radical sources or all antioxidants in a complex system [6]. Additionally, the lack in the reproducibility of results due to the great variability on the number of protocols and solvents used to perform the same assay, reflect the need to standardize these assays. It is also important to highlight the variability and instability of these probes due to external factors like light, temperature, or pH, which can degrade the probe during the time of analysis. Concerning biological methods, to have a full picture of antioxidant activity in the cellular redox environment, it seems appropriate to measure antioxidant blocking of ROS produced by different organelles. Indeed, ROS expression is affected by numerous chemical interactions and sensitivity of probes; low ROS levels require more sensitive probes than high levels.

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3 Chapter

Research article: Oxidative stress regulation on endothelial cells by hydrophilic astaxanthin complex: Chemical, biological and molecular antioxidant activity evaluation

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Based on the literature review and the biomaterials experiences of our team, an encapsulation system based on astaxanthin inclusion within hydroxypropyl-β-cyclodextrin (CD-A) was synthetized. The objective of this study was in a first place to produce the system and to evaluate it antioxidant capacities by chemical methods. Secondly, to corroborated the capacity of CD-A complex to deal with cellular and mitochondrial ROS imbalance on human endothelial cells (HUVEC) under chemically induced oxidative stress using different biological and molecular assays. Finally, an *in vivo* biocompatibility study was carried out. These results support the idea of using CD-A complex to treat oxidative stress related diseases.

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Résumé

Nous avons synthétisé et caractérisé des complexes d'hydroxypropyl-bêta-cyclodextrine-astaxanthine (CD-A). Ces complexes ont permis de stabiliser l'astaxanthine en solution aqueuse. D'autre part, leur activité antioxydante a été validée par des méthodes chimiques. Puis, nous avons induit un stress oxydatif exogène sur des cellules endothéliales humaines (HUVEC) et évalué les capacités des CD-A à piéger les ROS cellulaires et mitochondriales et à inhiber la peroxydation lipidique. De plus, l'activation des gènes antioxydants PTEN / AKT, Nrf2 / HO-1 et NQOI et l'expression des protéines associées ont été mesurées *in vitro* en présence du complexe CD-A. Enfin, aucun effet toxique sur les HUVEC supplémentés avec CD-A n'a été constaté.

En raison de ces résultats, le complexe CD-A semble un bon candidat pour être formulé dans un dispositif de libération locale destinée à une application cardiovasculaire.

Mots clés : Astaxanthine ; Stress oxydatif; Hydroxypropyl-β-Cyclodextrin; Cellules endothéliales humaines; Activité antioxydante; Espèces réactives oxygénées dans la mitochondrie, Peroxydation lipidique

Abstract

An imbalance in the reactive oxygen species (ROS) homeostasis is involved in the pathogenesis of oxidative stress related diseases. Astaxanthin, a xanthophyll carotenoid with high antioxidant capacities has been shown to prevent the first stages of oxidative stress. Here, we evaluate the antioxidant capacities of astaxanthin included within hydroxypropyl-beta-cyclodextrin (CD-A) to directly and indirectly reduce the induced ROS production. First, chemical methods were used to corroborate the preservation of astaxanthin antioxidant abilities after inclusion. Next, antioxidant scavenging properties of CD-A to inhibit the cellular and mitochondrial ROS by reducing the disturbance in the redox state of the cell and the infiltration of lipid peroxidation radicals were evaluated. Finally, the activation of endogenous antioxidant PTEN/AKT, Nrf2/HO-1 and NQOI gene and protein expression supported the protective effect of CD-A complex on human endothelial cells under stress conditions. Moreover, a non-toxic effect on HUVEC was registered after CD-A complex supplementation. The results reported here illustrate the need to continue exploring the interesting properties of this hydrophilic antioxidant complex to assist endogenous systems to counteract the ROS impact on the induction of cellular oxidative stress state.

Keywords: Astaxanthin; Oxidative stress; Hydroxypropyl-β-Cyclodextrin; Human endothelial cells; Antioxidant activity; Mitochondrial ROS, Lipid peroxidation.

1. Introduction

While reactive oxygen-derived species are the product of normal aerobic metabolism, they can also be produced at elevated rates under pathophysiological conditions [1]. As a consequence an alteration in the redox signaling leads to uncontrolled reactions between free radicals and neighboring molecules such as proteins, lipids, nucleic acids and carbohydrates, inducing an imbalance in the redox homeostasis [2,3] and thus originating a range of abnormalities further associated with chronic diseases. The apparition of abnormalities associated with vascular diseases were shown to be related with ROS production in the vessel wall [4]. Carotenoids like antioxidants have been investigated due to their capacity to moderate the damaging effects of ROS [5]. According to Britton G. [6], to be an effective antioxidant, carotenoids must react with free radicals in order to inhibit formation of harmful products by disrupting free-radical chain reactions. Additionally, carotenoids can serve as a lipid peroxyl radical quenching either by the addition or abstraction of a hydrogen atom, or by electron transfer [7]. H. Tapiero *et al*. [8] attributed the singlet molecular oxygen and peroxyl radical scavenger action against photooxidative process to carotenoids.

An important aspect to bear in mind is that some carotenoids can switch from antioxidants to pro-oxidants. Among the factors that may trigger such change are the excessive increase of carotenoids concentration, high partial pressure of oxygen and oxidative stress which speeds up ROS production, and the capacity of carotenoids to interact and localize within membranes [9]. Besides the toxic effect carried out by high carotenoids concentration, controlled amounts may lead to the activation of signaling pathways able to recognize potential threats [10], particularly the Nrf2 transcription factor, in which antioxidants are believed to exert an indirect action [11].

Astaxanthin, a xanthophyll carotenoid shows interesting and strong antioxidant and anti-inflammatory properties [12,13]. Among the available sources of astaxanthin, two are of relevant importance: the natural (from microalgae *Haematococcus pluvialis)* and the produced via chemical synthesis. In the present work, both sources of astaxanthin in free form (without esterification) and purified by high-performance liquid chromatography, containing different stereoisomers were used. Synthetic astaxanthin present the isomers 3-R, 3'-R', 3-S, 3'- S and 3-R, 3'-S while 3-S, 3'-S, is the only stereoisomer present in the natural source, Figure 1A [14–16]. Different antioxidant activity of both astaxanthin has been reviewed [17].

Owing to its structure, astaxanthin acts not only as a chain-breaking scavenger of free radicals but also as an inhibitor of lipid peroxidation [18]. In contrast to beta-carotene, the polar

characteristics of astaxanthin allows it to preserve the membranes structure showing a significant antioxidant activity while avoiding a pro-oxidant effect [19,20]. Additionally, the indirect antioxidant capacity of astaxanthin was also shown to potentially contribute to the regulation of gene expression [21–23]. As a highly unsaturated molecule, astaxanthin has a low water solubility and can be easily degraded by light, oxygen and temperature, leading to the decrease of its bioavailability and a diminution of its properties [24]. Coombes *et al.* reported the natural astaxanthin plasma absorption up to 0.19 μmol/L into the blood after 1 to 12 mg human intake for 1 year. Authors did not found significant effect on the oxidative stress reduction after oral ingestion in renal transplant recipients and suggested that the lack of effect could be due to the lower doses or to the length of the treatment [25].

At exposed by Forman *et al.* 2014 low antioxidant bioavailability could be convenient in order to prevent these molecules from acting as pro-oxidants, however, encapsulation systems besides being a limitation to this regard, protect the molecule externally before ingestion to avoid further degradations [10]. Among the encapsulation strategies attempted for astaxanthin protection [26–29]; the molecular inclusion with cyclodextrins has shown interesting results regarding astaxanthin solubility and stability [30,31]. This research focused on the direct and indirect evaluation of the protective effect of hydroxypropyl-β-cyclodextrin-Astaxanthin (CD-A) complex on human endothelial cells under exogenous oxidative stress. The synthesis and characterization of CD-A complex was successfully achieved. To verify the correct encapsulation of astaxanthin within the system and the sensitivity of this new complex to oxidation, direct, CD-A radical scavenging was quantified using AAPH, $ABTS^{\cdot+}$ and $DPPH^{\cdot}$ assays after *in tube* generation of peroxyl (ROO•), and alkoxyl (RO•) radicals. Additionally, *in vitro* tests allow the evaluation of CD-A complex interaction with target molecules such as protein side chain, unsaturated fatty acid and other reactive oxygen species after t-BuOOH stress induction [32,33]. Superoxide radicals production was generated after mitochondrial depolarization after induction by antimycin A, thus blocking the electron transfer [34], while lipid peroxyl radicals were initiated by addition of cumene hydroperoxide (CumOOH) [35]. An indirect antioxidant capacity evaluation was performed by understanding the molecular mechanisms involved in the regulation of endothelial cells gene expression by CD-A complex.

2. Materials and Methods

2.1. Chemical Reagents

Synthetic astaxanthin (SA; purity >97.3%, powder, Lot: 40816) was purchased from Dr. Ehrenstorfer Co. Ltd (LGC standars Germany). Natural astaxanthin (NA; purity >97% HPLC, powder, Lot: 5M4707V), Hydroxypropyl-β-cyclodextrin (CD; DS=0.67), MitoTEMPO (SML0737; >98% HPLC), N-Acetyl-L-cysteine (NAC; A9165) and Antimycin A (Streptomyces sp.) were purchased from Sigma-Aldrich Co. LLC (Saint-Louis, MO, USA); as well as the antioxidant standard 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Lot: BCBJ8170V), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, Lot: 061M538V), potassium persulfate (≥99%, Ref: 216224), Cumene hydroperoxide (≥99%, Ref: 247502), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), *ter*-butyl hydroperoxide (*t*-BuOOH, Lot: BCBJ2885V), 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH, Ref. 440914), Fluorescein (Ref. F6377), 2,2-Diphenyl-1-picrylhydrazyl (DPPH, Ref: D9132), Power syber green master mix (ABI biosystem, Ref : 4472908), dimethyl sulfoxide (DMSO, Lot: SZBD1830V), and Isopropanol (70% in H_2O, Ref: 563935). 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, Lot: 1600227) and C11-BODIPY® 581/591 (Lipid Peroxidation Sensor, D3861) were purchased from Life Technologies (Invitrogen, Eugene, OR, USA). MitoSOX red was purchased from ThermoFisher scientific (Oregon, USA). Acetone (HPLC gradient grade), Methanol (HPLC gradient grade), and chloroform (HPLC gradient grade) were purchased form Carlo Erba Reagents S.A.S (France). Cell culture reagents were all purchased from GIBCO (Life technologies, Carlsbad, CA, USA). Double distilled and deionized water was used for all the experimentation process.

2.2. Preparation of CD-A complex

Natural and synthetic astaxanthin (NA and SA) were included into CD according to the method presented by previous authors [30,36], with minor modifications. Briefly, 1 mL of NA or SA in acetone/chloroform (v/v 1:1) solution (1 mg/mL) was added to 250 mg of CD dissolved in 12.5 mL of 95% methanol (20 mg/mL) in a 25 mL flask filled with nitrogen. The mixture was sonicated (5 min Ultrasonic bath BANDELIN SONOREX RX-100-H) and stirred for 24 h at 35 ^{0}C in a dark chamber. Subsequently, the solution was subjected to a vacuum concentrator and recovered with distilled water. The final solution was frozen, lyophilized (Cryotec, Lyophilizer Crios, Saint Gely du Fesc, France) and stored at -4 ^{0}C.

2.3. Characterization of CD-A complex

To establish the astaxanthin content into the complexes, a calibration curve of SA (5 mg/10 mL DMSO) absorbance at 480 nm was plotted against concentrations (0-20 µM; UV/Vis spectrophotometric measurements, Perkin Elmer Lambda 12 spectrophotometer). Once the curve was established, the complexes (5 mg/1 mL DMSO) were analyzed by UV/Vis spectroscopy and the concentration and inclusion rate of astaxanthin into the complex were calculated according to Chen et *al*. [30] as seen in Eq.1. Where: $C_{astaxanthin}$ is the astaxanthin content in the inclusion complex (g/mL); W_i the total weight (g) of inclusion complex, V_c is the volume (mL) of the solvent used for quantification analysis; W_a is the weight of astaxanthin (g) used for inclusion complex preparation, and W_c is the weight of complex (g) used for quantification.

$$Inclusion\ rate\ (\%) = (C_{astaxanthin} * W_i * V_c)/(W_a * W_c) * 100 \qquad \text{Eq.1}$$

FT-IR characterization corroborates the astaxanthin inclusion within CD. A homogenized powder was obtained by mixing a sample of CD-NA or CD-SA with 200 mg of KBr. Then, each mix powder was placed into a sampling cup, smoothed, and compressed into the holder using a compression gauge. The obtained compact mixture was placed into the IR spectrometer (AVATAR 370, Thermo Spectra Tech Inc, Shelton, CT, USA) and spectral curves in the ranges of 400-4000 cm^{-1} were recorded using OMNIC Software (Thermo Fisher Scientific Inc, Waltham, MA, USA).

2.4. Direct CD-A antioxidant activity measurement

2.4.1. Trolox equivalent antioxidant capacity (TEAC) assay

Antioxidant capacity of CD-NA and CD-SA complexes were measured using the TEAC assay protocol presented by Re *et al*. [37] with slight modifications. Here, the ABTS radical cation ABTS•+ is obtained by the oxidation of ABTS (7 mM) with potassium persulfate ($K_2S_2O_8$, 2.45 mM) in distilled water (vol/vol reaction). The resulting solution was placed in the dark at room temperature for 12–16 h before use. CD-NA, CD-SA (0-5 µM, 50 µL) or Trolox standards (0-200 µM, 50 µL) were mixed with 1 mL of ABTS•+ (absorbance maximum of 0.70±0.02 at 734 nm), The absorbance of the mixture solution was recorded after 1 h of incubation at 30 °C (734 nm, UV/VIS Lambda 12, PerkinElmer Inc, Norwalk, CT, USA). Inhibition percentage was calculated using Eq.2. The Trolox equivalent antioxidant capacity (TEAC) was defined as the millimolar

concentration of Trolox with the same antioxidant activity as 1 mM concentration of the samples [38].

$$\textit{Scavenging rate (\%)} = \frac{[ABTS^{\cdot+}]_{initial} - [ABTS^{\cdot+}]_{final}}{[ABTS^{\cdot+}]_{initial}} * 100 \qquad \text{Eq.2}$$

2.4.2. *The oxygen radical antioxidant capacity (ORAC) assay*

The oxygen radical antioxidant capacity of CD-NA and CD-SA complexes was evaluated [32,39]. Fluorescein solution (4 nM, 150 μL) was mixed with CD-NA (0-5 μM, 25 μL), CD-SA (0-5 μM, 25 μL), Trolox standards (1-18 μM, 25 μL) or blanks, and AAPH (160 mM, 25 μL) in a 96-well microplate. The plate was incubated at 37 ^{0}C and fluorescence decay was monitored during 1 h with data taken every minute using an emission/excitation filter of 485-528 nm (i-control™ microplate reader software, TECAN Männedorf, Switzerland). The complexes antioxidant activity was determined from their ability to keep the fluorescence signal of the indicator in the presence of peroxyl radicals. The AUC and AUC_{net} were calculated using Eq.3, Eq.4. Where f_1 is the initial fluorescence reading at 0 min and f_i is the fluorescence measured at time i. Final ORAC values were calculated by using the regression equation between the Trolox concentration and the AUC_{net} and were expressed as Trolox equivalents (TEAC) as millimolar per liter.

$$AUC = 0.5 + \left(\frac{f_2}{f_1}\right) + \left(\frac{f_3}{f_1}\right) + \cdots + 0.5\left(\frac{f_n}{f_1}\right) \qquad \textit{Eq.3}$$

$$AUC_{net} = AUC_{sample} - AUC_{blank} \qquad \textit{Eq.4}$$

2.4.3. *CD-A antioxidant activity by DPPH*

CD-A complex antioxidant activity was evaluated using the DPPH free radical assay [40]. Briefly, DPPH (0.16 mM; 1.5 mL) was added to 2 mL of different samples CD-A complex (0–5 μM), Trolox standards, or blank in ethanol solution. The mixture was incubated for 30 min in the dark at 25 ^{0}C. Antioxidant effect was evaluated by following the decrease of UV absorption at 517 nm. The radical scavenging activity was calculated using Eq.5. Where A_c is DPPH solution absorption, A_i is the DPPH solution absorption after sample addition and A_J is the initial absorption of the experimental samples.

$$\textit{Scavenging rate (\%)} = \frac{A_c - (A_i - A_j)}{A_c} * 100 \qquad \textit{Eq.5}$$

2.5. Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from ATCC (CRL 1730). Cells were grown in minimum essential Medium-L-Glutamine (MEM), supplemented with 10% (v/v) fetal calf serum (FBS) and 1% penicillin–streptomycin-amphotericin (PSA). Cells were seeded in a T75 cell culture flask (Corning) and kept in a humidified incubator containing 5% CO_2 at 37 ^{0}C. The culture medium was replaced twice every week and the cells were split 1:3 every week.

2.6. Evaluation of CD-A cytotoxicity in HUVEC cells by MTT reduction assay

Cell viability was assessed using MTT assay. Briefly, HUVEC density of 2.10^4 cells/well were seeded and cultured overnight. Cells were treated with different samples: CD-NA and CD-SA (0-5 μM), any antioxidant (culture medium MEM, Negative Toxicity Control, NTC), or 10 % DMSO (Positive Toxicity Control, PTC) during 24 h or 48 h. Afterwards, all solutions were washed and cells were incubated during 3 h at 37 °C with 200 μL of MTT solution (0.5 mg/mL). Then, all wells were washed out of MTT solution and 200 μL of isopropanol were added for 20 min to solubilize the formazan crystals. The optical density was recorded at 490 nm (i-control™ microplate reader software, TECAN Männedorf, Switzerland). Not cytotoxicity of samples was considered if cellular viability was > 70% of the control (based on the ISO 10993:2009 regarding the biological evaluation of medical devises).

2.7. *In vitro inhibition of cellular reactive oxygen species and lipid peroxidation by CD-A complex*

The capacity of CD-NA and CD-SA complexes to reduce the cellular levels of ROS and lipid peroxides in human endothelial cells was determined using the fluorescence probes CM-H_2DCFDA, MitoSOX Red and C-11 BODIPY, respectively. Prior to tests, HUVEC were removed from growth media and detached with trypsin after reached the 80% of confluence. A density of 1.10^4 cells/well were seeded in a 96-well cell culture plates and incubated overnight at 37 ^{0}C with 5% CO_2. Low glucose-MEM was used to prepare antioxidant samples: CD-NA and CD-SA samples (0-5 μM), NAC (500 μM) and MitoTEMPO (500 nM). Hank's buffered salt solution (HBSS) was used to solubilize the probes.

2.7.1. Cellular ROS measurement

Cellular antioxidant activity of CD-NA and CD-SA complexes was quantified using the cell permeable probe CM-H_2DCFDA. This probe emits no fluorescence in its original state. After crossing the cell membrane, DCFH-DA is hydrolyzed by intracellular esterases into DCFH, which becomes fluorescent once oxidized to DCF in presence of ROS. The accumulation of DCF inside the cells is measured by the fluorescence increase at 530 nm using an excitation wavelength of 485 nm in a kinetic mode [41]. HUVEC were incubated overnight with medium containing CD-NA (5 μM), CD-SA (5 μM), or reference antioxidants NAC (500 μM), and MitoTEMPO (0.5 μM). After 24 h, the cells were washed twice with PBS to remove the medium and CM-H_2DCFDA was added to the wells (5 μM final concentration) during 1 h, under light protection. Oxidative stress was induced by the subsequent addition of t-BuOOH (100 μM) or Antimycin A (200 μM, during 20 min), excluding the blank wells. Variations in the fluorescence intensity were recorded during 60 min with data taken every 5 min (i-control™ microplate reader software, TECAN Männedorf, Switzerland). The capacity of CD-A to reduce the oxidative cell environment was evaluated as expressed in Eq.6: Where I_{c_n} and I_{s_n} represent the intensity of cells exposed to t-BuOOH or Antimycin A without and with antioxidant presence at time n, respectively.

$$CAA\ (\%) = \frac{I_{c_n} - I_{s_n}}{I_{c_n}} * 100 \qquad \textit{Eq.6}$$

2.7.2. Mitochondrial ROS detection

The capacity of CD-NA and CD-SA complexes to inactivate mitochondrial superoxide production was measured using MitoSOX Red fluorescent probe. MitoSOX Red after being target to the mitochondria, it is oxidized by superoxide to form 2-hydroxymitoethidium [42]. HUVEC were incubated overnight with medium containing CD-NA (5 μM), CD-SA (5 μM), or reference antioxidants NAC (500 μM), and MitoTEMPO (0.5 μM). After 24 h, the cells were washed twice with PBS to remove the medium and incubated with MitoSOX (5 μM), for 20 minutes at 37 °C, protected from light. Cells were gently washed three times with warm buffer. Reactive oxygen species were induced by the addition of Antimycin A (AA; 200 μM, during 20 min), excluding the blank wells. Fluoresce was monitored at λex/λem = 510/595 nm (Single-read measure; i-control™ microplate reader software, TECAN Männedorf, Switzerland). CD-A effect in

decreasing mitochondrial ROS generation was compared to the percentage of control (not stressor or antioxidant treatment).

2.7.3. Cellular lipid peroxidation analysis

C11-BODIPY581/591 is a fluorescent fatty acid analogue which allows the quantification of lipid peroxidation by indirect measure of mitochondrial ROS production [43]. Upon free radical-induced oxidation, its fluorescent properties shift from red to green [44]. Ninety-six well plates containing the cells were washed with PBS, before addition of C11-BODIPY (5 μM, during 30 min). Cells were incubated with 5 μM of CD-NA and CD-SA for 1 h, under light protection. Lipid peroxidation was initiated by addition of Cumene hydroperoxide (CumOOH, 50 μM). Fluoresce was monitored at red λex/λem = 590/7, 632/45 nm and green λex/λem = 485/14, 520/10 nm (i-control™ microplate reader software, TECAN Männedorf, Switzerland). Percent of CD-A cellular lipid peroxide activity inhibition was calculated relative to the positive control fluorescence intensity (PC; CumOOH without antioxidant).

2.8. Indirect CD-A antioxidant activity measurement

2.8.1. mRNA extraction and real time RT-PCR

HUVECs were seeded in 6 well plates. Then CD-A (8 μM) was added to the cells and incubated for 24 h. After discard of supernatant, the cells were washed twice with PBS and incubated with AAPH (5mM) or t-BuOOH (25 μM) for 24 h (the IC50 of AAPH and or t-BuOOH at 24 h were determined 5 mM and 25 μM respectively (data not showed). Total RNA was extracted using the TRIzol® reagent (Invitrogen TM, Life Technologies). The RNA yield and purity were determined using a NanoDrop ND-1000 spectrophotometer. For rt-PCR, 1 μg of RNA, 0.4 μm universal hexamer primer, 1 μLdNTP (10 Mm) and DEPC water mixed and incubated at 65 °C for 5 min and kept on ice. Then, 5U reverse transcriptase enzyme (MMLV), 1x RT buffer and 1U/μl RNase inhibitor were added to reaction and the solution was increased to a volume of 20 μL with DEPC water. Reverse transcription of mRNAs was performed at 25 °C for 10 min and 42 °C for 60 min. then, Real-time PCR was performed to measure expression levels of target genes (Table A) using a power SYBR Green Master Mix (ABI Biosystem) on a Bio-Rad IQ5 real-time PCR detection system.

2.8.2. Immunoblotting

Proteins were extracted from the treated HUVEC using radio-immunoprecipitation assay buffer (RIPA, 50 mM Tris-base, 1.0 mM EDTA, 150 mM NaCl, 0.1 % SDS, 1 % Triton X-100, 1 %

sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride). Extracts were separated by 12 % SDS–PAGE gel and transferred to a polyvinylidene difluoride membrane (Millipore, MA, USA) previously probed with primary antibodies (Abcam) specific to, NQO1 (ab80588), PTEN (ab137337), HO-1 (ab52947), Nrf2, and GAPDH (ab37168) before being incubated with horse radish peroxidase-conjugated secondary antibody (1:2000, Sigma–Aldrich). Bands were detected using a chemiluminescent kit (ECL, Thermo, 32106). Experiments were performed in triplicate.

2.9. Statistical analysis

All experiments were repeated at least three times to ensure the reproducibility of each test. Results were expressed as the mean ± SDE and statistical analysis was done using one-way ANOVA followed by Tukey's HSD *post hoc* test (JMP Software, Version 9; SAS Institute, Cary, NC, USA). Results were considered significantly different if p-value < 0.05.

Target genes	*Primer*	*Primer Sequence (5'-3')*	*Annealing Temperature (°C)*
PTEN	Forward	TCCCAGTCAGAGGCGCTATG	60
	Reverse	CACAAACTGAGGATTGCAAGTTC	
Nrf2	Forward	GAGACAGGTGAATTTCTCCCAAT	59
	Reverse	TTTGGGAATGTGGGCAAC	
HO1	Forward	ACGGCTTCAAGCTGGTGATG	61
	Reverse	TGCAGCTCTTCTGGGAAGTAG	
NQOl	Forward	ATGTATGACAAAGGACCCTTCC	62
	Reverse	TCCCTTGCAGAGAGTACATGG	
Bax	Forward	ATCCAGGATCGAGCAGGGCG	64
	Reverse	GGTTCTGATCAGTTCCGGCA	
AKT1	Forward	GTTTGCCGGAATCAATTTTC	60
	Reverse	AGCCAGAGCTGTGATCTCCTT	
GAPDH	Forward	GAGCCAAAAG GGTCATCATC	63
	Reverse	TAAGCAGTTGGTGGTGCAGG	
Caspase-3	Forward	TGTGAGGCGGTTGTGGAAGAGT	63
	Revers	AATGGGGGAAGAGGCAGGTGCA	
eNOS	Forward	ATCTCCGCCTCGCTCATG	61
	Revers	GAGCCATACAGGATTGTCGC	

Table A. The sequences of primers used for study of profile of RNAs using q-PCR.

For real time PCR, the level of expression was calculated based upon the PCR cycle number (C_T). The endogenous control GAPDH and RNU6 were used for normalization of mRNAs and micro-RNAs expression levels, respectively. C_T values were used to calculate relative

expression using SPSS Version 14.0 software by difference in the C_T values of the target RNAs after normalization to RNA input level. Relative quantification was represented by standard $2^{\Delta CT}$ calculations. $\Delta C_T = (C_{T\text{-target gene}} - C_{T\text{-GAPDH}})$. Each reaction was performed in triplicate.

3. Results and discussion

3.1. Characterization of CD-A complex

Here, both natural and synthetic astaxanthin were successfully included into the CD hydrophobic cavity (Figure 1SD), showing that stereoisomers did not constrain the inclusion process, as confirmed by FTIR measurements. Figure 1B, presents the representative absorption bands of astaxanthin at 1656-1652 cm^{-1} and 974-970 cm^{-1}, which indicate the C=O and C-H stretching vibrations respectively; after CD inclusion these bands were weaker, as reported by Qiu *et al*. [45].

According to the regression model suggested by Dong *et al*. [36], an inclusion rate of 12.05±0.96 % for CD-NA and 7.21±0.64 % in the case of CD-SA were obtained, with a weight recovery rate around 89% (Figure S1A-B). CD-NA and CD-SA concentration after inclusion were 3.4±0.4 μM and 2.6± 0.4 μM, respectively, calculated from the SA calibration curve and the CD-A OD curves at 480 nm (Figure S1C-D). No astaxanthin precipitation was noticed even when solubilized high amounts of CD-A complex in DI water, which is in accordance with similar works [31,46–48].

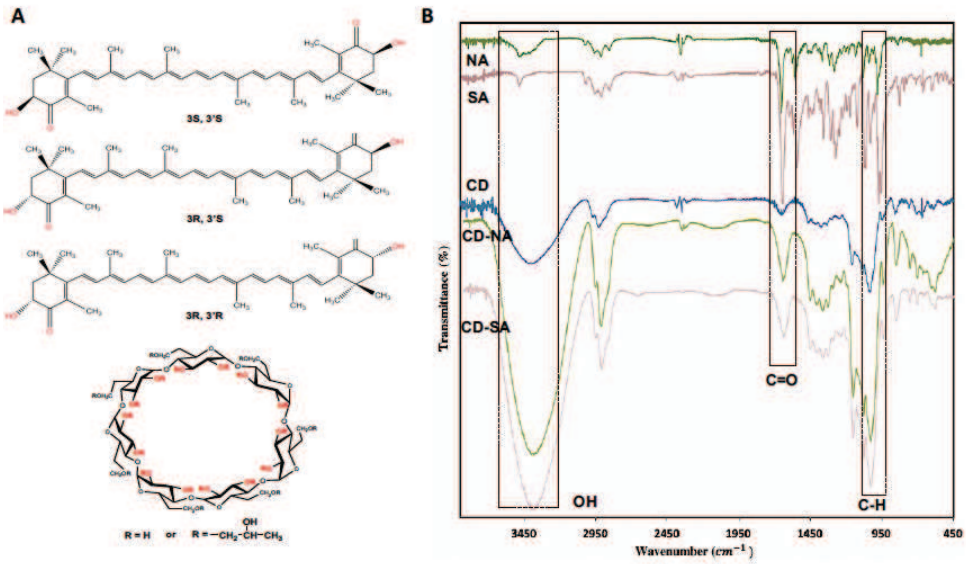

Figure 2. CD-A complex characterization. (A) Chemical structure of astaxanthin: 3S,3'S; 3R,3'S; 3R,3'R esterification's and Hydroxypropyl-ß-cyclodextrin (CD), respectively. (B) FT-IR spectrums of NA, SA, CD, CD-NA and CD-SA.

3.2. CD-A bioactivity quantification

Oxidative stress is produced by the action of different ROS; hence efficient methods able to quantify the influence of external substances like antioxidants in the prevention of radical's formation are needed. Available indirect probes provide valuable information on changes on the redox environment of the cell, but many of these methods are not specific, do not allow subcellular localization, and their response is affected by different chemical interactions [49]. Despite that, these methods represent a valuable tool to obtain an overview of the antioxidant ability of several molecules such as antioxidants.

The antioxidant activity of CD-NA and CD-SA complexes was directly assessed by TEAC, DPPH and ORAC assays. These chemical methods while being simple, sensitive and reproducible, provide useful information about the carotenoids antioxidant activity [50]. CD-NA and CD-SA scavenging capacities corresponded to 5.73±2.9 and 3.93±2.8 mM of Trolox, respectively (Figure 2A), evaluated by the $ABTS^{+\cdot}$ Assay. This result agreed with TEAC values reported by other authors for astaxanthin in its free form [39,51–54]. ORAC assay was used to measure the capacity of CD-NA and CD-SA to inhibit the thermal decomposition of AAPH against alkyl (R·), peroxyl (ROO·), and alkoxyl (RO·) radicals, (where R=H2N(HN)C) [32,39]. Here, ORAC values (5.73±2.1 and 5.10±3.10 mM of Trolox) were in the same range as those obtained by $ABTS^{+\cdot}$ assay (Figure 2C). It seems appropriate to refer to the scavenging capacities reported for esterified astaxanthin and synthetic astaxanthin, which according to the literature are in a range between 0.1±0.25 to 2.43±0.02 for natural and synthetic astaxanthin respectively using the ABTS assays and 1.68±0.25 to 8.1±1.12 using ORAC test [51,53,54], the variability of these capacities could probably be due to the low miscibility of hydrophilic components with chemical products. Additionally, the preservation of CD-A antioxidant capacity was evaluated after 6 months of complex storage at 6 ^{0}C under light protection. Figure 2D, presents a ORAC TEAC value in the order of 5 mM of Trolox for both CD-NA and CD-SA complexes, reflecting the successful conservation of astaxanthin in the CD cavity. Passed this time, a floc was observed in the vials, a behavior already described by Chen *et al*. [30], who observed a floc formation after 6 h of complex dispersion in water. However, the preservation of astaxanthin antioxidant capacity after 6 months revealed that even if astaxanthin precipitate, a new covalent bond could be induced by simply mixing the vial.

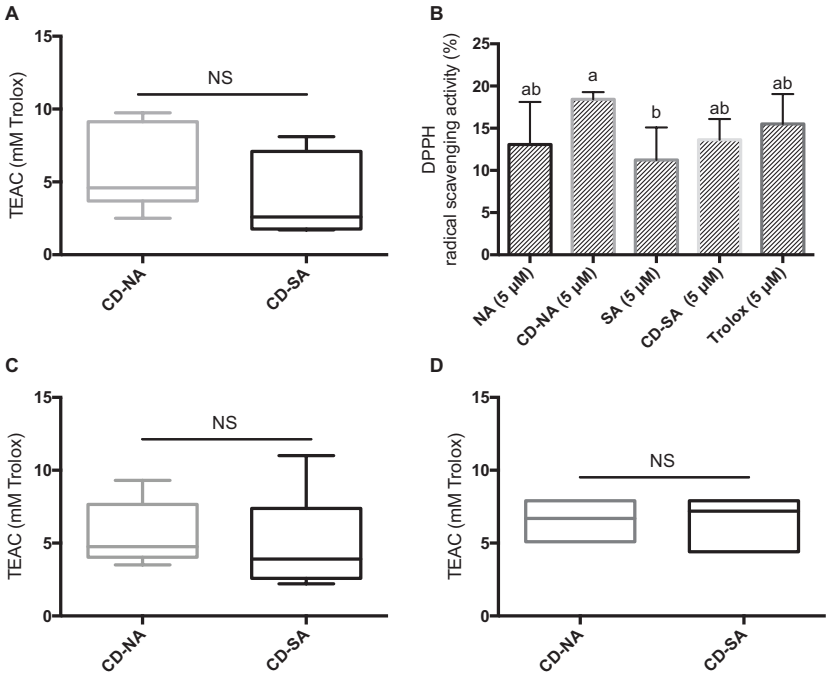

Figure 3. Biochemical assays measuring the antioxidant capacity of CD-A complex. (A) TEAC CD-NA and CD-SA complex measured by ABTS assay. (B) DPPH radical scavenging activity of free astaxanthin (NA and SA), CD-A complex and Trolox antioxidant reference molecule. (C) Oxygen radical absorbance capacity (ORAC) assay of both complexes expressed as the Trolox equivalent in Mm. (D) CD-A complex antioxidant stability study after 6 months of storage at 6 °C, under light protection, calculated by means of ORAC assay. Data are means ± SD of six experiments. Levels not connected by the same letter are significantly different ($p<0.05$).

Regarding the DPPH radical quenching, an inhibition percentage around 18% was found for CD-A complex at astaxanthin concentration of 5 μM; a not negligible value, since literature reported scavenging rate of 97% for a greater SA concentration (133 μM) [55]. Here, we report a complex inhibition capacity against ROS directly reliant to the astaxanthin concentration within the complex. CD-NA and CD-SA complexes presented a similar antioxidant scavenging capacity as expressed by the TEAC value, however their activity was stronger than the Trolox standard antioxidant molecule, evaluated by $ABTS^{+\cdot}$ and ORAC assays. Not significant difference was observed between astaxanthin radical quenching before and after inclusion into the CD and Trolox, in the DPPH test. Some authors attribute the antioxidant capacity of astaxanthin, to the activation of the hydroxyl group by the keto group allowing the hydrogen transfer to the peroxyl radical, and thus acting as a chain breaking in the free radical reaction [56,57]. The astaxanthin inclusion into the CD cavity took place due to a non-covalent link between the CD hydrophobic cavity and the hydrophobic molecule, enhancing the CD-drug interaction with the lipophilic environment [58], without affecting the antioxidant properties.

In this study, chemical probes were used in principle as a verification tool of the preservation of CD-A complex sensitivity to oxidation before the evaluation of biological antioxidant capabilities. However, authors want to recognize the variability and instability of these probes due to external factors like light, temperature, or pH, which can degrade the probe during the time of analysis. Despite the use of a reference antioxidant Trolox and expressing the results based on Trolox equivalents, obtained results using ORAC, TEAC and DPPH assays may lead to different conclusions, agreeing with data reported for some antioxidants [59]. It is worthy to highlight that CD-A reacts differently with the reagents used in each test for the determination of its antioxidant capacity, moreover its capabilities measured by these assays provide information about the chemical reactivity of these compounds to block ROS without referring to the *in vitro* or *in vivo* relevance on human health.

3.3. In vitro cytoprotective activity of CD-A complex

CD have been currently used as a solubilizer for different kinds of hydrophobic molecules and up to date any biocompatibility problem has been reported when exposed to diverse cell lines in concentrations not greater than 40 mg/mL [60–62]. Astaxanthin stability in culture medium is an important parameter conditioning cellular interaction and its antioxidant ability. Here, astaxanthin was included in CD complexes (CD-A) and thus stabilized in the culture medium at 37°C. As shown in Figure 3A-B, CD-A complex were non-cytotoxic to HUVEC cells, represented by a cell viability exceeding 70% at concentrations up to 5 μM after 24 and 48 h of exposure, showing a good biological acceptance of the complex by the HUVEC cells. A maximum decay of 20% of NTC was registered for the CD-SA at concentrations higher than 5 μM, while CD-NA showed a faster decay at 2.5 μM without exceeding 20% of NTC, indicating a suitable compatibility for both CD-A complex. These results agreed with the data reported for astaxanthin samples without inclusion, where a high cytoprotective potential was observed for concentrations lower than 25 μM in a cell population of HUVEC, HepG2 and MCF-7 cells [51,63]. Further, different CD concentrations were tested to verify their compatibility with HUVECs and no toxicity effect were remarked for concentrations lower than 40 mg/mL (data not shown).

3.4. Direct biological evaluation of CD-A antioxidant capability

In contrast to chemical assays which offer useful information of antioxidant activity of components, cellular tests take into account the bioavailability and metabolism of the tested compound providing information of ROS downstream effect in living cells [43]. Mechanisms of antioxidant/detoxifying protection are challenging. New approaches to detect ROS focused on the use of specific components to directly target organelles involve in ROS production. For instance, mitoSOX a red fluoresce probe is able to directly target complex III within the mitochondria to sense $O_2^{\bullet-}$ production, to form 2-hydroxy-mito-ethidium [42]. Also, C11-bodipy probe asses the indirect measure of mitochondrial ROS production in living cells to extent lipid peroxidation [44]. Moreover, the CM-H_2DCFDA assay provided information concerning general disturbance in the redox state of cells [49]. In this study, several methods and stressors were used to assess biological relevance and cellular availability of astaxanthin to block oxidative stress.

Additionally, the direct scavenger capacity of CD-A to neutralize the chemical-induced oxidative stress in HUVEC cells after either peroxyl (ROO•), or alkoxyl (RO•) radical generation using AAPH or t-BuOOH was evaluated. Additionally, superoxide radicals were induced by antimycin A, and lipid peroxyl radicals were initiated by addition of CumOOH. CD-A complex scavenging capacity was compared with two antioxidants that were shown to protect endothelial cells under induced oxidative stress [64,65]: (a) NAC, a scavenger of free radicals such as hydroxyl radical, hydrogen peroxide and superoxide [66] and (b) MitoTempo a mitochondria-targeted superoxide dismutase specific antioxidant.

Intracellular antioxidant capacity of CD-A evaluated by CM-H_2DCFDA showed that HUVEC supplementation with either CD-NA or CD-SA during 24 h, markedly inhibit superoxide radical induced by Antimycin A. Scavenging rate of astaxanthin complexes (40-50%) was higher than those measured after incubation with NAC or MitoTEMPO (10-20 %, $p<0.05$; Figure 3C). Besides, RO• and ROO• radical reduction after t-BuOOH induced stress were lower compared with CD-NA and CD-SA complexes (10-30%), NAC or MitoTEMPO (5-20 %) treatments (non-significant differences). MitoSOX Red probe showed a significantly increased mitochondrial $O_2^{\cdot-}$ levels in HUVEC under Antimycin A ROS induction (Figure 3D, $p<0.05$). The redox equilibrium was re-established after antioxidant supplementation (compared to non-stressed cells) showing similar levels for CD-NA, MitoTEMPO and NAC (Figure 3D). Additionally, CD-A ability to protect HUVEC from lipid peroxidation was evaluated. Lipophilic C-11 BODIPY probe was monitored as

indicator of peroxidative membrane damages after addition of CumOOH, a lipid peroxide radical initiator [22]. As showed in Figure 3E, a reduction in the cellular lipid peroxidation state was observed when HUVEC were supplemented with CD-NA and CD-SA complexes. The astaxanthin capacity to inhibit the penetration of oxidative substances across the lipid membrane by blocking the initiation of a lipid peroxidation process was confirmed by other authors [41,64]. Here, we demonstrate the preservation of astaxanthin activity into the complexes.

Interestingly, intracellular antioxidant activities of CD-NA and CD-SA complexes are higher than those reported for astaxanthin stereoisomers and free form without inclusion [51,67–69]. Moreover, we previously showed the CAA protective capacity exerted by algae natural astaxanthin in human endothelial cells subjected to oxidative stress *in vitro* [51]. Recently, Xue *et al*. [70], demonstrated that astaxanthin treatment markedly attenuated mitochondrial ROS produced after total body irradiation. Moreover, a potent mitochondrial ROS reduction had been noticed when using targeted than non-targeted mitochondrial antioxidants in different *in vitro* and *in vivo* models [64,70–74]. In contrast to synthetic astaxanthin, natural astaxanthin without purification also contain other carotenoids, and there is no much evidence confirming the fact that these carotenoids act in synergy, in an additive manner or possibly cancel each other [17]. The results showed here did not confirm or reject the anterior idea, but support the fact that similar antioxidant activities are obtained for either natural or synthetic astaxanthin in free and purified form included within CD.

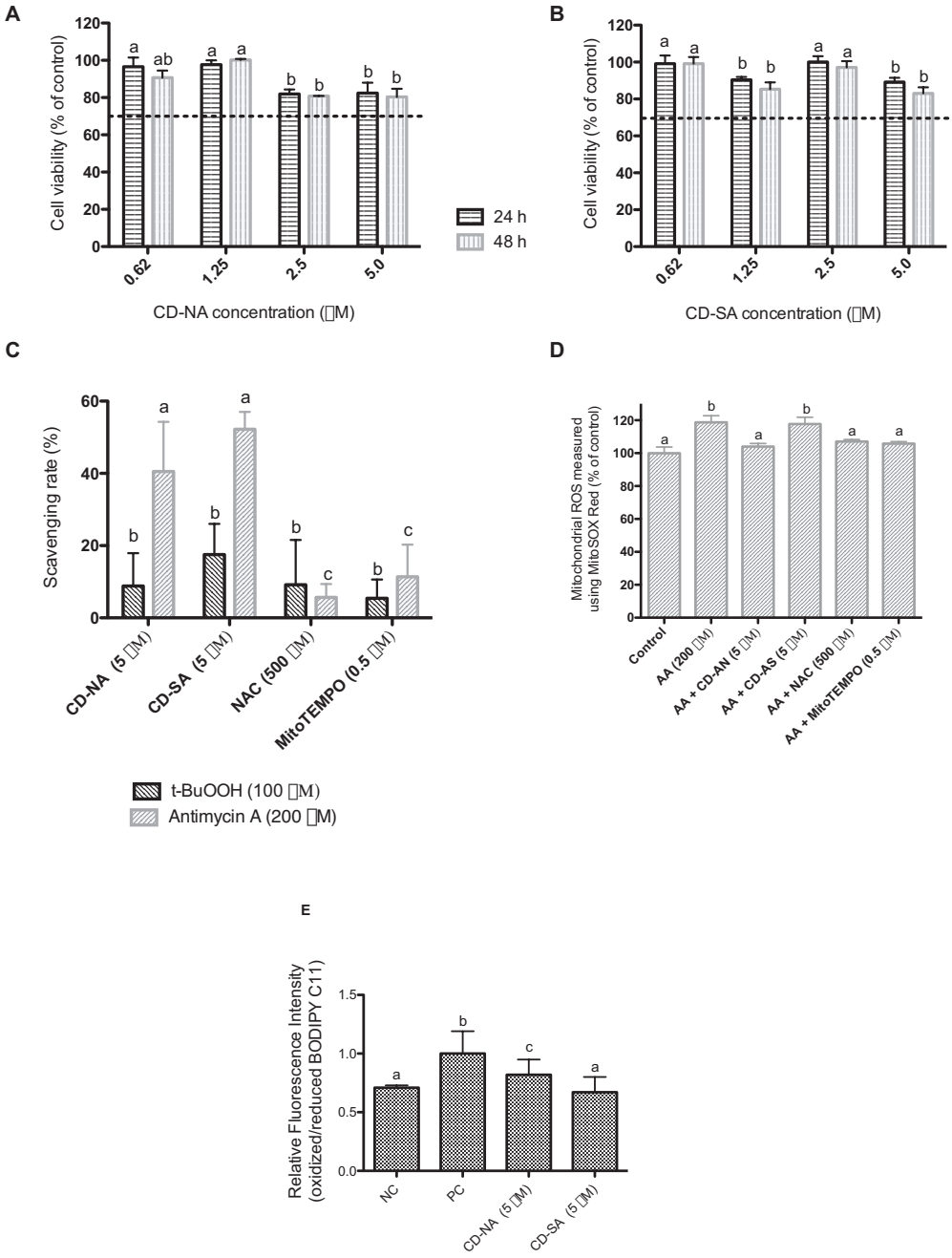

Figure 4. In vitro evaluation of CD-A complex cytocompatibility, CD-A complex protective effect against cellular ROS and lipid peroxidation. Cellular viability of HUVECs exposed to CD-A complex during (A) 24 h and (B) 48 h, doted lined represent a higher viability of 70% regarding to PTC (DMSO 10%), indicating a good cell-CD-A complex compatibility. Cellular antioxidant activity (CAA) of CD-A complex measured by (C) CM-H_2DCFDA and (D) MitoSOX Red, ROS were induced by either t-BuOOH or Antimycin A in both assays. NAC and MitoTEMPO were used as antioxidant references. (D) Cellular lipid peroxidation activity (CLPAA) results for CD-A complex compared to positive control (HUVEC exposed to CumOOH). Data are means ± SD of six experiments. Levels not connected by the same letter are significantly different ($p<0.05$).

Efficient probes able to quantify antioxidant scavenging capabilities as well as ROS levels provide a key to understand antioxidants action mechanisms and to regulate the redox balance in the body (Figure 1). However, reactive species present some characteristics that make difficult their detection, like their very short lifetime and the endogenous antioxidant mechanism which regulate their levels *in vivo* [75]. Furthermore, the response of antioxidants to different radicals or oxidant sources vary widely [76]. The ideal chemical or biological probe would be highly reactive at low concentrations, specific, nontoxic, easy to use and to load into organelles, cells, or tissues without subsequent leakage, readily available and inexpensive [49,77].

Up to now, there is not a unique probe filling all these criteria, therefore the simultaneous use of chemical and biological methods is advised to obtain a better screening of the tested molecule properties. Chemical methods "*in tube"* provide a first approach to validate the antioxidant capacities of a specific component. On the other hand, biological methods allow measuring the antioxidant ability of a component to regulate the redox environment of an organelle and provide information about oxidative markers, intracellular antioxidant capacities and endogenous antioxidant pathways [79].

Our results showed that the inclusion of astaxanthin within CD highly improved its lipid/aqueous affinity without affecting the intracellular antioxidant capacity against ROS. Moreover, positives antioxidant scavenging activities evidence astaxanthin cellular and mitochondrial-targeted action to reduce the disturbance in the redox state of endothelial cells.

3.5. Indirect CD-A antioxidant protection against ROS

3.5.1. CD-NA protects cells by activation of endogenous antioxidant systems by Nrf2/ HO-1/NQO1 pathway

Nrf2 (nuclear factor-erythroid 2-related factor 2), is a key transcription factor phisiologically attached to Keap1 protein within the cytoplasm in basal conditions. Under, oxidative stress, the Keap1-Nrf2 complex dissociates and Nrf2 translocates to the nucleus, inducing an endogenous antioxidant response of detoxifying enzymes and proteins such as heme oxygenase-1 (HO-1) and NAD (P) H: quinone oxidoreductase 1 (NQO1) [79]. Heme oxygenase-1 (HO-1), is a stress response protein induced in response to a variety of oxidative challenges and pathological stimuli having cytoprotective function. HO-1 mediates the anti-inflammatory effects [80] and has a central role in cardiovascular protection [81]. NQO1 has an anti-inflammatory action and encodes for a reductase enzyme preventing the reduction of quinones that result in the

production of radical species. Inflammatory cytokines that suppress NQO1 induce oxidative stress [82,83]; mutations in this gene have been associated with cardiovascular disorders [84].

In this research, we studied CD-NA protective effect on human endothelial cells. Two different stressors were examined: t-BuOOH and AAPH, frequently used as a free radical donors which generate a burst of ROS, thus inducing the dissociation of Nrf2/Keap1 complex [33,85]. An overexpression of HO-1 and NQO1 was noticed when oxidative stress was induced in endothelial cells by both stressors (Figure 4A-B). The CD-NA cell treatments led to the upregulation of HO-1 and NQO1 basal expression and downregulation in ROS excess conditions. HO-1 and NQO1 proteins expressions were analyzed after radical induction. A higher expression was detected when both stressors were present, while a similar upregulation trend using CD-NA complex was evidenced for both proteins expression (Figure 4A-B). In this study, the upregulation of HO-1 and NQO1 could be due to the separation of Nrf2/Keap1 by radical action; suggesting that in basal conditions CD-NA allows Nrf2 gene upregulation. Moreover, Nrf2 gene expression was not affected after oxidative stress induction. The protein expression study confirmed these results (Figure 4B).

Astaxanthin action on Nrf2/OH1/NQO1 pathway was previously described *in vitro* in animal cells, however revealing results were conflicting [22,86,87]. Increased cellular GSH due to astaxanthin treatment on Human hepatic cellular carcinoma cells (Huh7) was not mediated by an Nrf2 dependent signal transduction pathway [88]. In contrast, Nrf2 was activated in human retinal pigment epithelial cells (ARPE-19) showing a reduction in the oxidative damage due to astaxanthin treatment [89]. Recently, Lei Pan *et al.* showed that astaxanthin pretreatment significantly increased the expression of Nrf2, HO-1 and NQO1 mRNA in a cerebral ischemia rat model evidencing a protective effects against brain injuries [90]. The discrepancies between astaxanthin reported action could be related to differences in cellular types sensitivities upon carotenoids treatment, to the type of stress inducers and to the astaxanthin product composition [17].

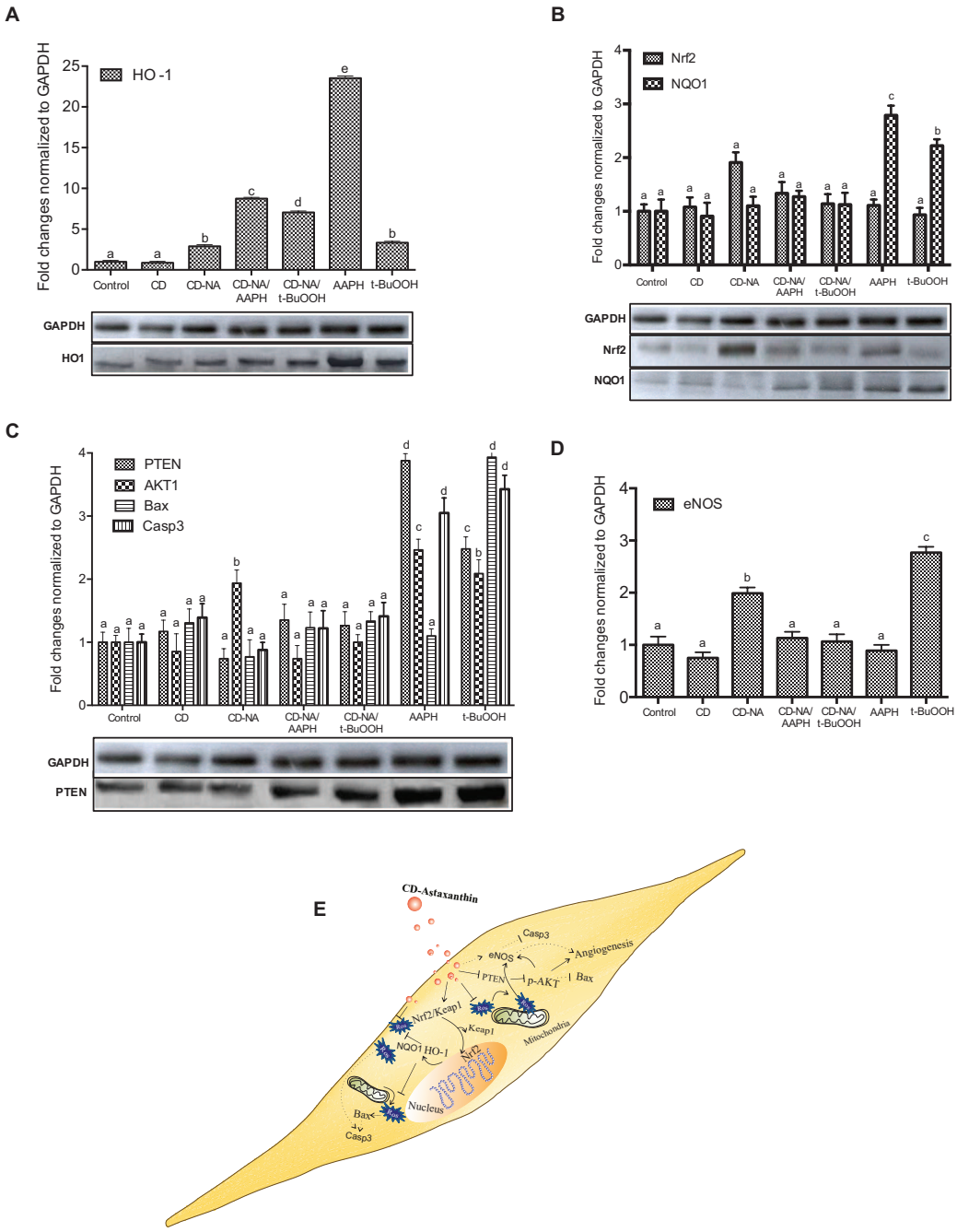

Figure 5. The molecular protection mechanism of CD-A complex against ROS. CD-A complex activates (A-B) Nrf2/HO-1/NQO1 and (C-D) PTEN/PI3K/AKT endogenous pathways. (E) Schematic representation of the molecular pathway of CD-A protective action on endothelial cells under oxidative stress. Levels not connected by the same letter are significantly different ($p<0.01$).

3.5.2. CD-NA prevents apoptosis of endothelial cells under oxidative stress by PTEN/ PI3K/AKT pathway

Previous studies revealed that intracellular ROS generation modulates the PTEN/PI3K/AKT pathway influencing the cell fate towards senescence and apoptosis [91]. AKT plays a vital role in vascular homeostasis; acting as a regulator of endothelial cell survival, growth and NO production [92]. Astaxanthin was shown to protect *in vivo* isoflurane-induced neuro-apoptosis in a rat model, supported by the diminution of brain damage, suppression of Casp3 activity and upregulation the PI3K/AKT pathway [93]. In this study ROS induction with AAPH and t-BuOOH upregulate PTEN gene expression probably to deactivate the AKT, guiding cells to apoptosis (Figure 4C). Conversely, CD-NA treatment significantly reduces the PTEN expression in endothelial cells under oxidative stress. Protein expression levels confirmed these results (Figure 4C). eNOS and Bax genes were upregulated when oxidative stress was induced using t-BuOOH stressor on endothelial cells. CD-NA treatment significantly reduced both eNOS and Bax genes expression. This effect was not observed after AAPH ROS induction (Figure 4C-D). Additionally, CD-A treatment downregulates AKT and Casp3 genes expression submitted to stress (Figure 4C). These results give some indications of a possible indirect CD-A cell protection against oxidative stress induced apoptosis.

4. Conclusion

The use of carotenoids like antioxidants can assist the natural mechanisms of cells in neutralizing oxidative stress. Particularly, the xanthophyll carotenoid astaxanthin is of special interest, due to its ability to interact with free radicals as a chain breaking molecule. Despite the positive results showed for astaxanthin *in vivo* treatment on induced oxidative stress related diseases, clinical trials have been disappointing due, among other factors, to the differences between the antioxidant systems of humans and rodents. Adequate carotenoids doses and determination of appropriate length treatments are not well established yet. In addition, several inconveniences have been attributed to their prone sensibility to degradation and lack of availability. Here, we showed the capacity of CD to enhance the astaxanthin solubility, improving the astaxanthin ability to re-establish the balance in the redox state of the cell. Additionally, the direct capacity of CD-A to inhibit the HUVEC and mitochondrial ROS and to reduce lipid peroxidation was demonstrated. Moreover, the CD-A indirect action to reduce ROS levels by reinforcing the Nrf2/HO-1/NQO1 endogenous antioxidant defenses was shown. Results presented in this research were performed in a human endothelial cell line and cannot be

generalized to primary cells or animal models. Owing to these results, CD-A complex appears to be highly suitable for a posterior evaluation of its antioxidants capacities to regulate the ROS production *in vivo,* as an oxidative stress regulator. However further research is necessary before considering the possibility of using this complex in human therapies.

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Supporting information

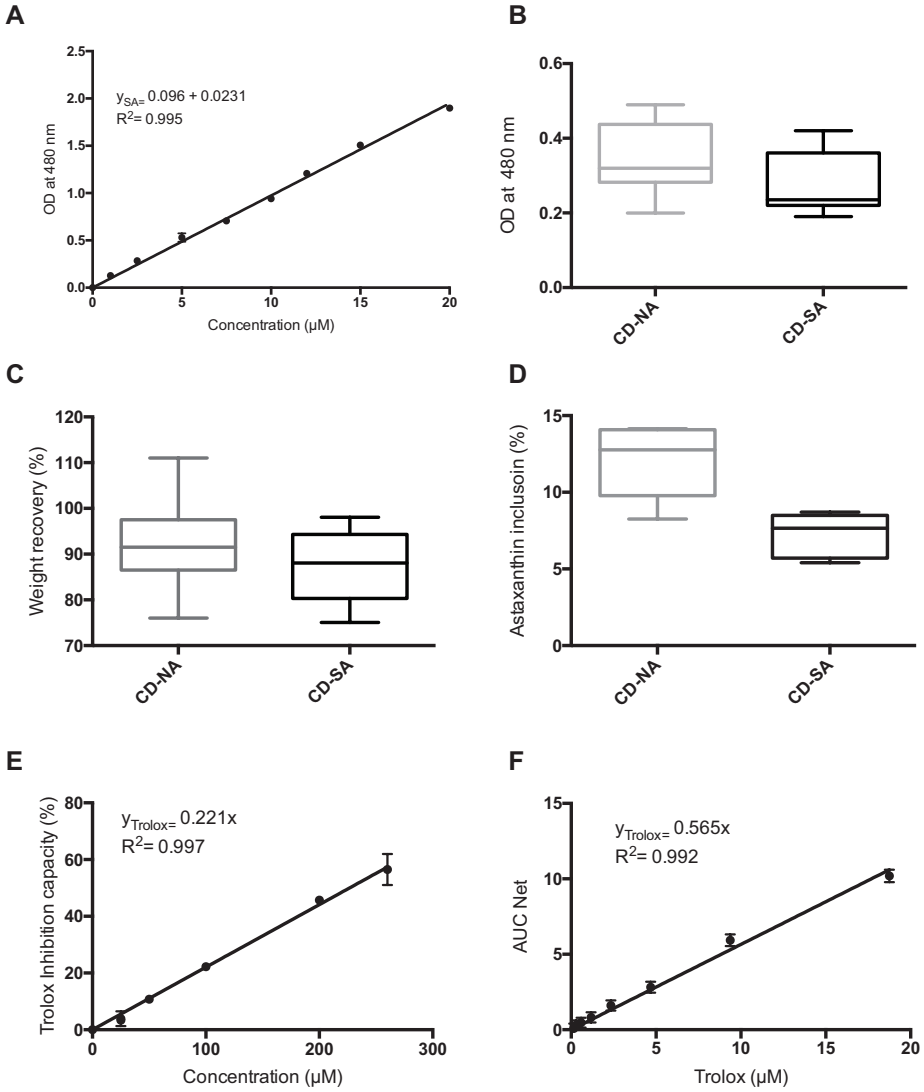

Figure S1. Characterization and astaxanthin quantification into CD complex. (A) SA calibration curve acquired by UV/VIS spectroscopy showed a good linear correlation between the concentration (X) and the absorbance (Y). (B) Concentrations of astaxanthin CD complex were established by recording the absorbance in DMSO at 480 nm. (C) Weight recovery rate and (D) CD-A inclusion capacity were calculated at the end of the procedure. Trolox calibration curves were plotted to calculate (E) Trolox inhibition capacity using TEAC/ABT assay and (F) Trolox AUC net against concentration by ORAC assay. Data are means ± SD of six experiments.

S2. In vivo biocompatibility evaluation of CD-A complex

Up to date the astaxanthin biological action after inclusion into CD cavity is not known. Here, we showed the tissue reaction after CD-A complex subcutaneous implantation during one week in a rat model (Figure A2b). The animal study was carried out under the approval of the Bichat University Institutional Animal Care and Use Committee. Adults Male Wistar rats weighing 240-250 g (ws/ws, purchased from Charles-Rivers, France) were anesthetized by intraperitoneal injection of sodium pentobarbital solution (30 mg/kg, Centravet, France). Animals were divided according to three selected conditions: CD-NA, CD-SA and Control. Two abdominal pockets of approximately 1x1 cm (Figure. A2a) were made on both left and right sides of the rat abdominal midline and each group received a subcutaneous implantation of 5 mg of sample per pocket. The skin was immediately sutured using VicrylIVR 4.0 (Ethicon). Rats were euthanized by an intraperitoneal injection of sodium pento-barbital (60 mg/kg) at day 5 post-surgery after tissue excision. Tissue portions were flushed with saline solution, fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Sections of 3 µm thickness were obtained using a microtome (HM 355S microtome, Thermo scientific Waltham, MA, USA). Histological HPS (hemalun-ploxine-safran) and CD68 immunohistochemical staining were realized. Aperio ScanScope (Leica Biosystems, France) and CaloPix Viewer software (TRIBVN, Châtillon France) were used to scan and analyze the slides respectively. These showed a good interaction between complex and tissue, that was confirmed by the immediately integration of the complex. After one week of daily visual monitoring of the animal, no modification their behavior was noticed, as well as, no tissue inflammatory reactions. Morphological observation of HPS stained tissues showed a regular morphology of tissues subjected to CD-A implantation when compared to control sample. Immune-histological staining did not reveal any proliferation of granulation tissue, or macrophage infiltration in treated tissues.

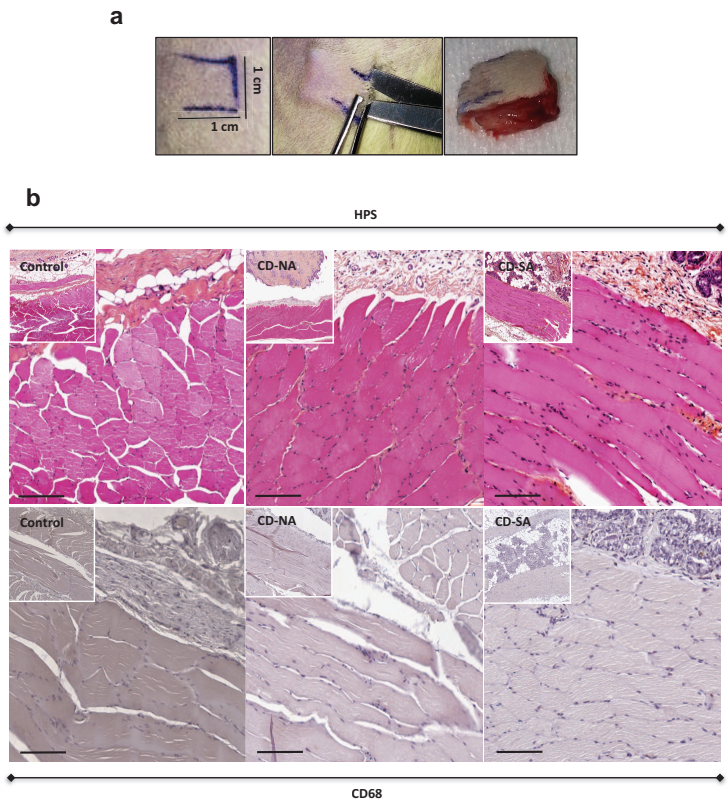

Figure A1. *In vivo* biocompatibility of CD-A complex. (a) Subcutaneous implantation procedure of CD-A complex. (b) Histological HPS and CD68 macrophages immuno-staining of implanted CD-A complex. Scale bars =100 μm.

Highlights

- CD improves the astaxanthin solubility without affecting its antioxidant scavenging capacities or compatibility.
- CD-A inhibit HUVEC cellular and mitochondrial ROS by reducing the disturbance in the redox state of the cell and the infiltration of lipid peroxidation radicals.
- CD-A complex can inhibit the oxidative stress in HUVEC cells by PTEN/AKT gene and protein expression and also enhance the Nrf2/HO-1 endogenous antioxidant defenses.

4 Chapter

Research article: PVA/Dextran patches as delivery system of antioxidant astaxanthin: A cardiovascular approach

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At this point of the study, the powerful antioxidant capacities of CD-A complex were granted. This following part wants to demonstrate that an *in situ* delivery strategy improves the ability of the CD-A complex to counteract ROS production in vivo. Here, a biomaterial strategy to directly deliver CD-A complex consisting on the use of synthetic polymer Poly(Vinyl Alcohol) PVA in combination Dextran, a natural polysaccharide was tested to achieve this objective. This study targeted a specific I/R pathology: Myocardial Infarction. An antioxidant-patch system is addressed.

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Résumé

Nous avons développé, en collaboration étroite avec le Pr P. Matricardi, des hydrogels élastiques à base d'alcool polyvinylique et dextrane (PVA/Dex) afin de libérer localement l'astaxanthine.

Ces biomatériaux ont été obtenus grâce à un procédé de congélation et décongélation. Les effets du poids moléculaire du PVA, du nombre de cycles de congélation-décongélation et de l'addition de Dex ont été étudiés par caractérisation rhéologique et dynamo-mécanique.

Les PVA145/Dex 1FT (Freeze-Thawing) et PVA47/Dex 3FT ont montré les meilleures propriétés rhéologiques et mécaniques par rapport aux autres systèmes étudiés, et ont donc été choisis comme les candidats les plus prometteurs. Le complexe CD-A a été chargé dans ces hydrogels, sans en affecter les propriétés mécaniques. Les images ESEM et confocale ont mis en évidence la structure poreuse des hydrogels qui permettrait la libération d'astaxanthine. La non-cytotoxicité des systèmes PVA/Dex/CD-A a été validée après 24 h de mise en contact avec des cellules HUVEC et 3T3. Par ailleurs, le système PVA145/Dex 1FT a été testé dans un modèle sous-cutané de biocompatibilité chez le rat. Enfin, la suturabilité de ces systèmes a été validée *ex vivo.* Les propriétés mécaniques et antioxydantes de ces systèmes permettent d'envisager leur utilisation dans une pathologie liée au stress oxydatif. Un type de patch visant à améliorer la reconstruction tissulaire pourrait donc être envisagé pour un développement futur, en particulier pour réduire le stress myofibrille inflige lors des lésions d'ischémie/reperfusion cardiaques.

Mots clés : Alcool polyvinylique; Dextrane; Astaxanthine; Hydroxypropyl-β-Cyclodextrin; Biocompatibilité.

Abstract

In collaboration with Professor P. Matricardi, we developed elastic hydrogels based on polyvinyl alcohol and dextran (PVA/Dex) aiming to locally deliver the astaxanthin antioxidant.

These biomaterials were obtained through freeze thawing (FT) process. Extensive rheological and dynamo-mechanical characterization of the effect of the PVA molecular weight, number of freeze-thawing cycles and Dex addition on the mechanical properties of the resulting hydrogels, were carried out. Hydrogel systems based on PVA 145kDa and PVA 47kDa blended with Dex 40kDa, showed the best rheological and mechanical properties and were thus selected as the most promising candidates. CD-A complexes were loaded within the hydrogels without affecting their mechanical properties. ESEM and confocal imaging evidenced a porous structure of the hydrogels allowing astaxanthin release. The no-toxicity of the PVA/Dex/CD-A system was validated after 24 h of HUVEC and 3T3 cells supplementation. Additionally, the PVA145/Dex 1FT hydrogels biocompatibility was tested *in vivo* using a model of subcutaneous implantation. Finally, the suturability of the system was validated *ex vivo*. The mechanical and antioxidants properties of these systems enable to envisage their use in an oxidative stress related pathology. A patch aiming to improve tissue reconstruction could be considered for a future development, as myocardial patches to reduce the myofibril stress induced after cardiac ischemia and reperfusion injury.

Keywords: Polyvinyl alcohol; Dextran; Astaxanthin; Hydroxypropyl-β-Cyclodextrin; Biocompatibility.

Abbreviations:

PVA: Polyvinyl alcohol

Dex: dextran

PVA/Dex: hydrogels based on PVA and dextran obtained by freeze-thawing

CD: Hydroxypropyl-β-Cyclodextrin

CD-A: Hydroxypropyl-β-Cyclodextrin Natural Astaxanthin complex

ROS: Reactive oxygen species

FT: Freeze-thawing

1. Introduction

Myocardial infarction (MI), is caused by the necrosis of the heart muscle, which is induced by the occlusion of one or more of the vessels in charge of the blood and oxygen supply. Reperfusion response carry out a burst of reactive oxygen species (ROS) which induce an imbalance in the regular cell environment, blocking the tissue recovery and damaging the muscle wall [1]. One complication, in case of a severe muscle damage, is the heart failure post-MI [2]. Up to date, early reperfusion therapy and heart transplantation are available treatments to MI, however induction of secondary reactions remains a concern. Therefore, ameliorating the interaction between heart viable tissue and the necrotic zone is still one of the most challenging issues in cardiac tissue engineering.

Antioxidants-like carotenoids have been investigated because of their capability to moderate the damaging effects of ROS [3–5]. Astaxanthin, a FDA approved lipophilic naturally derived xanthophyll carotenoid, has been tested as ROS regulator in regard to its antioxidant and anti-inflammatory properties. Several studies evaluate astaxanthin protective effect in cardiovascular pathologies after oral or intravenous supplementation using animal models or in clinical trials [6–8]. More interestingly, cyclic oligomers like hydroxypropyl-β-cyclodextrins (CD) have been suggested as a solubility enhancer of hydrophobic molecules such as astaxanthin [9]. In order to deliver this antioxidant molecule *in situ* in the myocardial tissue, the selected cardiac engineering material must reflect the mechanical and biological properties of the host tissue; while being biodegradable, easy to handle [10], with sufficient tensile strength and presenting elasticity properties similar to cardiac muscle [11,12]. Furthermore, it must have the capacity to enhance cell functioning without inducing an inflammatory response or rejection [13]. Hydrogel-based materials have been successfully used in cardiac engineering because of their interesting tissue-like properties. Polyvinylalcohol (PVA), due to its good biocompatibility and versatility, is one of the hydrogel-forming polymers most used in advanced biomedical applications. Its high-water content and tissue-like elasticity makes it a useful carrier in the present application. Moreover, thanks to its good biocompatibility and bioelimination, dextran (Dex) can be blended to PVA solutions in order to modulate the mechanical and physico-chemical properties of PVA hydrogels, showing a similar behavior like biological soft tissues [14,15]. Particularly, PVA based hydrogels have been used as cardiovascular grafts [16–18].

Here, we aimed to obtain a material with suitable physico-chemical and mechanical properties to be posteriorly used as a myocardial patch for the local delivery of the antioxidant astaxanthin. This PVA/Dex/CD-A patch will allow the stabilization of the oxidative stress state by blocking

ROS and the reduction of the myofibril stress generated after MI by improving the inner heart environment for repair and recovery of myocardial tissue functionalities.

2. Materials and Methods

2.1. Materials

PVA 145k (Mowiol® 28-99, Mw 145 kDa, 99.0% deacetylated), PVA 47k (Mowiol® 6-98, Mw 47 kDa, 98.0% deacetylated), Dextran 40k (Mw 40 kDa), Fluorescein isothiocyanate–Dextran (FITC-Dex Mw 40 kDa), Natural astaxanthin, hydroxypropyl-β-cyclodextrin (DS=0.67), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), (2,2-azobis(2-amidino-propane) dihydrochloride (AAPH, Ref. 440914), fluorescein (Ref. F6377), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), were provided by Sigma-Aldrich Co. LLC (Saint-Louis, MO, USA). Alexa Fluor-phalloidin was provided by Molecular Probes. Human umbilical vein endothelial cells (HUVEC, CRL 1730) and CCl 163 (Balb/3T3 clone A31) were purchased from ATCC-LGC Standards S.a.r.l. (Molsheim Cedex, France). Medium-L-Glutamine (MEM), fetal calf serum (FBS), penicillin–streptomycin-amphotericin (PSA), Phosphate Buffered Saline (PBS), were all purchased from GIBCO (Life technologies, Carlsbad, CA, USA). EnVision Dako kit (ref K4063 and ref K4010), Dako REAL peroxidase-blocking and Negative Control mouse IgG1 solution, were supplied by Dako (Dako, Carpinteria, CA, USA and Dako, Glostrup, Denmark). Mouse anti rat CD68 and CD163 primary antibodies were obtained from Bio-rad (MCA341A488, Marnes-la-Coquette, France). All other reagents, including chemical reagents were of analytical grade and used as received without any further purification. Double distilled and deionized water was used.

2.2. Methods

2.2.1. HPBCD/Astaxanthin complex preparation: HPBCD/Astaxanthin complex (CD-A) was prepared following the procedure describe elsewhere [19]. Briefly, natural astaxanthin (1 mg) in acetone/chloroform (v/v 1:1) solution (1 mg/mL) was added to 250 mg of CD previously dissolved under nitrogen in 12.5 mL of 95% methanol (20 mg/mL) in a 25 mL flask. The mixture was then sonicated for 5 min (Ultrasonic bath BANDELIN SONOREX RX-100-H) and stirred for 24 h at 35 ° C in a dark chamber; next, the solvents were removed by vacuum and the solid was recovered using double distilled water. The final solution was freeze-dried and stored at -4 °C. The water content of the solid CD-A was 12% w/w (by TGA, Mettler TG 50 thermobalance).

2.2.2. Hydrogels preparation: Hydrogels with a polymer composition of PVA 15% (w/w) and Dex 5% (w/w) were prepared. In a typical preparation, PVA (1.5 g, M_w 47k or M_w 145k) was suspended in 8 g of distilled water. The suspension was magnetically stirred for 2 h at 70 °C, then autoclaved for 20 min at 121 °C. 0.5 g of Dex were next added to the PVA solution and mixed at 70 °C for 30 min. To prepare PVA/Dex/CD-A hydrogels, a slightly different procedure was followed: in a typical preparation, 1.5 g of PVA (M_w 47k or M_w 145k) was suspended in 7.9 g of distilled water. The suspension was magnetically stirred for 2 h at 70 °C, then autoclaved for 20 min at 121 °C. 100 mg of CD-A and 0.5 g of Dex were then added to the PVA solution and mixed at 70 °C for 30 min. Finally, to prepare a blank hydrogel, i.e. without astaxanthin, the same procedure was followed but CD was added.

The polymer suspension was poured into 1 mm thickness mold and subjected to one to three freeze/thawing cycles (each cycle: 16 h at -20 °C and then 5 h at 20 °C). Mechanical characterizations were carried out on hydrogels prepared using the same procedure, but using specimens prepared in molds with different thickness: 2 mm for rheological characterizations and water uptake experiments, whereas 6-7 mm for uniaxial compression tests.

For the confocal microscopy, the different hydrogels were prepared using the same procedure, but FITC-Dex was added at final concentration 2 mg/g during the Dex adding. In the case of PVA 47k 3FT and PVA 145 1FT 0.005 % (w/w) of Dex FITC was added to the solution before poured into the mold and being subjected to FT process. All the samples prepared are summarized in Table 1.

2.3. Hydrogel characterization

2.3.1. Rheological measurements: Rheological experiments in shear regime were carried out by means of a controlled stress Haake RheoStress 300 Rotational Rheometer, equipped with a Haake DC10 thermostat. For the tests on the hydrogels, a cross-hatch plate device (Haake PP35 TI: diameter=35 mm) was adopted, in order to reduce the extent of the wall slippage phenomena [20]. The hydrogels, with a thickness of about 2 mm and a radius of 4 cm, were removed with the aid of a small spatula from the mold where they were prepared and laid with care on the lower plate of the rheometer. The upper plate was then lowered until it reached the hydrogel surface. Gap setting optimizations were undertaken according to the procedure described elsewhere [21]. Mechanical spectra of the hydrogels (frequency sweep experiments) were recorded at 25 °C in the range 0.01-10 Hz, in the linear viscoelastic regime, applying a constant deformation (γ=0.01), preliminarily assessed by stress sweeps experiments, performed at 25 °C and 1 Hz in the range 0.01-30 Pa. Flow curves of the solutions were obtained by using

a cone-plate geometry (Haake CP60Ti: diameter = 60 mm; cone = 1°; gap = 0.053 mm) in the range 0.001–1,000 s^{-1}. A stepwise increase of the stress was applied, with an equilibration time of 30 s. All measurements were carried out at 25 °C.

2.3.2. Uniaxial compression experiments: A software-controlled dynamometer, TA-XT2i Texture Analyzer (Stable Micro Systems, UK), equipped with a 5 kg load cell, was used for the uniaxial compression experiments. The samples, prepared as described above, were tested at room temperature, using an ebonite cylinder probe (P/10, diameter of 10 mm). The pre-test speed, the test speed and the post-test speed were set up at 2, 1 and 2 mm/s, respectively; the trigger force was set up at 0.005 N. Stress-Deformation curves, $\sigma = f(\gamma)$, were obtained, where: $\sigma = F/A$ (N/m^2); $\gamma = [(h_0 - h)/h_0] \times 100$; h_0 = initial height of the sample, h = final sample height, F = compression force (N) and A = cross-section area of the probe (m^2). The Young's modulus (E) was determined as the slope of the curve in the linear range of small deformations.

2.3.3. Needle penetration and suturability experiments: The needle penetration and suturability tests were performed with the TA-XT2i Texture Analyzer. For the needle penetration experiments, the samples, with a thickness of 1 mm, were tested at room temperature, using a steel cylinder probe connected to a syringe needle (27G) (Figure S1-a). The pre-test speed, the test speed and the post-test speed were set up at 2, 1 and 2 mm/s, respectively; the trigger force was set up at 0.005 N.

For the suturability tests, a modified procedure described by Barber *et al.* [22], i.e a resistance test in the tear of the suture thread, was adopted. The hydrogels were cut into strips (2 cm wide and 5 cm long); each specimen had a 5 mm horizontal mattress suture (3-0 Dexon II) placed 5 mm from the edge (Figure S1-b). The low side of the specimen was held with grips to prevent slippage. The suture ends were tied at the arm of testing machine, allowing the suture to be freely oriented in the vertical plane. The tensile suture measurements were carried out at room temperature: the pre-test speed, the test speed and the post-test speed were set up at 1, 0.1 and 1 mm/s, respectively; the trigger force was set up at 0.005 N. The ultimate suture load (Force at break, N) and displacement (mm) were recorded.

2.3.4. ESEM: Hydrogels morphology (5 mm diameter, 1 mm thickness) in their hydrated state were analyzed by environmental scanning electron microscopy (ESEM) using an Philips XL 30 ESEM-FEG (Eindhoven, The Netherlands) at an accelerating voltage of 15 keV at a pressure of 4 T. Hydrogels, after their preparation, were gently wiped using an absorbing paper and put on

the sample holder in order to analyze the hydrogel structure, samples were scanned horizontally (hydrogel surface) and after transversal cut (internal structure).

2.3.5. Confocal Microscopy: Optical sections of the PVA and PVA/Dextran hydrogels labelled with FITC-Dex (5 mm diameter, 1 mm thickness) were acquired using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Germany), equipped with a 10X Plan-NeoFluar objective lens (numerical aperture 0.3). FITC-labelled Dex was excited at 488 nm with an argon laser and its fluorescence emission selected by a 505–530 nm bandpass filter. 3-D visual images of confocal data were created using Amira® 3.0 software (Mercury Computer Systems Inc., Chelmsford, MA).

2.3.6. Water uptake/degradation experiments: The water uptake/degradation behavior of the hydrogels was determined gravimetrically. Five specimens of each hydrogel (thickness 2 mm) were prepared by cutting in rectangular aliquots (about 2 cm^2, corresponding to about 600 mg accurately weighted) and prepared according to the procedure described above; the starting weight of each aliquot (w_0) was recorded; then each specimen was immersed in 10 mL of PBS 0.1M, pH 7.4, and stored at 37 °C. At prefixed times, the supernatant was removed and samples were weighted (w_t). The water uptake value Q was evaluated by $(w_t-w_0)/w_0$. The different aliquots were then freeze-dried, and their dry weight (w^d_t) was compared with the dry weight of the fresh sample (w^d_0) to determine the percentage of solid residual.

2.3.7. Astaxanthin release: The CD-A loaded hydrogels (2 mm of thickness) were prepared according to the procedure described above and were then cut in rectangular samples (about 2 cm^2, corresponding to 600 mg accurately weighted). The samples were then immersed in 4 mL of PBS 0.1M, pH 7.4 at 37 °C; at fixed time intervals, the release medium was collected, freeze-dried and then suspended in 1 mL of dichloromethane/methanol 1/1. This suspension was centrifuged (4,000 rpm, 10 min) and the astaxanthin concentration in the supernatant was analyzed by HPLC. HPLC analyses were performed with a Eurospher II C18 column (250 mm x 4.6 mm, 5 μm particle size, Knauer) on a Clarity software controlled Knauer system, equipped with a binary pump (Azura Pump P 6.1L, Knauer) and a spectrophotometric UV/Vis detector (Azura UVD 2.1L, Knauer). The mobile phase was methanol/water/dichloromethane/acetonitrile (70:4:13:13, v/v/v/v). Under isocratic conditions, the analysis was carried out at a flow rate of 1.0 mL/min at room temperature, and the absorbance was monitored at 475 nm. A calibration curve was prepared with CD-A suspended in DCM/MeOH 1/1 and centrifuged (4,000 rpm, 10 min).

2.3.8 Astaxanthin antioxidant evaluation: The antioxidant capacity of PVA47/Dex/CD-A 3FT and PVA145/Dex/CD-A 1FT was evaluated using the ORAC method [23]. Briefly, PVA hydrogels were previously loaded with a complex astaxanthin concentration of 2.5 μM and immersed into 1 mL PBS solution for 24 h at 37 °C. Solutions of fluorescein (4 nM), AAPH (160 μM) and Trolox (0–5 μM, antioxidant standard reference) were prepared in PBS. Fluorescein solution (150 μL) was added to each well (96-well microplate), and then 25 μL of samples or blank (PBS) were distributed in the wells, prior to the addition of AAPH (25 μL). Fluorescence was monitored at 485 and 528 nm (excitation and emission wavelengths) at 37 °C every minute for 60 min (3 × 8 measurements par point). The area under the curve (AUC) of relative fluorescence was calculated. ORAC values were expressed as Trolox equivalent in μM and calculated as the slope ratio from curves, AUCnet, versus the concentration of antioxidant and Trolox, respectively (AUCnet = AUCsample − AUCblank).

2.3.9. Cell culture: Human umbilical vein endothelial cells (HUVEC) and mousse fibroblast cells (3T3) were grown in minimum essential Medium-L-Glutamine (MEM), supplemented with 10% (v/v) fetal calf serum (FBS) and 1% penicillin–streptomycin-amphotericin (PSA). Cells were seeded in a T75 cell culture flask (Corning) and kept in a humidified incubator containing 5% CO_2 at 37 ^{0}C. The culture medium was replaced twice every week and the cells were split 1:3 every week.

2.3.10. Cell viability and their morphology after hydrogels contact: Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, HUVECs or 3T3 cells density of $10x10^4$ cells/well were seeded and cultured overnight. MEM was washed with PBS and fresh MEM was added. Next, disks of 1 mm thickness (60 mg) of PVA/Dex, PVA/Dex/CD or PVA/Dex/CD-A were carefully placed in contact with cells in the center of the individual wells and incubated during 24 h. Two controls were carried out for all experimentations: Negative Toxicity Control (NTC, DMEM medium) and Positive Toxicity Control (PTC, 10 % DMSO in DMEM). Afterwards, all the solutions and hydrogels were discarded and cells were incubated during 3 h at 37 °C with 200 μL of MTT solution (0.5 mg/mL). Then, MTT solution was washed out and 200 μL of isopropanol were added for 20 min to solubilize the formazan crystals. The optical density was recorded at 570 nm (i-controlTM microplate reader software, TECAN Männedorf, Switzerland). No cytotoxicity of samples was considered if cellular viability was > 70% compared to PTC (based on the ISO 10993).

To evaluate cell morphology, HUVEC cells ($30x10^4$ cells/well) were cultured in Lab-Teck plates for 24 h. MEM, or 24 h release products (refer to section 2.3.7) from PVA47/Dex 3FT and PVA 145/Dex 1FT were added to the wells during 24 h. Then, cells were stained with Alexa Fluor-phalloidin to highlight the cytoskeleton and visualized under fluorescence microscope (Leica Biosystems, France).

***2.3.11. In vivo and ex vivo experiments*:** The animal study was carried out under the approval of the Bichat University Institutional Animal Care and Use Committee. Adults Male Wistar rats weighing 240-250 g (ws/ws, purchased from Charles-Rivers, France) were anesthetized by intraperitoneal injection of sodium pentobarbital solution (30 mg/kg, Centravet, France). Animals were divided according to selected conditions: PVA145/Dex 1FT, PVA145/Dex/CD 1FT, or PVA145/Dex/CD-A 1FT, or sham (same surgical procedure without material implantation). Four abdominal pockets of approximately 1x1 cm were made on both left and right sides of the rat abdominal midline and each group received subcutaneous implantation of PVA-based disks (5 mm diameter, 1 mm thickness) per pocket. The muscle was immediately sutured using VicrylIVR 6.0 (Ethicon) and the skin with VicrylIVR 4.0 (Ethicon). Rats were euthanized by an intraperitoneal injection of sodium pento-barbital (60 mg/kg) at day 8 post-surgery after tissue excision. Tissue was rinsed gently in saline solution, fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Sections of 4 µm thickness were obtained using a microtome (HM 355S microtome, Thermo scientific Waltham, MA, USA). Tissue morphology was visualized using hematoxylin-eosin staining and the total phagocytes and type-2 macrophages were identified by anti-CD68 and anti-CD163 antibodies labeling, respectively (1/30 working dilutions). Digital images were obtained and analyzed using Nanozoomer digital pathology software (Hamamatsu, Japan).

In order to verify the PVA145/Dex 1FT patch suturability on a rat heart, an *ex vivo* suture test was carried out as follows: the rat heart was exposed through a median sternotomy, and PVA145/Dex 1FT hydrogel (diameter 5 mm, thickness 1 mm) was sutured using a VicrylIVR 7.0 (Ethicon).

2.3.12. Statistical analysis: All experiments were repeated at least three times to ensure the reproducibility of each test. Results were expressed as the mean ± SDE and statistical analysis was done using one-way ANOVA followed by Tukey's HSD *post hoc* test. (JMP Software,

Version 9; SAS Institute, Cary, NC, USA). Results were considered to be significantly different if *p*-value < 0.05.

3. Results

3.1 PVA/Dex hydrogels preparation and CD-A loading

Preliminary experiments were carried out on a wide number of hydrogels obtained by varying different parameters, such as: deacetylation degree of PVA, molecular weights of PVA and Dex, polymer ratios and concentrations, number of freeze-thawing cycles. Finally, two M_w of fully deacetylated PVA (47 and 145 kDa) and one M_w of dEX (40 kDa) were selected as a good compromise between the mechanical properties and non-toxicity [24,25]. As a following step, a restricted set of experimental conditions were then chosen for a deep analysis of the biological and mechanical behavior of the system. The prepared samples are summarized in Table 1. The two selected formulations, PVA47/Dex 3FT and PVA145/Dex 1FT appeared to be the most promising formulations from the mechanical point of view and their ease handling.

Astaxanthin is a very lipophilic molecule; to improve its water solubility thus improving the homogeneity in dispersion in the above hydrogels, a complex with CD was formed without altering the astaxanthin properties [26].

Sample	PVA 15 % (w/w)		Dex40K 5 % (w/w)	FT cycles			CD	CD-A
	PVA47K	PVA145K		1	2	3		
PVA47/Dex 1FT	*		*	*				
PVA47/Dex 2FT	*		*		*			
PVA47/Dex 3FT	*		*			*		
PVA47/Dex/CD 3FT	*		*			*	*	
PVA47/Dex/CD-A 3FT	*		*			*		*
PVA145/Dex 1FT		*	*	*				
PVA145/Dex 2FT		*	*		*			
PVA145/Dex 3FT		*	*			*		
PVA145/Dex/CD 1FT		*	*	*			*	
PVA145/Dex/CD-A 1FT		*	*	*				*

Table 1: Acronyms and compositions of the different tested samples.

3.2. Rheological and mechanical characterization of samples

In order to study the effect of the M_w of PVA, freeze-thawing cycles number and Dex on the mechanical properties of the different hydrogels, an extensive rheological and dynamo-mechanical characterization was carried out.

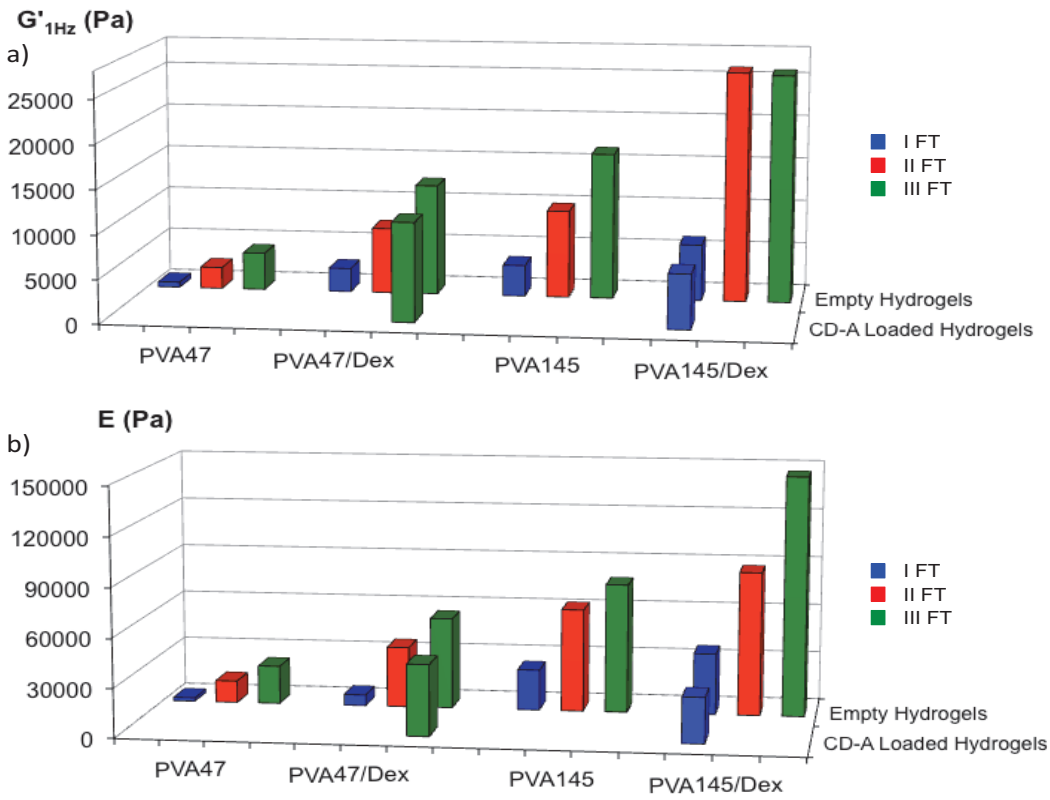

Figure 1: PVA-based hydrogels characterization. a) Elastic modulus (G') values, measured at the frequency of 1Hz, and b) Young modulus values for PVA47k, PVA47k/Dex, PVA145k and PVA145k/Dex and CD-A loaded hydrogels at 25°C. All the measurements were carried out at 25°C.

The starting solutions (PVA 15% w/w, both 47k and 145k, and Dex 5% w/w) and blends (PVA47/Dex and PVA145/Dex) showed the typical shear thinning behavior of polymer solutions (Figure S1). Shear flow experiments showed that the rheological properties are not additive; adding Dex to both samples of PVA led to a not additive increase of the viscosity at *quasi* zero shear. In Figures S2-S5 mechanical spectra of starting solutions, blends and hydrogels (1, 2 and 3 FT cycles) were also reported. The solutions and blends showed the typical behavior of concentrated polymer systems. The hydrogels, prepared according to the procedure described in the experimental part, instead showed a strong gel behavior (G' more than one order of magnitude larger than the viscous modulus G'', and both moduli essentially independent of frequency over the range 10^{-2}-10 Hz). The mechanical properties of the different hydrogels were

also tested in uniaxial compression experiments, to determine their Young modulus values. The elastic moduli G' (obtained at 1 Hz by means of frequency sweep measurements) and the Young moduli E (obtained by means of uniaxial compressions) are summarized in Figure 1a-b. The CD-A loaded samples (PVA47/Dex/CD-A 3FT and PVA145/Dex/CD-A 1FT) were also prepared for both rheological and dynamo-mechanical characterization: the effect of the loaded drug on the mechanical properties resulted to be negligible.

3.3. Water uptake/degradation experiments

In Figure S8, the water uptake/degradation behaviors of the different samples are reported. During the first hours, the water uptake capability of the samples resulted to be dependent on the FT cycles and on the Dex content: samples PVA47 1FT and PVA145/Dex 1FT, with a lower crosslinking density, showed a higher water uptake in comparison with the corresponding PVA47 3FT and PVA145/Dex 3FT. Moreover, the presence of Dex within the hydrogelS led to the formation of a more hydrophilic network able to absorb a higher water amount. Nevertheless, the degradation kinetics of PVA hydrogels containing or not Dex were very similar, showing a fast degradation during the first 5 days and a constant weight loss for 25 days. In Table 2, the percentage of residual solid is reported. The obtained data show that PVA47 hydrogels, even if more cross-linked, have a lower residual solid, at the end of the experiments, when compared to the PVA145 samples.

Sample	8 days	16 days	22 days	30 days
PVA47 3FT	60 ± 3	44 ± 2	43 ± 3	42 ± 2
PVA47/Dex 3FT	55 ± 4	45 ± 1	37 ± 5	35 ± 5
PVA145 1FT	70 ± 2	58 ± 4	56 ± 1	53 ± 3
PVA145/Dex 1FT	65 ± 5	53 ± 3	51 ± 3	47 ± 2

Table 2: Residual solid (% by weight) of the different samples in water uptake/degradation experiments (PBS 0.1M pH 7.4, 37°C).

3.4. Astaxanthin loading and release

To determine the release properties of the prepared hydrogel systems, the astaxanthin release profiles from PVA47 and PVA145 in water environment were collected. CD-A was loaded within the various hydrogels and the release profiles of CD-A from two hydrogels are reported in Figure 2a. PVA145/Dex 1FT hydrogel showed a faster release profile, in comparison to PVA47/Dex 3FT. The former, formed by the high M_W PVA, released all the loaded astaxanthin over 5 hours, while the latter, formed by low M_W PVA 8 h to release 80% of the loaded antioxidant.

3.5 Astaxanthin antioxidant capability evaluation

After 24 h of astaxanthin release from the PVA-based hydrogels, its antioxidant-scavenging activity was evaluated as the capability to retain fluorescein intensity in the presence of AAPH. CD-A (0-5 μM) and Trolox (0–5 μM) were previously used to perform a calibration curve by plotting the area under the curve against sample concentrations (Figure S9). Antioxidant activity was expressed as Trolox equivalent (TE) μM. CD-A calibration curve presented a slope of 1.4 while 0.6 for Trolox, thus representing a scavenging activity of 2.3 μM TE. By analyzing the AUCnet of CD-A release product from the hydrogels after 24 h, a similar CD-A AUCnet value was obtained for both PVA47/Dex/CD-A 1FT and PVA145/Dex/CD-A 3FT (Figure 2 b). CD-A activity at a concentration of 2.5 μM was the same before and after loading within the hydrogels, indicating the release of the molecule and the preservation of its antioxidant activity.

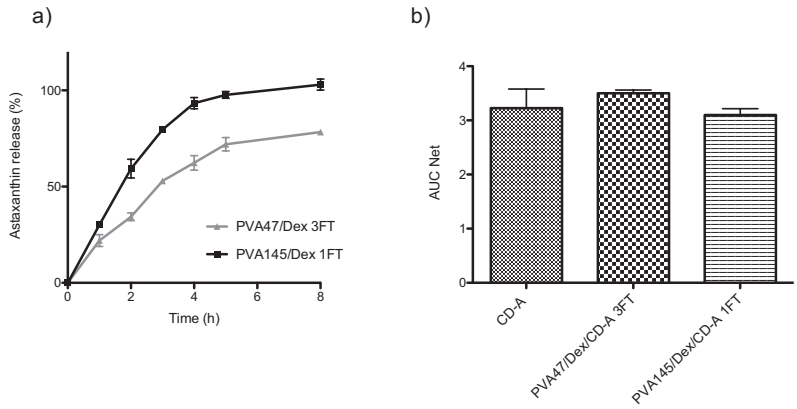

Figure 2: (a) CD-A release from PVA47/Dex 3FT and PVA/Dex 1FT hydrogels in 8 h (PBS 0.1M pH 7.4, 37°C). (b) Antioxidant scavenging capacity of the release environments by PVA47/Dex/CD-A 3FT and PVA145/Dex/CD-A 1FT loaded with an astaxanthin concentration of 2.5 μM expressed by means of AUC net calculated by the ORAC method. All tests were performed in triplicate and the obtained values always lay within 10% of the mean.

3.6. Hydrogel morphology

The various samples prepared were characterized in terms of their morphology. Environmental scanning electron microscopy (ESEM) of the hydrogels revealed that even if the external surface of PVA47/Dex 3FT and PVA145/Dex 1FT appeared to be very similar, internally, a heterogeneous interconnected porous structure was present (Figure 3 a-h), suggesting that Dex and CD act as porous agents. Particularly, samples based on the lower PVA M_w, showed a pore diameter in the range 11-26 μm, and the higher PVA molecular weight presented a pore diameter in the range 4-12 μm. It is interesting to note that when adding either CD or CD-A, the

porosity of both the PVA47/Dex 3FT and PVA145/Dex 1FT increases, creating a more heterogeneous porous distribution when compared with the no drug loaded hydrogels (Figure S10).

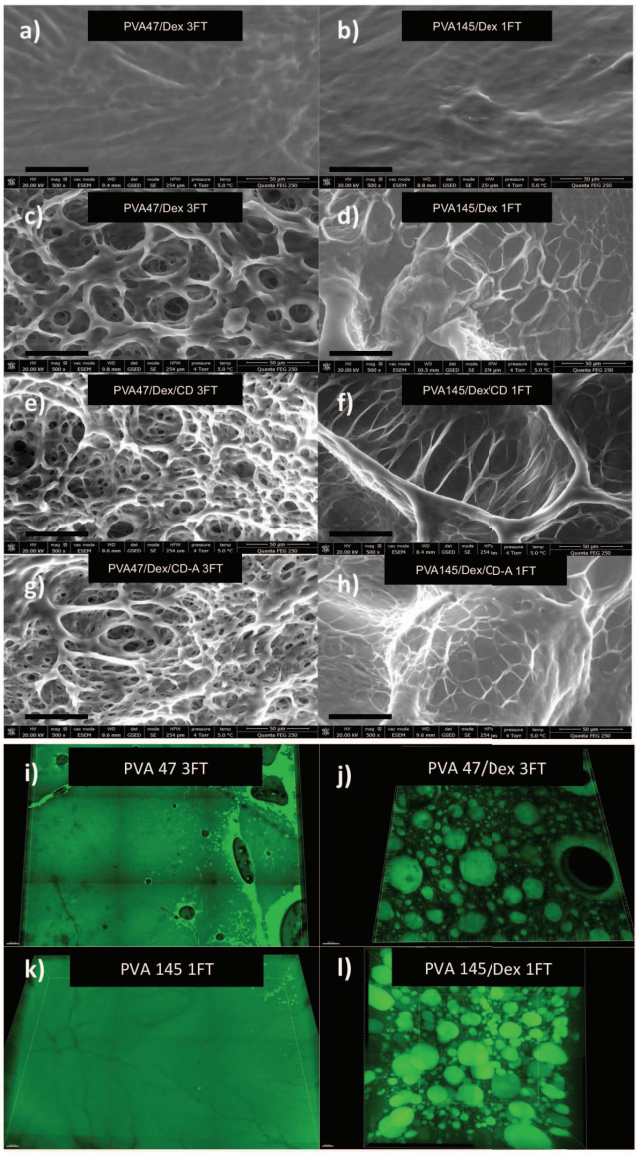

Figure 3: ESEM and Confocal analysis of PVA based hydrogels, (a-h) ESEM micrographs of: (a) PVA47/Dex 3FT and (b) PVA145/Dex 1FT, horizontal surfaces. (c-h) Transversal images reflecting the internal structure of the different hydrogels conditions. Scale bars: 50 μm and magnification x500. (i-l) Confocal microscopy images of PVA hydrogels surface structure of (i) PVA47 3FT and (k) PVA145 1FT and internal structure of (j) PVA47/Dex 3FT, and (l) PVA145/Dex 1FT.

To confirm these results, the same polymer systems were analyzed by confocal microscopy, using Dex-FITC (M_w 40 KDa) as florescent probe. The hypothesis is that this polymer distributes in the overall matrix as Dex, thus evidencing, by fluorescence, the mixing or the demixing of the two polymer systems used, i.e. PVA and Dex. At this point, we decided to use Dex and Dex-FITC, having the same molecular weight; in these conditions, it can be assumed that the probe behaves as Dex chains, thus dissolving at the same rate and at the same extent in the various phases of the blend.

Confocal images reported in Figure 3 i-l, for PVA47 1FT and PVA145 3FT showed a morphology quite similar to that obtained by ESEM. In the present images, the hydrogels showed a homogeneous surface and a heterogeneous structure in the inner part, due to the dispersion of Dex in PVA47 3FT and PVA145 1FT. In PVA47 and PVA145 the Dex-FITC was distributed homogeneously, as expected in blends of PVA and Dex at low concentration [5, 22].

3.7. Needle penetration and *ex vivo* suturability experiments

The ability of the hydrogel patches to be sutured was evaluated by simulating the suture using a penetration test with a needle (30G). The obtained data (reported in Figure S7) showed an increase of the maximum force (N) required for the needle penetration as the molecular weight of PVA and the FT cycles increased. The same trends of data are obtained by adding Dex (5% w/w) to PVA. The samples PVA47 (1, 2 and 3 FT) resulted to be too weak and not suitable to be tested (the force that should be used was below the instrument's sensitivity). The samples were also tested for suture retention strength: one critical property for these materials is the adequate strength to withstand mechanical solicitations at the suture-patch interface. In Figure 4a, the ultimate suture load (N) and deformation are reported for the different samples (PVA47/Dex 3FT and PVA145/Dex 1FT). Here, PVA145/Dex 1FT showed the best mechanical performances (Young modulus in between 30-150 kPa, high elasticity and biocompatible). As displayed in Figure 4b-e, the *ex vivo* suturability test showed that PVA145/Dex 1FT was easily sutured to the heart myocardium without complications.

a)

Sample	Ultimate suture load (N)	Ultimate suture displacement (mm)
PVA47/Dex 3FT	0.081 ± 0.007	2.3± 0.2
PVA145/Dex 1FT	1.232 ± 0.021	38.7± 2.3

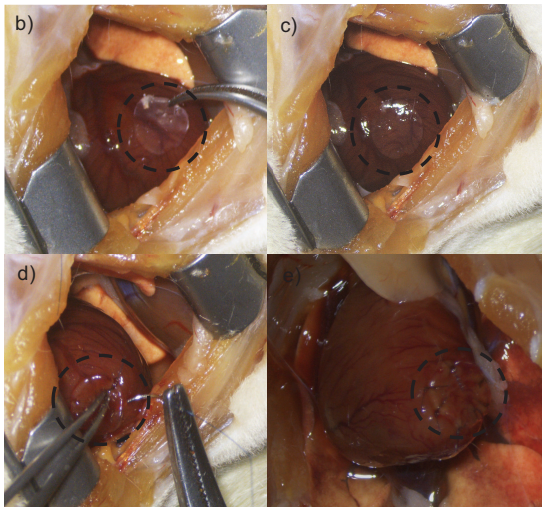

Figure 4: (a) Ultimate suture load (N) and displacement (mm) for the different samples. Each specimen (2 cm wide and 5 cm long) had a 5 mm horizontal mattress suture (3-0 Dexon II) placed 5 mm from the upper edge. (b-e) *Ex vivo* suturability assay of the PVA145/Dex 1FT on rat heart performed with suture7.0 (Ethicon SAS).

3.8. *In vitro* toxicity evaluation

In vitro cell compatibility of samples was analyzed using two cell lines: (i) a mouse fibroblast cell line (3T3), proposed by the ISO N°10993.1-12 for the biological evaluation of medical devices due to its high sensitivity; (ii) a human cell line representing the cardiovascular system (HUVEC) [27], after 24 h of cell-hydrogels contact. As shown in Figure 5, all samples did not significantly decrease the cellular viability up to 70%, compared with PTC, showing a good biological acceptance of the material by the cells. Moreover, as illustrated in Figure 6 the HUVEC cell morphology was not modified after PVA47/Dex/CD-A 3FT and PVA145/Dex/CD-A 1FT patches exposure.

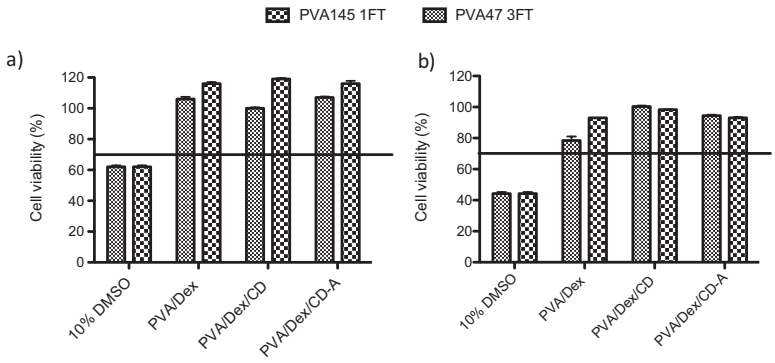

Figure 5: *In vitro* compatibility study of (a) HUVEC and (b) 3T3 cell lines after being exposed to the hydrogels for 24 h. All conditions are significantly different when compared to control ($p < 0.01$). Results are mean ±SEM (n=3).

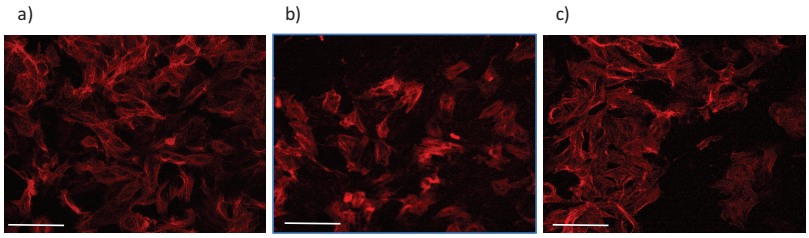

Figure 6: Fluorescence microscopy images of HUVEC stained with Alexa Fluor-phalloidin and cultured with (a) MEM, or release products from (b) PVA47/Dex/CD-A 3FT, or (c) PVA145/Dex/CD-A 1FT, during 24 h.

3.9. *In vivo* compatibility

Any sign of inflammation was noted externally in the animal tissue during the entire evaluation time. After 8 days post-implantation, PVA145/Dex 1FT, PVA145/Dex/CD-A 1FT did not undergo complete degradation as observed in the hematoxylin-eosin stained sections (Figure 7). Further, the hydrogels remained in the subcutaneous pocket, without presenting cell colonization.

Macrophages are heterogeneous cell populations that can rapidly adapt their function in response to local microenvironmental signals [28] and are subtyped according to their functionality as M1 or pro-inflammatory and M2 or tissue repair macrophages [29]. In order to evaluate the macrophages response generated by the implantation of foreign biomaterials such as the PVA patches, two immunohistochemical markers were used. CD68 antigen positivity is attributed to a classic sign of inflammation [30] and is expressed in both M1 and M2 populations; while CD163 represents a specific marker for M2 macrophages phenotype [31]. A standard foreign body reaction constituted by macrophages localized around the hydrogels was observed after CD68 immunolabelling in both PVA145/Dex 1FT (Figure 7i) and PVA145/Dex/CD-A 1FT (Figure 7j). These observations represent a classic reaction to implanted biomaterials [32,33].

Concerning the CD163 marker, a positive staining was noted in all the samples, but the staining spread within the tissue area (Figure 7l-m). Sham samples presented a similar positivity distributed along the tissue using both markers (Figure 7k,n). These results could be attributed to the constructive tissue remodeling process [30,34,35].

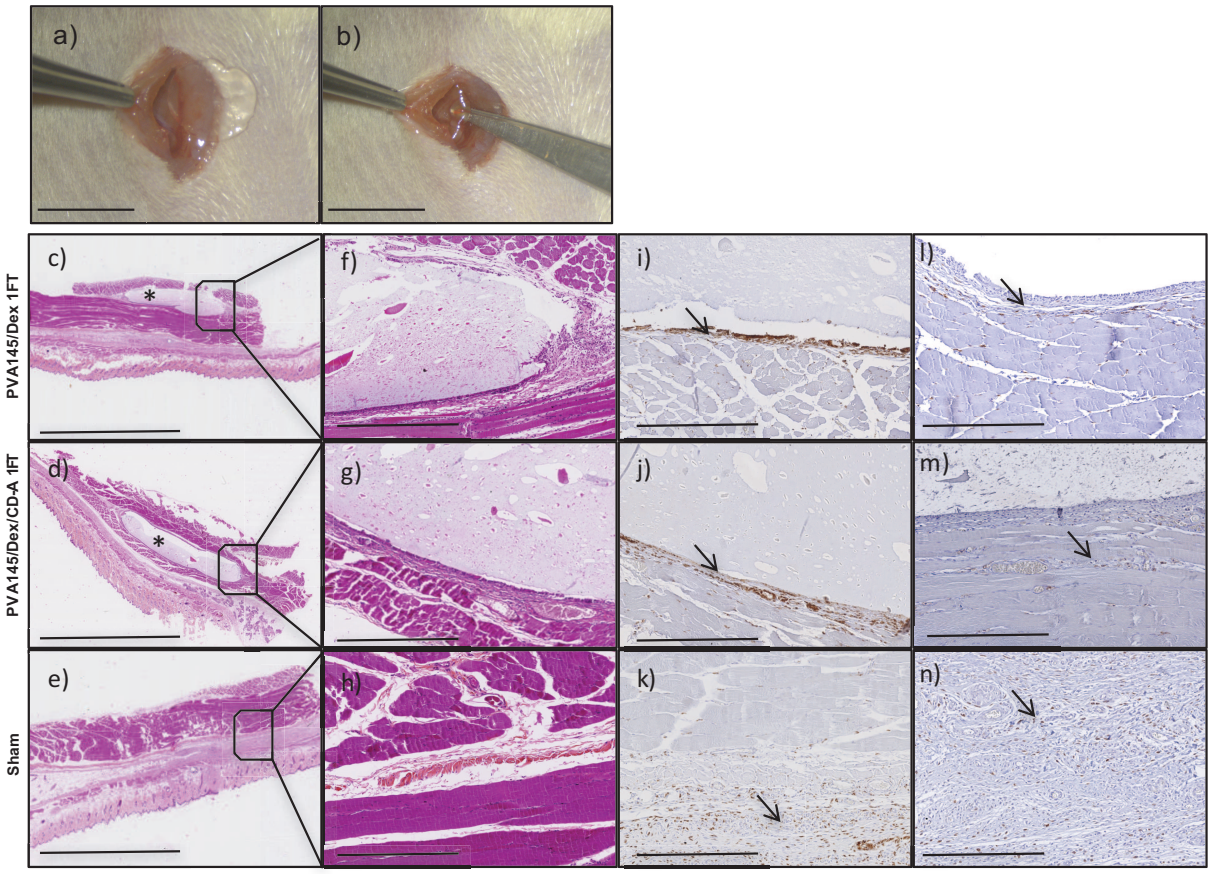

Figure 7: (a, b) In vivo intramuscular pocket was made in the abdomen of the rat allowing the implantation of the selected hydrogel disks (5 mm diameter and 1 mm thickness) and sutured by two stitches (suture 6.0). Note that the hydrogels can be easily handled. Histological analysis of (c,f,i,l) PVA145/Dex 1FT, (b,g,j,m) PVA145/Dex/CD-A, and (e,h,k,n) sham, after one week of subcutaneous implantation in rat. Sections (c-h) were histologically stained with hematoxylin- eosin. (i-k) Immuno-histological CD68 marker was used to assess macrophages infiltration after one week of implantation (black arrows) and (l-m) Immuno-histological CD163 marker was used to assess the attenuation of chronic inflammation after one week of implantation (black arrows). Stars indicate the implanted hydrogel. Scale bars: (a-e) 5 mm; (f-n) 0.4 mm and magnification 10x.

4. Discussion

Improving the myocardial environment after an ischemic event is one of the most challenging issues in cardiovascular tissue engineering. Different polymer systems have demonstrated to be effective in the recovery of the heart mechanical properties after myocardial infarction. According

to theoretical simulations and thanks to their elasticity, these devises could decrease heart wall stress when used as cardiac patches; [36]. However, under ischemic conditions, oxidative stress-status takes place, limiting the recovery of the affected myocardial tissue, leading to post-MI effects. Therefore, the local delivery of an antioxidant molecule to regulate ROS levels through the use of an elastic hydrogel could represent an interesting strategy. This system could act simultaneously, as a myocardial patch bringing mechanical stability and support to the non-ischemic heart muscle area, allowing a long-term elasticity, required to support the constant and cycling movements of the myocardial tissue. In the present work, a PVA/Dex hydrogel matrix was developed aiming to reduce the oxidative stress status by the delivery of natural astaxanthin, a potent antioxidant extracted from microalgae.

As extensively reported in the literature, when PVA aqueous solutions are subjected to repetitive freezing–thawing cycles, without using chemical crosslinking agents and reagents, a polymer crystallization can result in an ordered hydrogel structure. This property can be investigated to prepare hydrogel systems with a potential to be used as cardiac patches, due to their mechanical properties and biocompatibility. Cascone *et al.* [37], studied the enhancement in the crystallization process of PVA due to the presence of Dex, allowing the formation of a more ordered and homogeneous structure. Moreover, the absence of modification on PVA melting temperature (Tm), suggests that no major changes occur in the nature of PVA crystallites due to the presence of the polysaccharide. On the contrary, the biopolymer is able to modulate the PVA crystalline degree, which increases as the Dex content increases. In this respect, Fathi *et al*. [14] showed that, the Dex content did not significantly affect the Tg of PVA/Dex hydrogels, whereas crystal size distribution and channeled morphology were evidenced for PVA/Dex samples of higher Dex contents. As observed by ESEM experiments carried out in the present work (Figure 3) this porosity is due to the partial immiscibility of Dex and PVA in solution, when the two polymers are blended at high concentrations [14,38], agreeing with previous reported data [14]. Additionally, the confocal images confirmed the porogenic behavior of Dex solutions. In this case, the different morphology of the surfaces and the inner part of the hydrogels can be explained in terms of a combination of the sample treatment before the experiment and the interfacial tension air/sample.

The rheological characterization of the hydrogels in shear flow experiments showed a not additive increase of the viscosity, when mixing Dex to PVA solutions. These results could be attributed to the contemporary effect of the polymers concentration increase and the formation of micro-droplets of Dex within the PVA solution. At the same time, an enhancement of the elastic and Young modulus was observed by increasing the molecular weight of PVA and the number of

FT cycles. Further, the presence of Dex within the hydrogel led to an increase in the mechanical properties, probably due to a polymer concentration effect and to the increase in the FT cycles, which resulted in the formation of a strongest hydrogel as already reported by [39]. As reported by other authors, human myocardium presents a Young modulus in between 20-500 kPa [40–42] whereas rat myocardium between 1-140 kPa [43,44]. The system proposed here, allows adjusting the mechanical properties from below 30-60 kPa when using PVA47/Dex and 30-150 kPa for PVA145/Dex. Nevertheless, these values can be modified by increasing the freezing-thawing cycles. Regarding the water uptake/degradation experiments, PVA 47 hydrogels showed a lower residual solid percentage, likely due to the loss of low M_w fraction of the polymer sample during the experiments; whereas the presence of Dex led to a higher loss of solid mass, probably because a higher percentage of Dex diffuses out of the network during the experiments.

This mechanical workability leads to consider PVA/Dex based hydrogel as a possible system useful to support the mechanical movement of the affected heart. Moreover, the loading of an antioxidant molecule within the hydrogel can act in synergy by inducing the reduction of oxidative stresses generated by the recruitment of inflammatory cells and ROS in the damaged tissues. It is important to note that literature data already showed an improvement of the ischemic conditions thanks to the oral administration of astaxanthin or astaxanthin derivatives treatment [6,7,45]. Nevertheless, the lack of good delivery methods able to stabilize the molecule is a limitation in using astaxanthin as ROS target [19,46]. Here, we propose PVA/Dex hydrogels as delivery systems to allow the *in situ* release of the astaxanthin molecule, optimizing in this way the anti-inflammatory and antioxidant action mechanism of the molecule in the myocardium.

As presented in Figure 1, the addition of either CD or CD-A to the PVA hydrogels did not affect their physico-chemical or mechanical behavior. Additionally, astaxanthin was released from the PVA/Dex hydrogels in less than 8 hours, a positive behavior to counteract the burst of ROS produced in ischemic conditions, while limiting the risk of a generation of pro-oxidant action [47]. Furthermore, the activity of astaxanthin released from the patches was analyzed for its scavenging capability, by ORAC method, showing a similar Trolox equivalent capability compared with the CD-A before loading and two times higher than the reference molecule, confirming the release of the molecule from the hydrogels after 24 h in an active antioxidant form.

Cell viability test confirmed the non-toxicity of all the tested PVA/Dex formulations. It is worth noticing that, each component of the hydrogels, at the adopted concentrations, is widely

reported in literature as safe [48–50]. Finally, after subcutaneous implantation, a positive CD68 immune-histological staining reveals an inflammatory tissue response due to macrophages recognition of the PVA patches as a foreign material. This behavior was previously reported in other works as a normal response at first stages of the repair process [51,52]. Additionally, the CD163 positive staining revels the start of the regenerative process within the damaged tissue. Moreover, hematoxylin-eosin staining allowed the hydrogel visualization after 8 days of implantation; at that time, just 40% of the patch degraded, reaching 60% up to 30 days under the *in vitro* conditions that can be considered very drastic in comparison to the *in vivo* environment. The penetration and suturability tests, already described for PVA patches [16], attribute to the PVA145/Dex 1FT a better surgical handling and adequate suture holding strength. As confirmed by the *ex vivo* suturability test, PVA/Dex hydrogels are good candidates to be tested as myocardial patches, as verified by several suture assays. It is worthy to point out that, the hydrogels stay attached to the heart during the whole procedure, despite of the remaining contractile heart reflexes implying the feasibility of this procedure even under *in vivo* conditions. The results presented here show that PVA/Dex/CD-A can be further explored in MI animal models as myocardial patches to assist infarcted heart mechanical function and to reduce oxidative stress by the *in situ* release of astaxanthin.

5. Conclusions.

In the present work, the development and the physico-chemical and mechanical characteristics of a hydrogel system as a myocardial patch were described. Indicating that this new elastic hydrogel material can be obtained from PVA and Dex blends, allowing astaxanthin loading, without affecting its antioxidant capability. The hydrogel patches demonstrated high *in vitro* compatibility, as well as mechanical and stability properties. In addition, the *ex vivo* suturability test showed a potential to considerer these systems for a cardiovascular application. The antioxidant was released and the *in vivo* conditions demonstrated that the hydrogel patches could be implanted in rat without altering the surrounding tissues or inducing inflammation. To conclude, this device could be studied for therapies that aim to decrease the myofibril stress generated after MI and to delivery an antioxidant molecule in damaged heart tissue, reducing the oxidative stress status induced after an ischemic episode.

Acknowledgements

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Supporting information

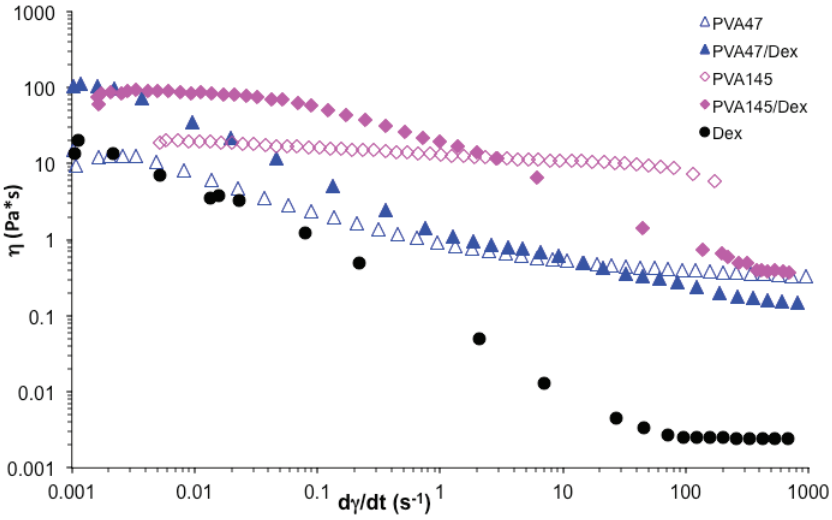

Figure S1: Flow curve of starting solutions: Dex 5% w/w (black circle); PVA47 15% w/w (empty blue triangle); PVA47/Dex (filled blue symbol); PVA145 15% w/w (empty purple diamond); PVA145/Dex (filled purple diamond).

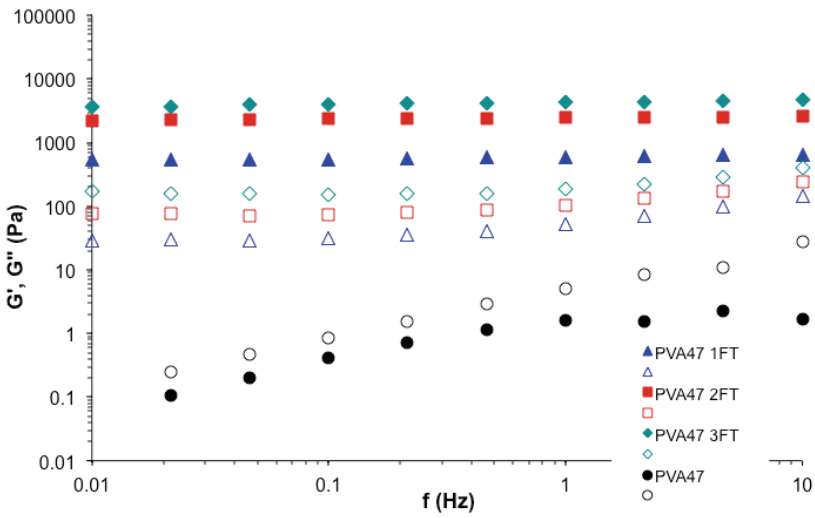

Figure S2: Mechanical spectra of PVA47: solution (black); 1 FT (blue); 2 FT (red); 3 FT (green). G': filled symbols; G'': empty symbols.

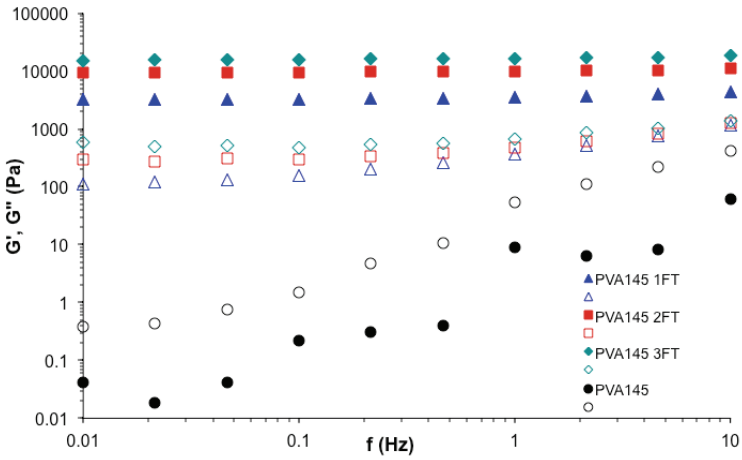

Figure S3: Mechanical spectra of: PVA145: solution (black); 1 FT (blue); 2 FT (red); 3 FT (green). G': filled symbols; G'': empty symbols.

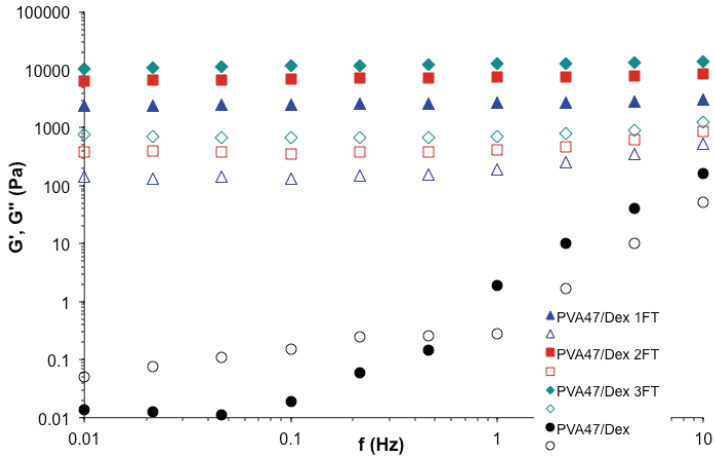

Figure S4: Mechanical spectra of PVA47/Dex: solution (black); 1 FT (blue); 2 FT (red); 3 FT (green). G': filled symbols; G'': empty symbols.

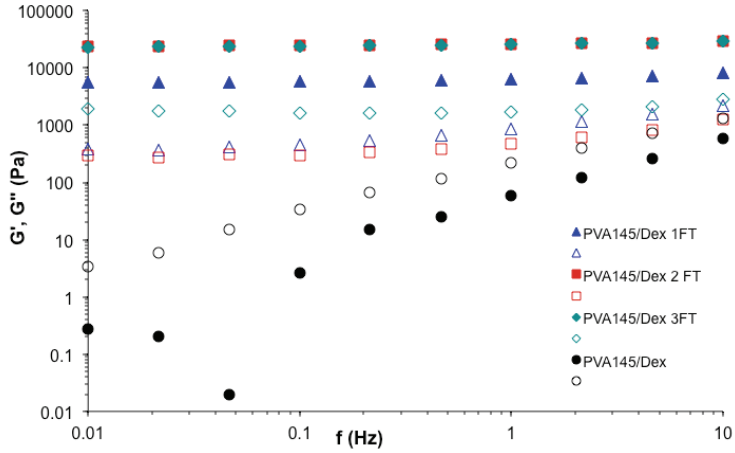

Figure S5: Mechanical spectra of PVA145/Dex: solution (black); 1 FT (blue); 2 FT (red); 3 FT (green). G': filled symbols; G'': empty symbols.

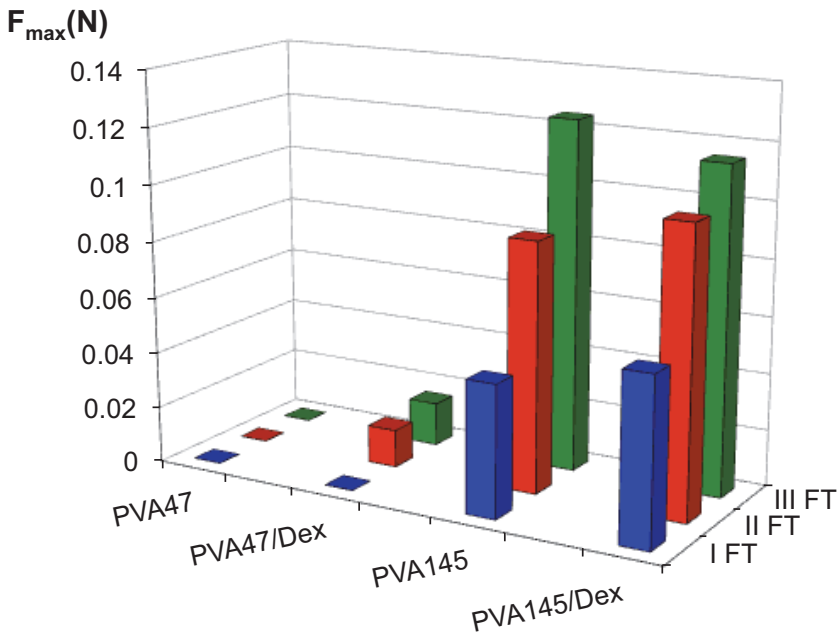

Figure S6: Penetration experiment for PVA47, PVA47/Dex, PVA145 and PVA145/Dex hydrogels at 25°C. All the measurements were collected at 25°C. All tests were performed in triplicate and the obtained values always lay within 10% of the mean.

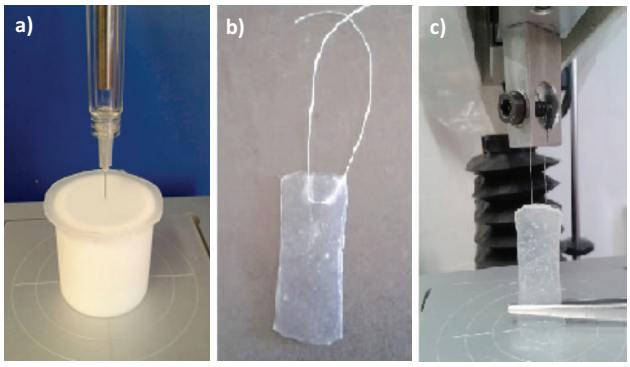

Figure S7: a) Texture Analyzer configuration for penetration tests; b) typical patch strip for suturability tests; c) Texture Analyzer configuration for suturability tests.

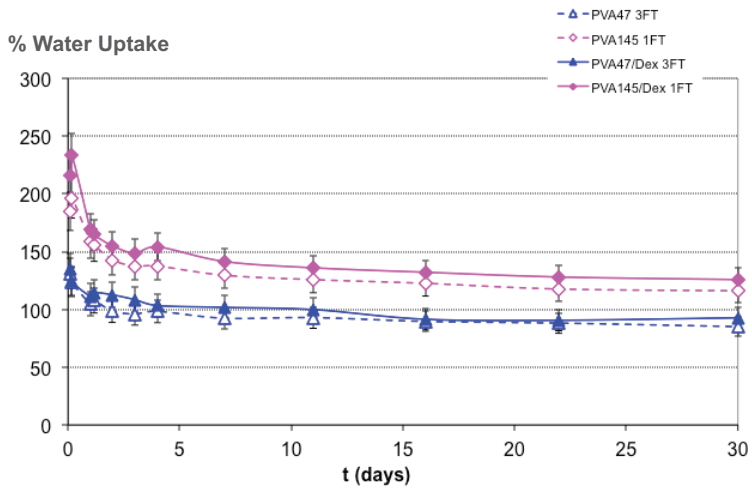

Figure S8: Water uptake/degradation experiments for the different hydrogels PVA47 3FT (empty blue triangle); PVA47/Dex 3FT (filled blue symbol); PVA145 1FT(empty purple diamond); PVA145/Dex 1FT (filled purple diamond). PBS 0.1M pH 7.4, 37°C. All tests were performed in triplicate, and SD is reported.

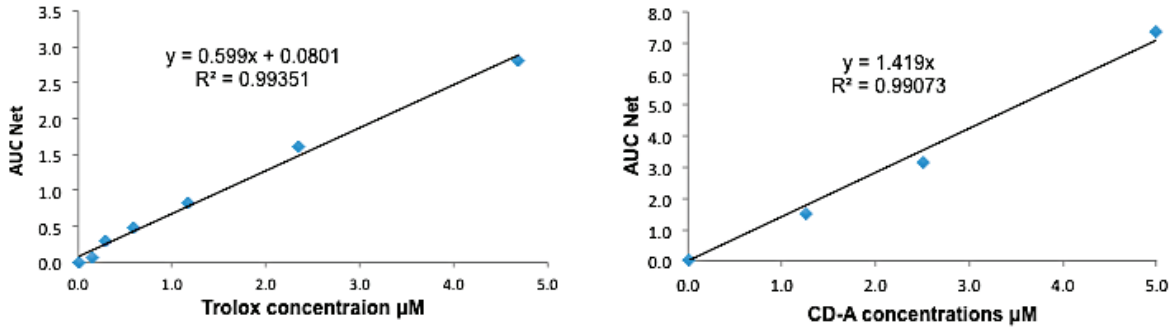

Figure S9: Calibration curve of (a) Trolox antioxidant (reference molecule) and (b) CD-A in a range concentration between 0-5 µm.

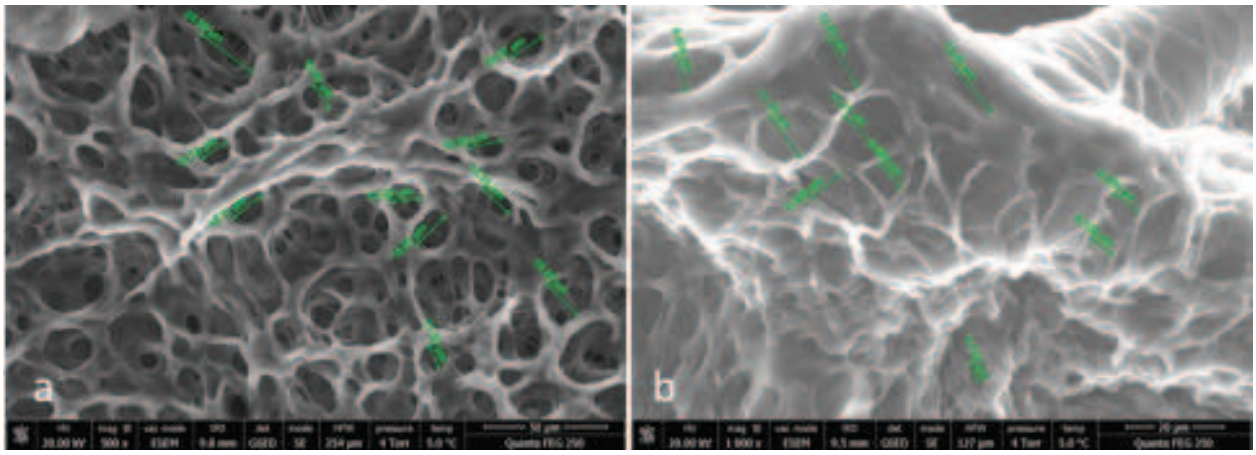

Figure S10: ESEM images of a) PVA47/Dex 3FT (pore diameter in the range 11-26 µm) and b) PVA145/Dex 1FT (pore diameter in the range 4-12 µm).

Highlights

- PVA47/Dex 3FT and PVA145/Dex 1FT appeared to present the best mechanical properties.
- PVA47/Dex 3FT and PVA145/Dex 1FT allowed the CD-A release without altering astaxanthin radical scavenging capacities or compatibility.
- PVA/Dextran/CD-A mechanical workability and antioxidant capacities support the next application of the system as a patch for the reduction of myofibril stress.

5 Chapter

Research article: *In situ* Astaxanthin Delivery to Attenuate the Muscle Damage after Ischemia-Reperfusion Injury

M. Zuluaga, L. Choudat, R. Aid-Launais, O. Thibaudeau, L. Louedec D. Letourneur, V. Gueguen, A. Meddahi-Pellé, A. Couvelard, G. Pavon-Djavid.

Under revision in the journal of Frontiers in Physiology

The purpose of this fifth chapter was to evaluate a second biomaterial strategy based on two natural polysaccharides: Pullulan and Dextran as astaxanthin delivery system.
An in vivo proof of concept of in situ antioxidant delivery system using a model of induced I/R in the femoral artery aimed to reduce lower hind limb muscle injury.

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Résumé

Cette partie décrit l'évaluation d'un de nos systèmes de matrices polymères chargées en CD-A dans un modèle d'ischémie/reperfusion (I/R) de l'artère fémorale. Une matrice polysaccharide à base de pullulane et dextrane a été chargée en CD-A (P/D/CD-A). La capacité antioxydante du CD-A chargé dans la matrice et la non-toxicité du système ont été validées *in vitro* avec succès. Cette matrice a alors été implantée dans le muscle gracilis du membre inférieur chez le rat. Le modèle a consisté à induire l'ischémie de l'artère pendant 45 min et permettre sa reperfusion pendant 60 min, 7 ou 15 jours. Les animaux ont été divisés en quatre groupes : contrôle, contrôle I/R, sham et antioxydant. À la fin de la procédure, l'étude histologique et immunohistologique de la réponse tissulaire a permis d'évaluer la formation fibreuse, l'infiltration des cellules phagocytaires et l'état de stress oxydatif. Aucune altération n'a été observée chez les animaux témoins. Les groupes I/R ont montré, autour de la matrice, une réaction inflammatoire composée des macrophages positifs aux CD68 et CD163 et d'un infiltrat inflammatoire dans le périmysium. Il s'agit d'une réaction classique aux biomatériaux implantés indiquant un processus de remodelage. Le dépôt de fer non-heme dans les groupes I/R à 15 jours de perfusion indique une population cellulaire susceptible de subir un dommage radicalaire. Le marquage nucléaire de Nrf2 qui reflète un état de stress s'est avéré plus faible dans les groupes traités avec le P/D/CD-A. Ces résultats pourraient indiquer une réduction du stress oxydatif dans le muscle gracilis. Ce travail est une première étape dans la mise au point d'un système de libération locale d'astaxanthine.

Les expériences réalisées ont permis d'aborder l'étude de la réponse tissulaire au stress dans un modèle d'I/R du membre inférieur et d'évaluer le bénéfice de la libération *in situ* d'une molécule antioxydante.

Mots clés: Astaxanthine; Ischémie/reperfusion; ROS; Stress oxidatif; Hydroxypropyl-β-Cyclodextrin.

Abstract

This part of the project presents the evaluation of a polymeric system loaded within CD-A in an animal model of femoral hind-limb ischemia/reperfusion (I/R). Polysaccharide scaffolds based on pullulan and dextran were loaded within CD-A (P/D/CD-A). The CD-A antioxidant capacity and the non-toxicity of the system were successfully validated *in vitro.* Then, P/D/CD-A scaffolds were implanted into the gracilis muscle of the rat. Femoral ischemia was induced for 45 min and perfused during 60 min, 7 or 15 days. Animals were divided in four groups: control, control I/R, sham and antioxidant. At the end of the procedure, the gracilis muscle was histologically and immunohistologically evaluated to assess tissue response regarding inflammatory cells infiltration, toxicity, and oxidative stress status. No alterations were found in the control animals. The I/R groups showed an inflammatory reaction surrounded the scaffolds positive labeling for CD68 and CD163 macrophages markers, and an inflammatory infiltrate within the perimysium. These results were attributed to a classic reaction to implanted biomaterials indicating a remodeling process. A non-heme iron deposition within the I/R groups after 15 days of perfusion, reflects a cell population susceptible to free radical damage. The nuclear Nrf2 positivity expressed under oxidative stress was weaker in the P/D/CD-groups. These results could reflect a reduction of the oxidative stress damage in the gracilis muscle. This works is a first step towards the development of a local astaxanthin delivery system. The performed experiments addressed to evaluate the tissue behavior under stress in a model of femoral hind-limb I/R and to evaluate the beneficial effect of an *in situ* release of an antioxidant molecule.

Keywords: Astaxanthin; Ischemia/Reperfusion; ROS; Oxidative stress; Hydroxypropyl-β-Cyclodextrin.

1 Introduction

An ischemic condition results in an imbalance in the oxygen production and consumption in the cell and tissues, generating a constant influx of pro-inflammatory reactions that renders tissue vulnerable to microvascular dysfunction (Madamanchi et al., 2005) and to additional injury (Collard and Gelman, 2001). Consequently, an oxidative stress status is generated, characterized by an overconcentration of reactive oxygen species (ROS) (Schieber and Chandel, 2014). An increase of tissue injury and high inflammatory response (Yellon and Hausenloy, 2007) has been associated with the reperfusion process, due to the endogenous antioxidant defense system inability to handle the radical load and to restore the affected environment (Guillot et al., 2014; McCord, 1985). In the case of lower limb where muscles represent the primary mass of tissue damage triggered by I/R represents the most critical effect of the inflicted injury (Blaisdell, 2002). According to Gardner et al. (Gardner et al., 2015) antioxidant and anti-inflammatory strategies may be useful to treat lower I/R pathologies. Moreover, animal studies have demonstrated the efficacy of antioxidant therapy in preventing or attenuating I/R injury (Charles et al., 2017; Collard and Gelman, 2001).

Natural carotenoids have shown particular antioxidants capabilities to scavenge ROS and enhance the cell's ability to prevent oxidative stress (Ranga Rao et al., 2013). Astaxanthin, a xanthophyll carotenoid, has been studied due to its antioxidants and anti-inflammatory properties (Pashkow et al., 2008). Astaxanthin oral supplementation pretreatment have shown to reduce oxidative stress and inflammation on rodent animals presenting ischemic renal injury (Qiu et al., 2015) and to reduce apoptosis and autophagy in animal with hepatic ischemia (Li et al., 2015). Furthermore, the effect in myocardial I/R injury of either astaxanthin or vitamin E oral ingestion for 21 days, reported a higher cardioprotection when treated with astaxanthin (Adluri et al., 2013). Intracerebroventricular injection of astaxanthin revealed a reduction in ischemia-related injury in brain tissue through the inhibition of oxidative stress, reduction of glutamate release, and antiapoptosis (Shen et al., 2009). Besides, these positive results some drawbacks regarding astaxanthin stability and bioavailability *in vivo* have also been reported (Coombes et al., 2016). A strategy based on the use of carriers for *in situ* delivery of astaxanthin represents a possible solution to enhance its *in vivo* effect. In this study, we first challenged the efficacy of a polysaccharide system for the *in situ* delivery of astaxanthin. Two natural polysaccharides pullulan and dextran were selected. This choice was supported by previous works, which showed pullulan and dextran good biocompatibility, biodegradability and flexibility in the cardiovascular field, when used as delivery systems (Abed et al., 2011; Chaouat et al., 2006;

Derkaoui et al., 2008; Purnama et al., 2013). Then, the preservation of antioxidant activity of astaxanthin after being loaded into the scaffolds and the non-toxicity of the system were confirmed. Finally, an animal model of I/R was selected as proof of concept. The occlusion of the femoral artery was chosen since the superficial femoral and popliteal arteries are continuously affected by ischemic and reperfusion periods (Vogt et al., 1992). Astaxanthin capacity to compensate muscle damages was determined after 45 min of ischemia induced by femoral artery clamping followed by varied periods of reperfusion (60 min, 7 or 15 days), as compared with the sham (I/R muscles without antioxidant treatment) and control groups. The gracilis muscle was explanted and histologically evaluated to assess tissue response regarding inflammatory cells infiltration, absence of toxicity, and indirect oxidative stress status.

2 Materials and Methods

2.1 Chemical and Biological reagents

Natural astaxanthin (purity >97% HPLC, powder, Lot: 5M4707V), Hydroxypropyl-β-cyclodextrin (DS=0.67) and Potassium ferrocyanide (BioUltra, >99.5%) were purchased from Sigma-Aldrich Co. LLC (Saint-Louis, MO, USA); as well as 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), trisodium trimetaphosphate (STMP, lot# MKBQ7691V), sodium chloride (NaCl, lot# SZBA0490) and Isopropanol (70% in H_2O, Ref: 563935). Pullulan, MW 200000 was purchased from Hayashibara Inc., Okayama, Japan. Dextran MW 500000, 5% degree of branching was obtained from Leuconostoc mesenteroids; Pharmacia, Uppsala, Sweden. Acetone, Methanol, Hydrochloric acid, Hydrogen peroxide and chloroform were all purchased form Carlo Erba Reagents S.A.S (France). EnVision Dako kit (ref K4063 and ref K4010), Dako REAL peroxidase-blocking and Negative Control mouse IgG1 solution, were supplied by Dako (Dako, Carpinteria, CA, USA and Dako, Glostrup, Denmark). Mouse anti rat CD68 and CD163 primary antibodies were obtained from Bio-rad (MCA341A488, Marnes-la-Coquette, France). Phospho anti-Nrf2 (S40) antibody was purchased from Abcam (ab76026, Paris, France). The water used was double distilled and deionized. Mouse fibroblasts cells CCl 163 (Balb/3T3 clone A31) were purchased from ATCC-LGC Standards S.a.r.l. (Molsheim Cedex, France). Minimum essential Medium-L-Glutamine (MEM), fetal calf serum (FBS), penicillin–streptomycin- amphotericin (PSA), and Trypsin/EDTA solution (TE), were purchased from GIBCO (Life technologies, Carlsbad, CA, USA).

2.2 Pullulan/Dextran/CycloDextrin-Astaxanthin (P/D/CD-A) scaffold preparation and loading

2.2.1 P/D scaffold preparation

Polysaccharide-based scaffolds were prepared according to the protocol proposed by Autissier et al. (Autissier et al., 2010). Briefly, pullulan, dextran and NaCl (3:1:4) were mixed in 40% water (w/v). Then, 10 g of the polysaccharide solution were withdrawn and 1 mL of NaOH 10 M was added, and incubated at 50°C for 20 min. Next, 300 mg of STMP (30% w/v) in 1 ml of water was added as a chemical crosslinking agent. Finally, the mixture was poured into glass mold of 10 cm x5 cm x1 mm. The resulted hydrogel was washed extensively in PBS pH 7.4, freeze dried (Cryotec, Lyophilizer Crios, France), sterilized under UV light and stored at RT until use.

2.2.2 CD-A preparation

To facilitate natural astaxanthin loading into the scaffolds, hydroxypropyl-β-cyclodextrin was previously mixed with astaxanthin accordingly with the following preparation. Natural astaxanthin (A, 1 mg) in acetone/chloroform (v/v 1:1) solution was mixed with hydroxypropyl-β-cyclodextrin (CD, 250 mg) dissolved in 12.5 mL of 95% methanol and filled with nitrogen. The mixture was sonicated during 5 min at 35°C (Ultrasonic bath bandelin sonorex rx-100-h) and stirred under light protection overnight. The solution was dried under vacuum, recovered with double distilled water, freeze dried and stored at -4°C until use.

2.2.3 Scaffold loading and characterization

Sterilized P/D scaffolds were cut in 5 x 5 mm squares and loaded with either 10 µL of saline solution in the case of sham group (P/D) or 2.5 mg CD-A reconstituted in 10 µL saline solution (12.5 µM) for the antioxidant group (P/D/CD-A). Five minutes were necessary for the solutions to be completely absorbed by the scaffolds. P/D and P/D/CD-A scaffolds were characterized by environmental scanning electron microscopy (ESEM) using a Philips XL 30 ESEM-FEG (Eindhoven, The Netherlands) at an accelerating voltage of 15 keV and at a pressure of 3.5 Torr.

2.3 Release kinetics evaluation

To determine the release of CD-A complex from the hydrogel, a calibration curve by plotting the CD-A complex concentration (0-15 µM) against absorbance (OD) at 472 nm was established (i-controlTM microplate reader software, TECAN Männedorf, Switzerland). The CD-A complex release from the scaffolds was determined as follow: P/D/CD-A scaffolds were immersed in PBS

0.1 M, pH 7.4 at 37° to allow the release of CD-A into the solution. Then, 50 µL of release solutions were collected at 5-10-20-30-40 and 60 min. The absorbance of P/D/CD-A release products was recorded at 472 nm, and OD values were reported to the percentage of CD-A concentration based on the calibration curve.

2.4 CD-A antioxidant activity evaluation

The antioxidant scavenging capacity of CD-A before and after released from P/D scaffolds were evaluated using the ORAC method (Sueishi et al., 2012). Briefly, P/D scaffolds were previously loaded with CD-A (12.5 Mm) and immersed into 1 mL PBS solution at 37°C during 60 min.

Solutions of fluorescein (4 nM), AAPH (160 µM, stressor) and Trolox (0–50 µM, antioxidant standard reference) were prepared in PBS. Fluorescein solution (150 µL) was added to each well (96-well microplate), and then 25 µL of P/D/CD-A scaffold release product, blank (PBS) or Trolox were distributed in the wells, prior to the addition of AAPH (25 µL). Then fluorescence decay was monitored (485/ 528 nm Ex/Em) at 37°C for 60 min, and data taken every minute.

The area under the curve (AUC) of relative fluorescence was calculated. The AUC values for CD-A release products and Trolox were reported to the AUC value calculated for AAPH (stressor), to indicate the AUC net values. ORAC values were expressed as Trolox equivalent in µM (TEAC, µM) and calculated as the slope ratio from curves, AUCnet, versus the concentration of antioxidant and Trolox, respectively (AUCnet = AUCsample − AUCblank).

2.5 Cell viability assay

Cell viability was assessed using the MTT assay. Mouse fibroblast cells (3T3) were grown in MEM, supplemented with 10% (v/v) FBS and 1% PSA. Cells were seeded in a 96-well microplate at a cellular density of $1x10^4$ cells/well and cultured overnight. After MEM removal, CD-A (1.25 to 5 µM) or P/D/CD-A scaffold release product, MEM (Positive Control, PC), or 10 % DMSO (Negative Control, NC) were added. After 24 h incubation in cell culture conditions, the solutions were discarded and the MTT assay was performed. MTT solution (200 µL, 0.5 mg/mL) were added to cells and incubated during 3 h at 37°C. Then, 200 µL of isopropanol were added for 20 min. Absorbance was recorded at 570 nm (i-controlTM microplate reader software, TECAN Männedorf, Switzerland). Non-toxicity of samples was considered if cellular viability was higher than 70% compared to NC (based on the ISO 10993:2009 1-12 regarding the biological evaluation of medical devises).

2.6 Surgical procedure and Experimental design

Animal surgeries were performed in accordance with the “principles of laboratory animal care” and were approved by the animal ethic committee of the Bichat laboratory. Hind-limb I/R surgeries were performed in male rats weighted 250-300 g (Charles River Laboratory, Wilmington, MA) using an I/R model adapted from (Luyt et al., 2003). Animals were anaesthetized using pentobarbital injection of 30 mg/kg (Centravet, France). Animals were randomly assigned to three experimental groups: control (n=12), sham (n=12), and antioxidant (n=12) (Figure 2A). Control animals underwent 2 h of general anesthesia. The sham and antioxidant groups underwent 45 min of ischemia induced by the ligature of femoral artery with a 6-0 silk suture. Then an intramuscular incision, perpendicular to the muscle fibers was done in the gracilis muscle, allowing the implantation and holding of the P/D scaffold charged with 10 µL saline solution (sham group) or P/D scaffold loaded with 12.5 µM of the antioxidant molecule (P/D/CD-A), five minutes prior to artery reperfusion. Then arterial blood flow was restored and reperfusion periods of 60 min, 7 or 15 days (Figure 2B) were evaluated. Finally, rats were sacrificed with an intraperitoneal injection of overdose of sodium pentobarbital (150 mg/kg) and gracilis muscles were explanted and histologically evaluated.

2.7 Histological and immunological evaluation

Animals were sacrificed 1 h, 7 days or 15 days post-surgery. Gracilis muscles were collected and immersed in 4% formaldehyde during 24 h, dehydrated and embedded in paraffin according to standard protocols. Sections of 4 µm thickness (HM 355S microtome, Thermo scientific Waltham, MA, USA) were stained with hematoxylin and eosin. Total phagocytes and type-2 macrophages were identified by anti-CD68 and anti-CD163 antibodies labeling respectively (1/30 working dilutions). Anti-phosphoNrf2 (1/100 working dilution) immunomarker were used to collect information about the muscle general oxidative stress status after I/R. DAKO EnVision+ System and 3,3'-diaminobenzidine was used as a chromogen. Iron accumulation in healthy and I/R muscles was quantified by Perl’s reaction amplified by 3,3'-diaminobenzidine (DAB). After antigen retrieval, samples were incubated in a solution (pH 0.5–0.6, 25°C) containing 1% HCl and 1% potassium ferrocyanide, followed by H_2O_2 incubation (Terzian et al., 2017). All slides were counterstained with hematoxylin and digital images were obtained and analyzed using Nanozoomer digital pathology software (Hamamatsu, Japan).

Samples images were analyzed by two independent methods:

(i) a mathematical quantitative program: a saturation analysis which highlighted the positive stains in the images given by the different immunostaining markers using Matlab software (The MathWorks, Inc., Natick, Massachusetts, United States). Each image was separated into its components hue, saturation, and value color bands. Then thresholds were defined to determine the mask for the region of color corresponding to the positively stained cells represented by brown color spots. Total image pixels of each image were used to calculate the percentage of positive staining as a pixel difference.

(ii) a qualitative method: the analysis of the proximal and distal section of the muscle was performed by a physician/pathologist in an independent and random manner. The tissue response was scored (macrophages, cellular infiltration, giant cells) according to a relative scoring system (Gibson-Corley et al., 2013): - = no observation, + = Mild and ++=moderate.

2.8 Statistical analysis

All experiments were repeated at least three times to ensure the reproducibility of each test. Results were expressed as the mean ± SD and statistical analysis was done using one-way ANOVA followed by Tukey's HSD *post hoc* test (JMP Software, Version 9; SAS Institute, Cary, NC, USA). Statistical significance was set at p-value < 0.05.

3. Results

3.1 P/D/CD-A loading and antioxidant activity evaluation

ESEM images reflect a heterogeneous microporosity structure of the P/D based scaffold (Figure 1A-B), spread all over the surface. Interestingly, the addition of the CD-A within the P/D scaffold smoothed the scaffold pores (Figure 1B). As presented in figure 1C the freeze-dried process allowed an easy handle to the scaffold and a high and fast molecule loading capacity. Five minutes were required for the complete absorption of the molecule (10 µL/scaffold). The volume needed to charge the scaffold was determined as the swollen maximum capacity without volume saturation (data not shown). The initial dimension of the scaffold was reduced after the complete absorption of the different conditions, varying from 5*5 mm to 3*3 mm.

P/D/CD-A release product was quantified regarding its concentration and antioxidant capacity. First, a calibration curve using different concentrations of CD-A (0-12.5 µM astaxanthin concentration) vs. absorbance at 472 nm was plotted (Figure S1A), then P/D scaffold was charged with the maximal amount used for CD-A quantification (2.5 mg; astaxanthin concentration 12.5 µM) and P/D/CD-A controlled kinetics release was reported. Figure 1D indicated a fast release during the first 20 min (50%) and a total release after 60 min. Then,

calibration curves using Trolox (antioxidant standard, 0-50 μM) and CD-A (0-50 μM), were plotted vs. AUC net (Figure S1B). Slope ratio from both curves, indicated a high antioxidant capacity of CD-A when compared to the antioxidant standard (2.8 mM TEAC). Figure 1E shows a lower AUC net value for CD-A released product 11.2 against 12.7 expected for a total CD-A release, thus indicating a slight retention of the CD-A antioxidant activity by the P/D scaffold. However, when analyzing all antioxidants at the same concentrations, CD-A alone or as a release product, both presented a higher antioxidant activity than the reference antioxidant molecule, Trolox.

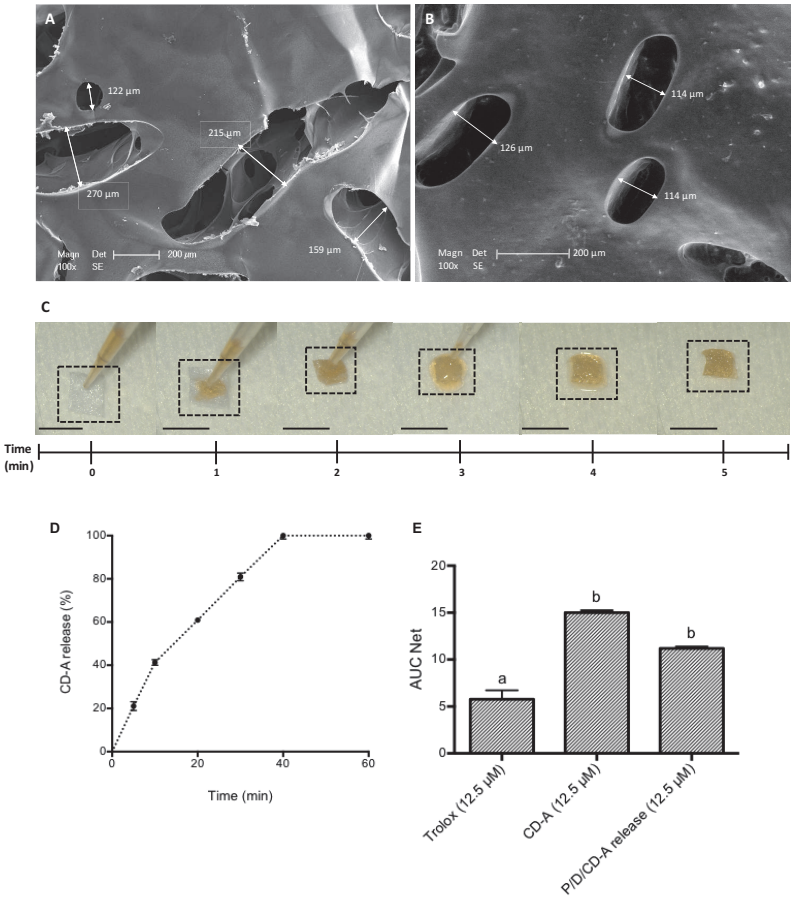


Figure 1: (A) Environmental scanning microscopy (ESEM) of the P/D scaffold and (B) P/D/CD-A scaffold. (C) P/D scaffolds were loaded with 10 μL of either saline solution or CD-A until complete absorption. (D) Release kinetics of CD-A from the P/D scaffolds during 60 min in PBS (pH 7.4). (E) Antioxidant activity evaluation of CD-A and P/D/CD-A release product at 12.5 μM compared with Trolox at 12.5 μM antioxidant standard; results are presented as AUC net values. Values connected with the same letter are not significantly different

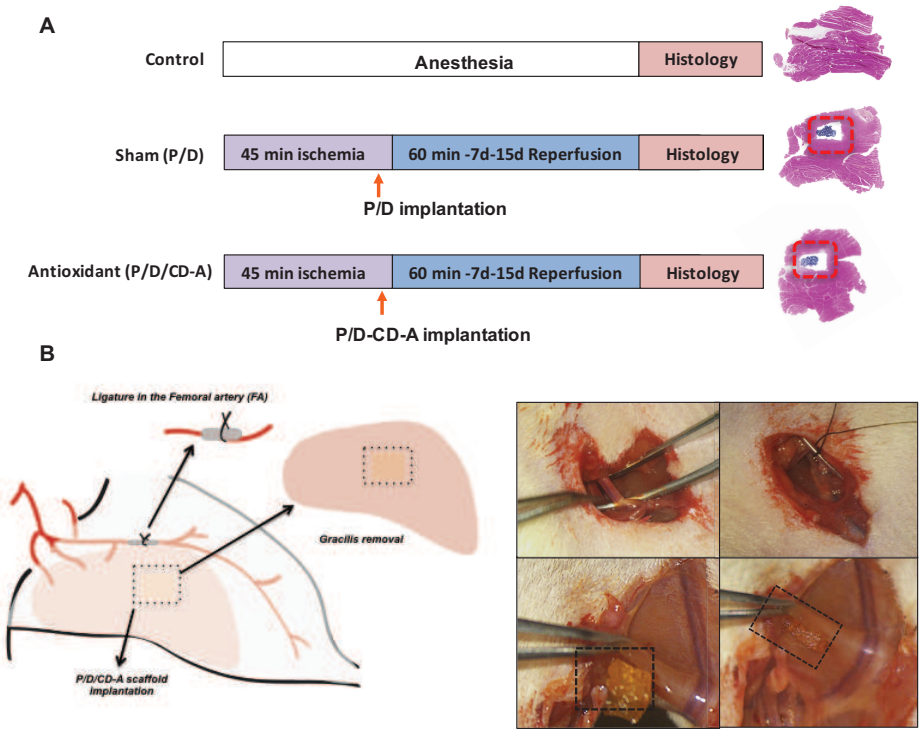


Figure 2: *In vivo* experimental design and surgical I/R procedure. (A) The study was divided into three different groups: control (n=12), sham (n=12), and antioxidant (n=12). (B) Control animals underwent 2 h of general anesthesia. The sham and the antioxidant groups underwent 45 min of ischemia induced by the ligature of femoral artery. Then an intramuscular incision, perpendicular to the muscle fibers was done in the gracilis muscle, allowing the implantation and holding of the P/D scaffold charged with saline solution (sham group) or P/D scaffold loaded with the antioxidant molecule (P/D/CD-A), prior to artery reperfusion for either 60 min, 7 or 15 days. Finally, gracilis muscles were explanted and histologically evaluated.

3.2 P/D/CD-A scaffold *in vitro* and *in vivo* toxicity evaluation

P/D based scaffolds were autoclaved by UV light and evaluated to determine any sign of *in vitro* and *in vivo* toxicity. The toxicity of CD-A and P/D/CD-A release product (12.5 μM) were evaluated after being in contact with 3T3 cells during 24 h. This cell line allows the evaluation of samples toxicity thanks to its high sensitivity. Figure 3A shows an acceptable cell viability (superior to 70% of NC), when 3T3 cells were incubated with CD-A (0-5 μM) or with CD-A released products.

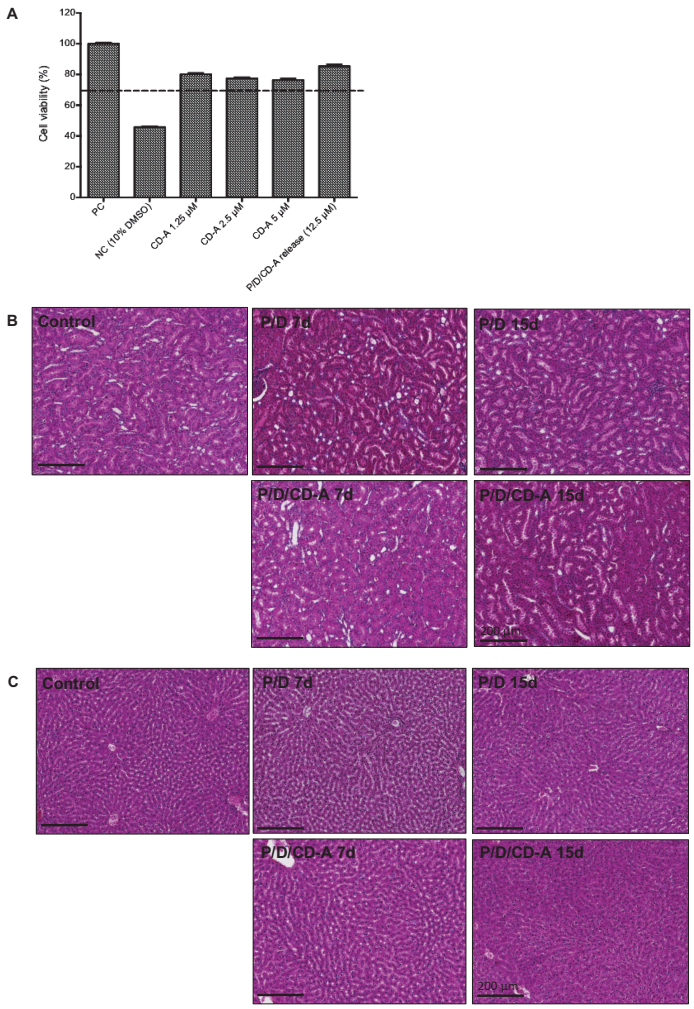


Figure 3: (A) Cell viability study of CD-A and P/D/CD-A release product by MTT assay. Antioxidant samples were significantly different compared to the positive toxicity control (PC). (B) Hematoxylin-eosin staining of liver and kidney from control and I/R groups which underwent 45 min of ischemia and were subjected to 7 or 15 days of reperfusion. Scale bars, 200 µm; magnification 10X. Images are representing 3 independent experiments (n=4 animals per group).

In vivo toxicity was evaluated by monitoring the response of vital organs distant to the implant. No noticeable systemic reaction was triggered by the presence of either the P/D scaffold or the P/D/CD-A antioxidant in the kidney (Figure 3B) and liver (Figure 3C) in animals subjected to 45 min of ischemia and 7 or 15 days of reperfusion when compared to control group.

3.3 *In vivo* evaluation of P/D/CD-A scaffolds in femoral I/R model

3.3.1 Histological and Immunohistological analysis

During the *in vivo* implantation study, animals did not present any alteration in their normal behavior or any dietary changes that reflects suffering. No sign of motion restriction or external

acute inflammation was observed during the entire duration of the I/R protocol. After 45 min of ischemia, artery blood flow was gradually restored in sham and antioxidant groups (confirmed by patency test) by different reperfusion periods (60 min, 7 or 15 days). P/D scaffolds remained in the site of implantation in both P/D (sham) and P/D/CD-A (antioxidant) groups, even after 15 days of analysis allowing the delivery of the antioxidant molecule astaxanthin from the scaffold in the surrounded I/R area.

Hematoxylin eosin staining of control muscles showed a normal morphology composed by grouped fibers in pattern fascicles (figure 4A). Conversely, P/D and P/D/CD-A groups that were subjected to I/R presented muscle fibers with a mild hypertrophic aspect, particularly after 7 and 15 days of reperfusion compared to control fibers (Figure 4B-C). A cellular infiltration that surrounded all P/D based scaffolds-tissue interfaces after 7 and 15 days of reperfusion, was observed. The thickness of this cellular aggregation was similar in P/D and P/D/CD-A groups, subjected to the same reperfusion periods.

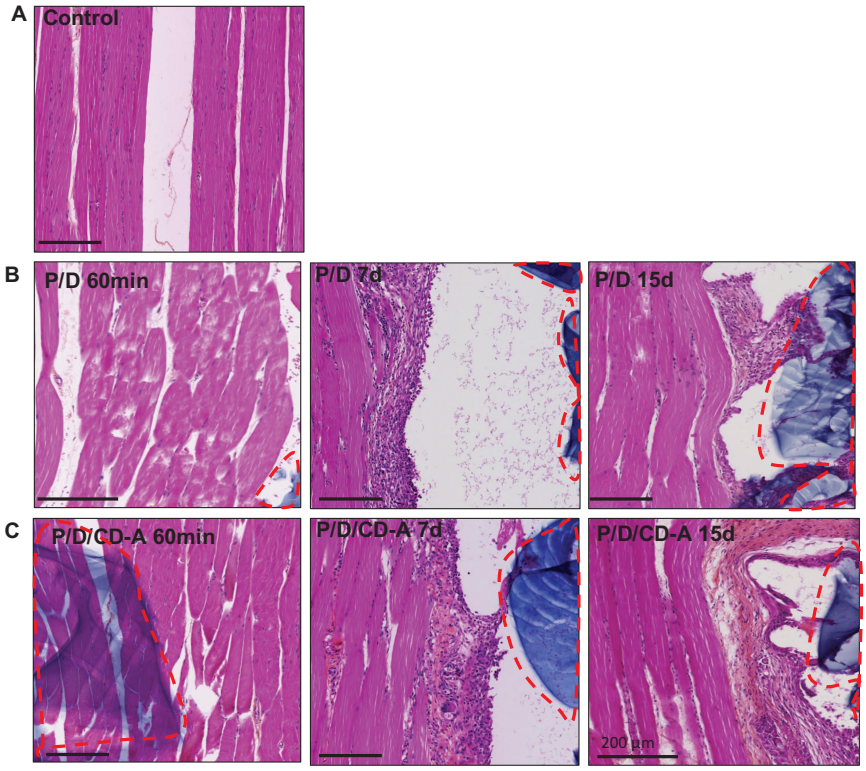
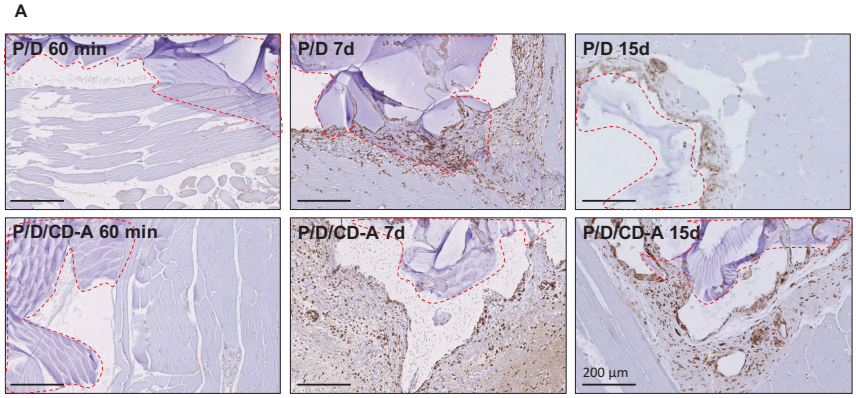
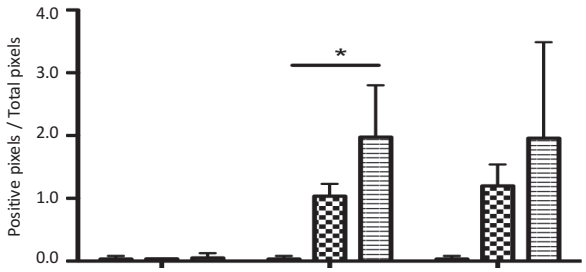


Figure 4: Hematoxylin-Eosin staining of gracilis muscle of (A) control, (B) sham, and (C) antioxidant groups. The sham and the antioxidant group which underwent 45 min of occlusion of the femoral artery, followed by 60 min, 7 or 15 days' reperfusion and treated with P/D scaffold charged with saline solution or CD-A (antioxidant group), respectively. Scaffolds are stained in purple and gracilis muscle in pink, indicated by red dots. Scale bars, 200 µm; magnification 10X. Images are representing 3 independent experiments (n=4 animals per group).

3.3.1.1 Phagocytic cells reaction to scaffold

Evaluation of 60 min of reperfusion demonstrated no significant inflammatory reaction given by positive CD68 or CD163 markers in any of the I/R groups. A significant difference ($p<0.05$) in the total CD68 monocyte/macrophage positive population was found at 7 days of reperfusion between the P/D and the antioxidant-loaded scaffold P/D/CD-A (Figure 5A), being almost two times higher according to the image quantification. After 15 days of reperfusion a non-significant difference between I/R groups was achieved by a slight increase in the P/D CD68 positivity. Few cases of P/D/CD-A scaffolds presented cell colonization composed of CD68 positive marked cells observed after 15 days of implantation. CD163 positive cells were observed surrounding the scaffold and in the gracilis muscle's periphery whereby the scaffold was present (Figure 5B, Figure S2A). Compared to sham group, there were no significant changes in the number of cells positively stained by CD163 marker after 7 days of reperfusion in the P/D/CD-A group. However, a significantly increase of M2 repair macrophages ($p<0.05$) was observed in the P/D/CD-A group after 15 days.





CD68	Reperfusion 60min			Reperfusion 7d			Reperfusion 15d		
Condition	Control	P/D	P/D/CD-A	Control	P/D	P/D/CD-A	Control	P/D	P/D/CD-A
Proximal to patch	-	-	-	-	+	+	-	+	+
Muscle's periphery	-	-	-	-	-	+	-	++	++

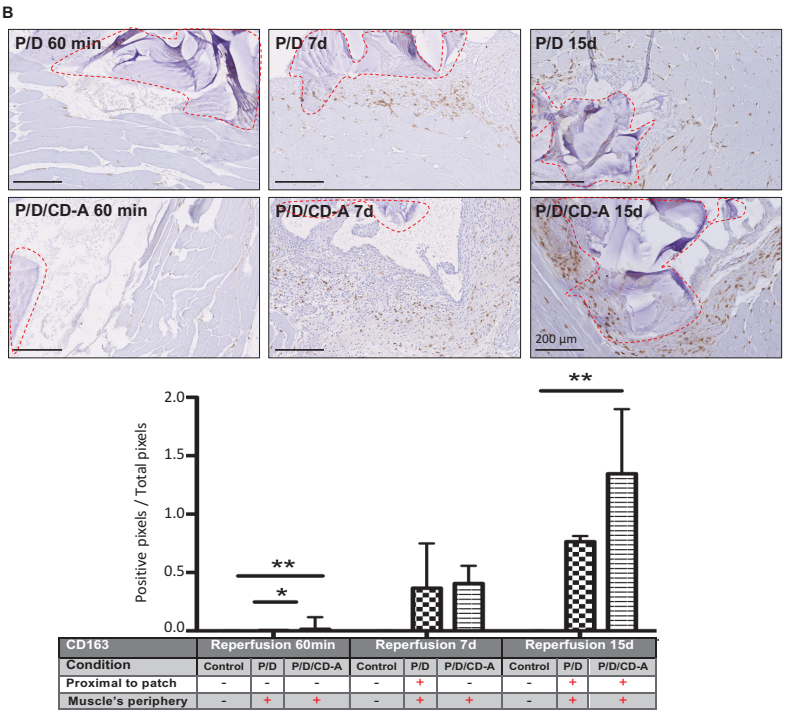
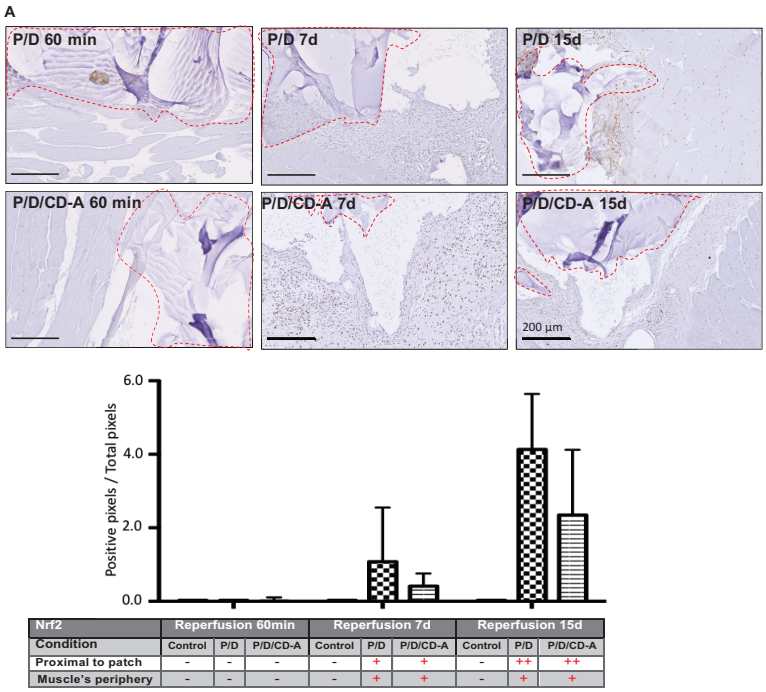


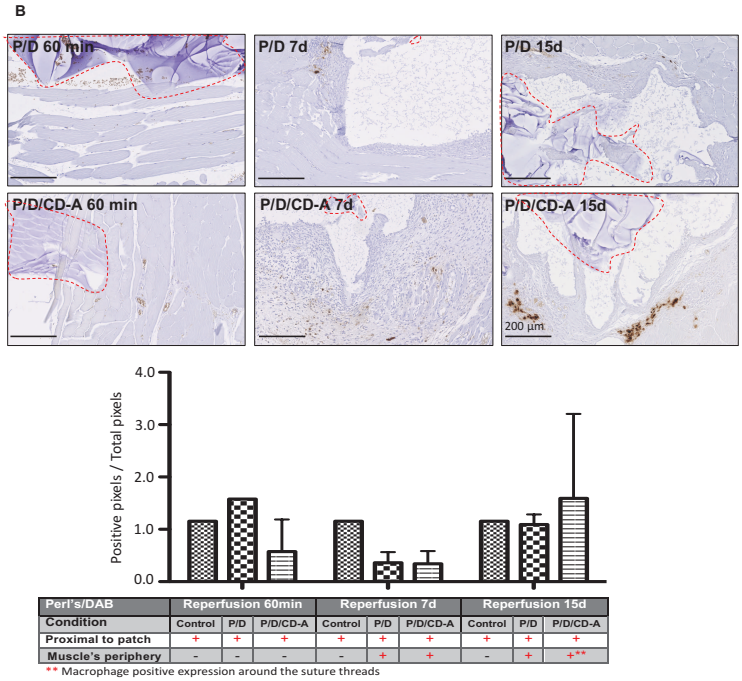
Figure 5: Immunohistochemical detection of monocytes/macrophages populations by CD68 and CD163 markers. Immunological staining and quantification of gracilis muscles containing a P/D implanted scaffold or P/D/CD-A scaffolds using (A) CD68 and (B) CD163 markers. In all cases the muscle was subjected to 45 min ischemia and reperfused during 60 min, 7 or 15 days. The quantification was done using Matlab and contrasted with the pathologist analysis. Red arrows indicate the scaffold localization. Scale bars: 200 µm/10X magnification. Images are representing 3 independent experiments (4 animals per group).

3.3.1.2 Translocation of the transcription factor Nrf2

The effect of CD-A local treatment on Nrf2 translocation in the gracilis muscle under I/R conditions was studied. Immunohistochemical detection showed a positive phosphorylated Nrf2 nuclear staining at 7 and 15 days of reperfusion in P/D/CD-A group (Figure 6A). The quantitative analysis reflected a positivity staining that gradually increased at increased reperfusion periods in both groups (Figure 6B, Figure S2B). It is worth to notice that even after 15 days P/D/CD-A groups showed a less Nrf2 positivity compared to the sham group in which Nrf2 expression was more pronounced. Nonetheless, the variability of the responses between both groups did not lead to significant differences.



Nrf2	Reperfusion 60min			Reperfusion 7d			Reperfusion 15d		
Condition	Control	P/D	P/D/CD-A	Control	P/D	P/D/CD-A	Control	P/D	P/D/CD-A
Proximal to patch	-	-	-	-	+	+	-	++	++
Muscle's periphery	-	-	-	-	+	+	-	+	+



Perl's/DAB	Reperfusion 60min			Reperfusion 7d			Reperfusion 15d		
Condition	Control	P/D	P/D/CD-A	Control	P/D	P/D/CD-A	Control	P/D	P/D/CD-A
Proximal to patch	+	+	+	+	+	+	+	+	+
Muscle's periphery	-	-	-	-	+	+	-	+	+**

** Macrophage positive expression around the suture threads

Figure 6: Nrf2 and Perl's/DAB positive retention by the gracilis muscle containing P/D scaffold or P/D/CD-A scaffold at different reperfusion periods. Immunohistochemical detection and quantification of (A) Nrf2 and (B) Perl's/DAB, which was evaluated for positive retention of iron in the region proximal to the scaffold of by the red blood cells. The quantification was done using Matlab and contrasted with the pathologist analysis. Red arrows indicate the scaffold localization. Scale bars: 200 µm/10X magnification. Images are representing 3 independent experiments (4 animals per group).

3.3.1.3 Oxidative stress detection by means of iron deposition into the tissues

A positive tissue stain to Perl's/DAB was differentiated from red blood cells-heme or not heme iron fixation. After 60 min and 7 days of reperfusion, the P/D/CD-A group showed a decrease in the Perl's/DAB positive staining compared to the sham group. However, after 15 days of reperfusion, a slight increase in the non-heme iron fixation in the muscle area next to the implanted scaffold in either the sham or the antioxidant group suggested a retention by macrophages (Figure 6B, Figure S2B). Positive heme iron fixations of red blood cells were noticed in all groups (Figure 6B) and a negative expression was found when Perl's was not amplified by the DAB component in all conditions Figure S3A, contrasting with the positive stained observed after DAB staining attributed to the presence of red blood cells (heme iron staining). Nevertheless, any retention in the gracilis muscle or surrounded the scaffold was found using both Perl's or DAB markers independently in all groups (Figure S2C, Figure S2B).

4 Discussion

The main findings of this study are first the delivery of an antioxidant molecule such as astaxanthin from a polymer system composed by a mixture of two polysaccharides pullulan and dextran in an easy and controlled manner. This system could be used as an *in situ* antioxidant treatment to enhance the muscle's damaged environment induced after I/R injury.

In this study, the I/R protocol consisted of the femoral artery clamping in the section between the popliteal and the deep femoral artery during 45 min was chosen. This model was described as the most suitable model of chronic mild ischemia and the most representative for patients-like ischemic degrees (Goto et al., 2006). Under lower limb I/R conditions the peripheral muscles are subjected to environmental alterations produced by a higher ROS influx that leads to the depression of the inner body defense mechanism, inducing an imbalance between a burst of ROS and the inability of re-oxygenated cells to handle this radical load, leading to cell degeneration process (McCord, 1985), thus disturbing their redox stability. Moreover, although muscular damages are induced after ischemic periods, circulation re-establishment after reperfusion represent a mayor alteration in the morphological structure (Carmo-Araújo et al., 2007). As reported by other authors, after 60 min of reperfusion a muscular lesion is induced (Grisotto et al., 2000; Harman, 1946; Sagara et al., 2009).

Previous studies have reported the capacity of antioxidants to attenuate the oxidative process after hind-limb I/R (Huk et al., 1997; Koksal et al., 2003, 2004; Santos et al., 2012; Yoshihara et al., 2016). Particularly, several studies, intended to evaluate the antioxidant effect of astaxanthin in I/R models by oral and intravenous administration pathways (Deng et al., 2017; Gross and Lockwood, 2004; Li et al., 2015; Pan et al., 2017) nevertheless limitations regarding the biodistribution and stability of the molecule once inside the human or animal body must be considered. An astaxanthin *in situ* delivery in the site of interest in a controlled manner using a biocompatible scaffold system intend to address these limitations. The polymer network used in this study was obtained through the formation of phosphoester linkages between pullulan and dextran by STMP chemical cross-linking and scaffold porosity was achieved by the addition of sodium chloride (Abed et al., 2011). The fact that the implanted scaffolds remained in the site of implantation within the gracilis muscle even after 15 days of implantation confirmed the feasibility of using this system for the *in situ* delivery of an antioxidant molecule during a specific period of time. These results are in accordance with a previous work that showed the preservation of the scaffold after 30 days of incubation in a simulated physiological environment (Abed et al., 2011).

Further, the biocompatibility of P/D scaffolds had been widely evaluated *in vitro* and *in vivo*, and no negative side effects have been reported in a wide range of applications (Abed et al., 2011; Chaouat et al., 2006; Derkaoui et al., 2012; Purnama et al., 2013), but there is no data concerning the toxicity of P/D scaffolds containing the CD-A molecule. The findings presented here support the non-toxicity of the interaction between the polymer system and the antioxidant molecule evaluated. Moreover, the foreign body reactions against biomaterials such as scaffolds are controlled by macrophages responses. Macrophages are heterogeneous cell populations subtype according to their functionality as M1, which represents pro-inflammatory and tumorogenic activity macrophages, and M2 that refers to tissue repair macrophages (Barros et al., 2013; Klopfleisch, 2016). CD68 antigen is expressed in both M1 and M2 populations, representing a classic sign of inflammation (Lau et al., 2004); while CD163 is a specific marker for M2 macrophages phenotype (Barbosa et al., 2015). The histological results revealed a cellular reaction particularly important in the sham and the antioxidant groups within the areas surrounding the scaffold. This reaction represents a classic response to implanted biomaterials composed mainly of macrophages and giant cells, which play a central role in the tissue response against the presence of foreign bodies (Anderson, 2001; Anderson et al., 2008; Racz et al., 1997; Wokalek and Ruh, 1991). Additionally, a partial colocalization of CD163 surface marker with the zones also positive for CD68 in the 7 and 15 days of reperfusion, accompanied

by a cellular positivity spread along the muscle area particularly higher in the antioxidant group could suggest an immunoregulation and constructive tissue remodeling process (Badylak et al., 2008; Lau et al., 2004; Mantovani et al., 2004).

One of the most important cytoprotective system against oxidative stress is the Keap1-Nrf2 complex (Holmström and Finkel, 2014). Nrf2 is a pivotal transcription factor that regulates gene expression of intracellular antioxidants, detoxifying enzymes, and several other proteins that neutralize ROS (Li et al., 2016). Under physiological conditions, Nrf2 remains inactive in the Keap1-Nrf2 complex near the cell membrane. However, upon high ROS exposure, Nrf2 detach from Keap1-Nrf2 complex and translocates to the nucleus, stimulating antioxidant response gene expression (Mann, 2014). Nrf2 translocation promotes cell survival, preserves cellular redox homeostasis and also plays a key role in reducing inflammation (Kensler et al., 2007). In this study, the immunohistological results suggest an indirect reduction of the oxidative stress response in the antioxidant treated groups, corroborating our previous findings where we showed the capacity of CD-A to indirectly activate the endogenous antioxidant system of human endothelial cells subjected to exogenous oxidative stress by Nrf2/HO-1/NQO1 pathway. Moreover, our previous findings showed an upregulation of HO-1 and NQO-1 protein expression levels when cells received the antioxidant CD-A treatment (Zuluaga et al., 2017). Similarly, a protective effect by astaxanthin pretreatment against brain injuries expressed by a significant increase of the of Nrf2, HO-1 and NQO1 mRNA expression in a cerebral ischemia model was also reported by Lei Pan et al. (Pan et al., 2017).

Iron is a crucial component in living organisms. It plays a vital role as component of heme-containing proteins (Fe^{2+}) such as hemoglobin, myoglobin and cytochromes, and as a metal cofactor (non-heme iron, Fe^{3+}) present in several enzymes including Fe-S cluster proteins in mitochondria. Iron homeostasis has been associated with oxidative stress and ROS generation (Latunde-Dada, 2017). One of the characteristic reactions of I/R is the reduction of non-heme Fe^{3+} and release of Fe^{2+} ions (Kim et al., 2017), that are able to react with H_2O_2 to generate highly reactive hydroxyl radicals resulting in cellular injury (Balla et al., 1991; Nagy et al., 2010). Owing to its high sensitivity, the Perl's/DAB method was used to provide information about non-heme iron deposition regardless of oxidation states in normal and ischemic conditions by revealing cell population susceptible to free radical damage (Iwatsuki et al., 2008; Meguro et al., 2003). The results obtained here, indicate a response to ROS generation linked to the reperfusion duration and to the non-heme ferrous iron production, which is considered to be critically involved in free radical generation.

Although, we believe that an *in situ* delivery approach must be considered when using astaxanthin molecule to treat I/R related pathologies like chronic arterial occlusive disease of the lower extremities, we likewise recognize the limitations it imposes. The I/R model here evaluated, is in accordance with the ischemic degrees presented in patients (Goto et al., 2006). However, by inducing a mild ischemia in a specific segment of the femoral artery, it seems essential to consider the collateral arteries irrigation which could reduce the ischemic impact in the adjacent muscles such as the gracilis, and thus, making difficult to evidence the oxidative damage induced in the hind-limb and limiting the quantification of the possible treatment reached by means of antioxidant. Moreover, as reported here, even if *in vitro* results showed a high scavenging activity at 12.5 μM astaxanthin concentration, *in vivo* results reflect a preliminary finding suggesting an improvement by *in situ* release of CD-A antioxidant molecule in the oxidative stress muscle's environment generated under I/R injury but also evidenced that an extensive evaluation is still required to select the most appropriate dose needed in order to maximize the antioxidant effect.

In conclusion, the release of CD-A antioxidant through a P/D system in a damaged I/R environment induce a positive immunological response reflected by an increase in the tissue repair macrophage markers and by the indirect modulation of the Nrf2 transcription factor and the expression of a population susceptible to oxidative stress. Thereby, this study suggests that the local release of astaxanthin by a polysaccharide scaffold could decrease the damages inflicted in the muscles by I/R injury by increasing the antioxidant endogenous system environment.

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Supplementary data

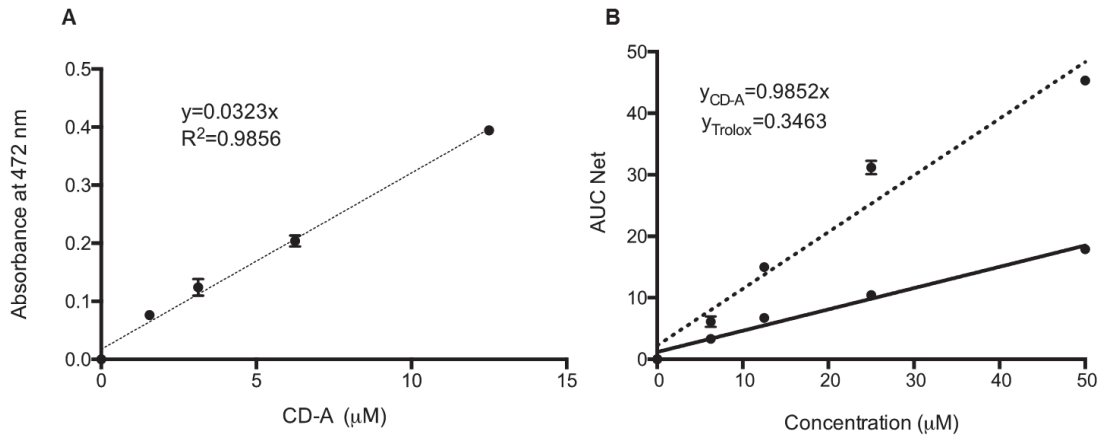


Figure S1: (A) Calibration curve of CD-A at concentration ranging from 0-15 μM measure at an absorbance of 472nm. (B) Calibration curve of CD-A and Trolox antioxidant scavenging capacities were analyzed by ORAC method; values are represented as means of AUC net against CD-A or Trolox concentration between 0-50 μM.

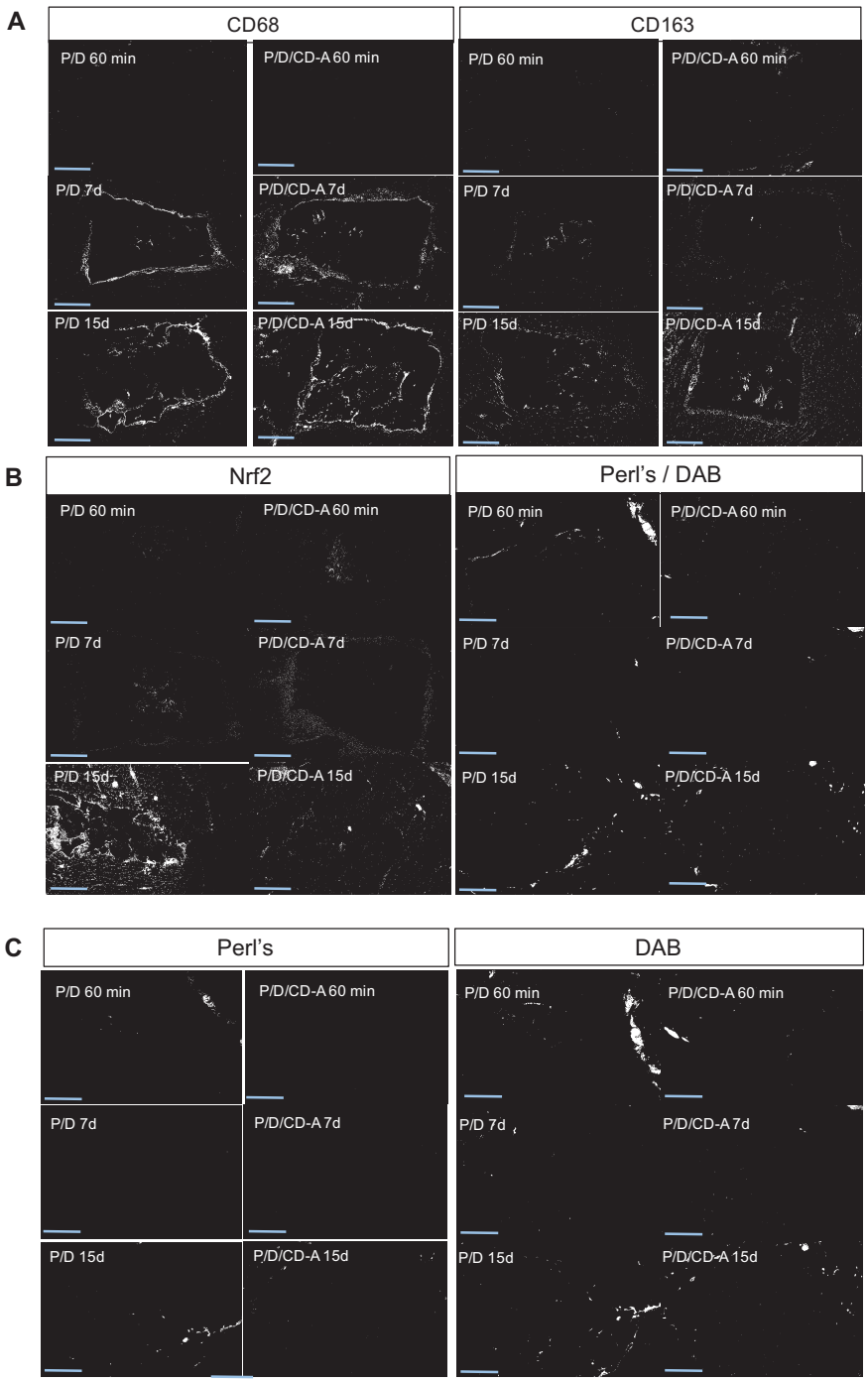
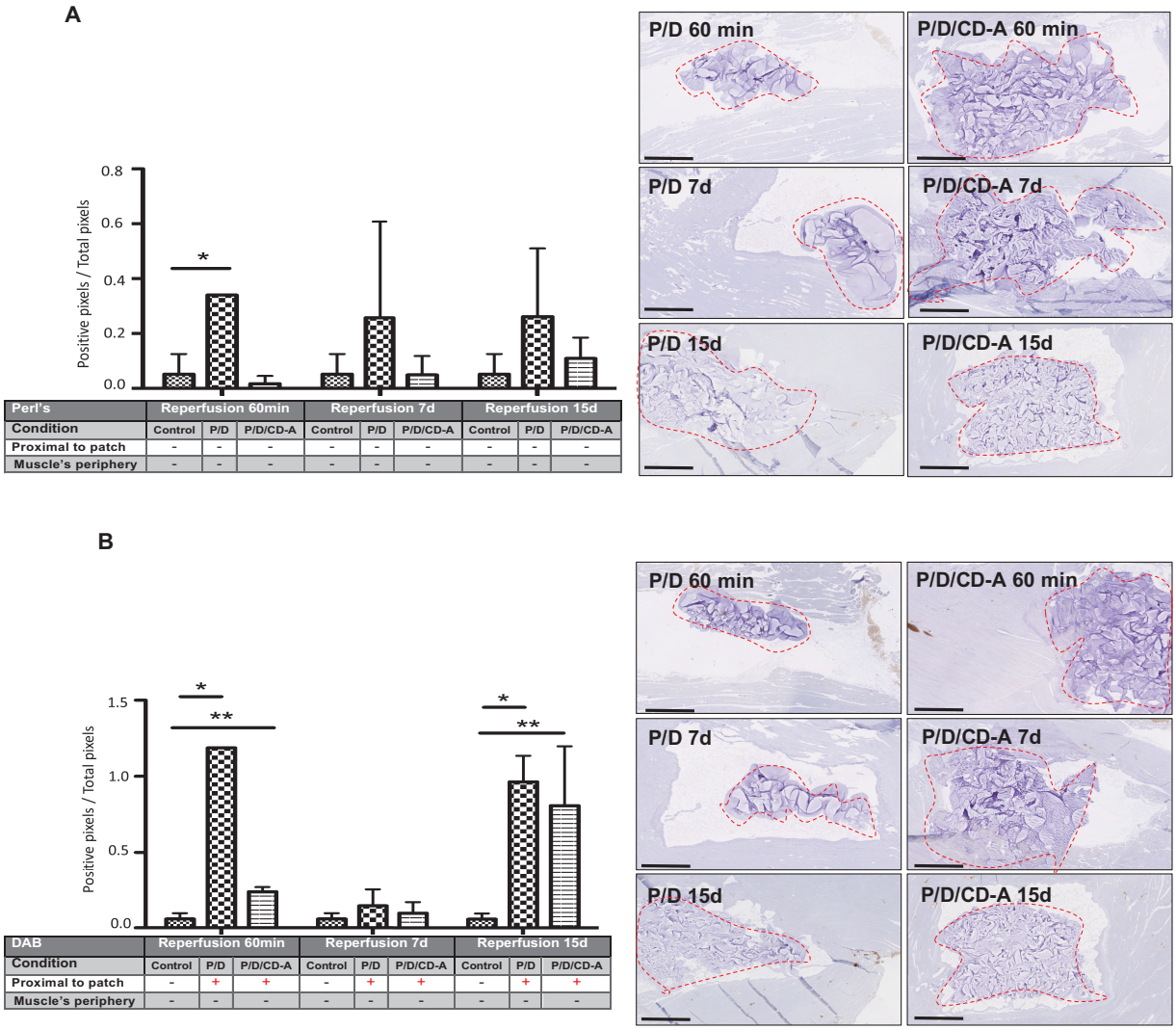


Figure S2: Quantification of positive staining using Matlab. (A) CD68 and CD163 samples, (B) Nrf2 and Perl's/DAB samples, and (C) Perl's and DAB samples. White dots in the images represent the positivity of the respective marker. Scale bars, 2 mm. Images are representing of 4 independent experiments.



Perl's	Reperfusion 60min			Reperfusion 7d			Reperfusion 15d		
Condition	Control	P/D	P/D/CD-A	Control	P/D	P/D/CD-A	Control	P/D	P/D/CD-A
Proximal to patch	-	-	-	-	-	-	-	-	-
Muscle's periphery	-	-	-	-	-	-	-	-	-

DAB	Reperfusion 60min			Reperfusion 7d			Reperfusion 15d		
Condition	Control	P/D	P/D/CD-A	Control	P/D	P/D/CD-A	Control	P/D	P/D/CD-A
Proximal to patch	-	+	+	-	+	+	-	+	+
Muscle's periphery	-	-	-	-	-	-	-	-	-

Figure S3: (A) Perl's and (B) DAB staining of gracilis muscle containing P/D and P/D/CD-A scaffold at different perfusion periods. A positivity Perl's and DAB staining was attributed to iron retention by red blood cells. Red arrows indicate the scaffold localization. Scale bars, 2 mm. Images are representing of 4 independent experiments.

General Conclusion and Future Directions

This work helps to better understand the production of ROS and the generation of cellular oxidative stress. Emphasis was placed on the potential applications of antioxidants on oxidative stress related diseases. Experimental *in vivo* and *in vitro* studies, as well as epidemiological studies, suggest an inverse correlation between severity of the disease and levels of antioxidants. Particularly, researches on carotenoids suggest that these pigmented components may be able to regulate ROS levels by acting as antioxidants, though their unique physicochemical properties or radical quenching. This work was centered on the study of a potent antioxidant carotenoid, astaxanthin which is able to counteract ROS influx *in vitro and in vivo*. Natural astaxanthin is accepted as a dietary supplement by the Unites States Food and Drug Administration (FDA) and the European regulatory authorities. Astaxanthin polar structure influence the molecule solubility in hydrophilic medium and render astaxanthin highly instable to external factors such as oxygen, light and pH, thus affecting its activity and bioavailability and compromising its possible applications. Different encapsulation systems have been proposed to breakthroughs on these issues, increasing astaxanthin protection and solubility. However, the literature lacks in the demonstrations of astaxanthin antioxidant properties after encapsulation. Reliable information on the successful performance of the proposed methods is necessary and must be carried out in order to search for a future translation in the industrial and medical fields.

Considering these limitations, an inclusion system based on hydroxypropyl-β-cyclodextrin (CD-A) was developed. Physicochemical CD-A complex characterization confirmed the successful improvement on solubility and stability properties of astaxanthin. Then, several chemical and biological tests were adapted to determine the preservation of antioxidant capabilities of complexes. Chemical methods used such as TEAC/ABTS, DPPH and ORAC, provided a first confirmation of the no degradation of astaxanthin scavenging capabilities after inclusion. These properties were stable after six months or storage at 4°C in a freeze-dried form. It is important to highlight the variability and instability of these probes due to external factors like light and temperature which can degrade the probe during the time of analysis. Other point to take into account is the lack of normalization in the protocols used for antioxidant determinations and a great variability on the solvents used to perform the same assay. For example, DPPH results widely vary dependently of the solution used to perform the assay, and there are a considerable number of protocols variations in the literature.

The results obtained on CD-A complexes were encouraging to study their biological capabilities to regulate the cellular redox state. First, we confirmed the good acceptance of these complexes

by human endothelial cells (Huvec). Then, biological detection methods of antioxidant capacities of CD-A complexes were studied using DCFH-DA, MitoSOX and C11-Bodipy probes. These assays allowed the evaluation of redox cellular levels, mitochondrial superoxide production and lipid peroxidation, respectively. Oxidative stress was induced on Huvec using different exogenous stressors, particularly antimycin A, a well know stressor of mitochondrial complex III. CD-A complex counteracted the oxidative stress induced by antimycin A, however for future works different stressor such as rotenone, which is directed against complex I, will provide additional information about CD-A effect against mitochondrial ROS regulation. Additionally, the general oxidative stress of the cell and mitochondria was analyzed, but as pointed out in chapter 1, there are other cellular organelles implicated in ROS regulation, so it will be interesting to target them using directed-ROS stressors. Moreover, direct astaxanthin antioxidant capacity was analyzed by the study of molecular signal transduction pathways. The results indicated a high scavenging capabilities of CD-A complex to target and reduce ROS levels and an indirect effect modulating the genes in charge of the regulation of endogenous antioxidant defenses. It is clearly stablished that ROS are important for cell signaling, thus to regulate their levels instead of eliminate them represent a more appropriate strategy to treat oxidative stress related pathologies.

Regarding the above information and the results presented in this thesis, it is strongly advisable to perform several chemical and biological tests to quantify either the antioxidant capacity of a component or to evaluate the scavenging ability of such component to target divers ROS and sites of production in order to reduce cellular radicals and prevent oxidative stress. Independently on how different and how sensitive to degradation these mechanisms are, all of them represent a tool to break the gap in the knowledge of radical functions and in the understanding of their action mechanisms.

Based on the positive results of CD-A obtained *in vitro* and considering the improvement of antioxidants levels in patients with I/R diseases after astaxanthin supplementation, we explored the therapeutic potential of CD-A complex to target ROS in an *in vivo* model of I/R. The first I/R approach (chapter 4), addresses the possible use of a PVA/Dex/CD-A hydrogel patches as an alternative to ameliorate the condition of the myocardial muscle after ischemia. Currently different strategies to treat I/R injury including the use of percutaneous coronary interventions are under development.

After verification of the mechanical properties of the PVA/Dex patch, a feasibility study on the load and deliver of CD-A, while preserving its scavenging capabilities was conducted. Here, a new antioxidant system able to regulate the induced oxidative stress environment was drawn up. The hydrogel patches showed high cell compatibility, *ex vivo* suturability and allowed the *in vivo* astaxanthin release. The next steps for the PVA/Dextran/CD-A patches evaluation will consist in the validation of the system using a myocardial I/R *in vivo* model.

The second approach (chapter 5) consisted in an *in vivo* proof of concept of Pullulan/Dextran/CD-A scaffolds as antioxidant delivery system. It is important to recall that this is the first time that the protective action of a CD-A system is tested in an *in vivo* I/R model. This system was developed for the treatment of the lower hind limb muscle damage induced by arterial occlusion and reperfusion in a rat model. Pullulan/Dextran/CD-A scaffolds generated an immune response attributed to a normal body reaction to the presence of foreign materials, showing a tendency to the reduction of oxidative stress status when CD-A complex was locally delivered into the I/R muscle. As a proof of concept there are still some parameters to be settled such as the correct dose required to obtain a higher ROS regulation, or the most suitable delivery system used to treat this kind of pathologies.

A future approach to improve the target of ROS influx *in vivo* in a model of hind limb I/R, could consist in the injection of nanosystems as CD-A delivery carriers, which are less invasive, and could decrease the inner immunological response induced but the scaffold systems. It seems important to considerer that animal models do not represent the human pathologies because of the anatomic/pathological differences, particularly, rodents have a vascular structure constantly irrigated by diffusion, which limits the ischemic effect. However, animal models still represent a first critical approach to the understanding of the pathophysiology of human conditions such as oxidative stress related diseases.

For the future of this project, it will be necessary to consider the use of astaxanthin not only to treat diseases but also as a preventive agent by including astaxanthin as supplement in the animal diet. This would probably increase the scavenging capabilities of the molecule and by consequence reduce the impact of oxidative stress diseases. Using astaxanthin as supplement in the animal diet combined with a mixed drug delivery strategy incorporating different target molecules like anti-thrombotic agents or a mixture of antioxidants to improve the regulation of ROS, represents a future approach to be considered as a support to reduce the generation of

oxidative stress diseases. Additionally, besides the already known positive effect showed after astaxanthin dietary intake as supplement, there is still a gap in the understanding of astaxanthin antioxidant mechanism to counteract the damaging effect induced by ROS overconcentration in oxidative stress related diseases before being used as potential clinical targets.

During the development of this thesis, different people expressed their beliefs that using antioxidant as target agent do not represent a good strategy to treat diseases, as well as other who strongly believe in the antioxidant potential of these molecules. Using antioxidants will not represent the “miraculous” strategy to be adopted specially under redox homeostasis, but as the human body has a powerful endogenous antioxidant machinery, it will be able to eliminate them if they are not required to ensure the correct functioning in the body. Whereas in case of necessity these molecules will boost the antioxidant inner action working in synergy to protect the body from oxidative stress related diseases.

After three years working on antioxidant astaxanthin, this manuscript provides support evidence to the powerful antioxidant capabilities of astaxanthin *in vitro and in vivo*, and it paves the way to the use of carotenoid like antioxidant systems to treat oxidative stress related diseases like cardiovascular pathologies.

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Résumé :
Une présence en excès d'espèces réactives oxygénées induit un déséquilibre redox cellulaire pouvant conduire à des pathologies liées au stress oxydatif, notamment les pathologies cardiovasculaires. Connue et étudiée pour ses propriétés antioxydantes, l'astaxanthine, molécule de la famille des caroténoïdes, présente un intérêt thérapeutique potentiel. Cependant, sa structure chimique lui confère un caractère hydrophobe ainsi qu'une sensibilité à l'air, à la lumière et à la chaleur. Dans cette thèse, tout d'abord, un système de complexation de l'astaxanthine avec l'hydroxypropyl-β-cyclodextrine a été élaboré (CD-A). Nous démontrons que cette complexation améliore la stabilité de l'astaxanthine en solution aqueuse tout en préservant ses activités antioxydantes, mesurées par des méthodes chimiques et biologiques. L'action du CD-A semble être médiée par les voies de signalisation PTEN/AKT, Nrf2/OH1/NQO1 dans des cellules endothéliales soumises au stress oxydatif. Puis, afin de libérer l'astaxanthine in *situ* sur le site du stress, nous avons élaboré deux systèmes de type matriciel en PVA/dextrane ou en pullulane/dextrane chargés en CD-A. Nous avons évalué, comme preuve de concept, la faisabilité de ces dispositifs pour le traitement local de la pathologie d'ischémie/reperfusion. Les patchs de PVA/dextrane/CD-A ont montré une bonne compatibilité *in vitro*, ainsi qu'une grande stabilité et tenue mécanique sans modification des propriétés antioxydantes. Leur biocompatibilité in *vivo* et suturabilité au muscle cardiaque ont aussi été étudiées. Le deuxième système à base de pullulane/dextrane/CD-A a été évalué *in vitro* et *in vivo* dans un modèle d'ischémie/reperfusion du membre inférieur à différentes périodes d'implantation. Les résultats ont montré l'activation d'un mécanisme de défense normal lié à la présence d'un matériel étranger et une diminution de la translocation du Nrf2 pouvant indiquer un effet protecteur dans les tissus traités par le CD-A. Ce manuscrit présente des arguments en faveur du potentiel thérapeutique de systèmes de libération d'astaxanthine agissant au niveau du stress oxydatif lié aux pathologies cardiovasculaires.

Mot clés : Espèces réactives oxygénées; Stress oxydatif; Antioxydants; Astaxanthine; Ischémie/Reperfusion; Biomatériaux, Systèmes de libération.

Abstract
An overconcentration of reactive oxygen species induces a redox imbalance within the cell inducing oxidative tissue damage and leading to oxidative stress related diseases, particularly cardiovascular pathologies. Astaxanthin, a well-known and studied antioxidant molecule, member of the xanthophyll carotenoid family, presents an important therapeutic potential. However, the chemical structure confers to astaxanthin a hydrophobic character and renders it susceptible to air, light and temperate degradation. During this thesis, a carrier system based on astaxanthin inclusion within hydroxypropyl-β-cyclodextrin (CD-A) was developed. We demonstrate that after astaxanthin inclusion, not only its stability was enhanced by also the antioxidant scavenging capabilities were preserved, confirmed by chemical and biological tests. The action of CD-A seems to be mediated by PTEN/AKT, Nrf2/OH1/NQO1 signaling pathways of endothelial cells submitted to oxidative stress. Then, two systems based on PVA/dextran and Pullulan/Dextran loaded within CD-A were evaluated for astaxanthin *in situ* delivery in the stressed environment. The feasibility of using these systems in the local treatment of ischemia/reperfusion injury was evaluated as a proof of concept. PVA/Dextran patches showed good *in vitro* compatibility, high mechanical and stability properties, while preserving CD-A antioxidant capabilities, also the path suturability to the cardiac muscle and the *in vivo* biocompatibility were studied. The second system based on pullulan/dextran scaffolds were evaluated in vitro and in vivo in an ischemic/reperfusion model at different implantation periods. Results showed an inner body defense mechanism to foreign materials. Additionally, the Nrf2 translocation could indicate a protective effect of CD-A in treated tissues. This manuscript provides a support evidence of the therapeutic potential of CD astaxanthin delivery system, to act against oxidative stress linked to cardiovascular conditions.

Key words: Reactive oxygen species; Oxidative stress; Antioxidants, Astaxanthin, Ischemia/reperfusion; Biomaterials; Drug delivery systems.