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Inflammatory pathways in granulosa cells: exploring pharmacological significance of chalcones

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Abstract

The inflammatory state found in the ovaries during ovulation can disrupt normal follicular dynamics, being associated with reduced oocyte quality and infertility. Neutrophils and macrophages are the first immune cells to be recruited to the inflammation site. These cells are involved in the production of reactive oxygen and nitrogen species and induction of cyclooxygenase-2 (COX-2) and 5-, 12-, and 15-lipoxygenases (LOXs) expression by granulosa cells (GCs). As consequence, occurs an increase in bioactive lipids synthesis that may exhibit pro-inflammatory actions, such as eicosanoids, or anti-inflammatory, such as specialized pro-resolving mediators. Since GCs are directly in contact with the oocyte, they play a key role in their development and maturation. However, imbalanced production of either pro- or anti-inflammatory mediators can impact oocyte quality. Additionally, the persistence of the inflammatory state in the ovaries may trigger pathologies associated with the reproductive system, such as endometriosis and polycystic ovary syndrome. Since chalcones show antioxidant and anti-inflammatory properties, they may be useful in the nutritional supplementation of women undergoing assisted reproductive techniques, thus modulating the oxidative/nitrosative stresses and inflammatory states occurring in GCs. In this sense, the first aim of this thesis was to investigate the antioxidant activity of a panel of 34 structurally related chalcones, in terms of their ability to scavenge and/or inhibit the formation of hypochlorous acid (HOCl), the most powerful ROS produced during the neutrophils' oxidative burst. According to the results obtained, it was possible to observe that the ability of chalcones to scavenge HOCl depends on the position and number of hydroxyl groups present in aromatic rings A and B. Regarding the inhibition of the neutrophils' oxidative burst, the activity of chalcones depends on the presence of a hydroxyl group at C-2' of aromatic ring A and groups/atoms, such as methoxyl, hydroxyl, nitro, and/or chlorine, at C-2, -3 and/or -4 of aromatic ring B. Following, the ability of M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophages in the activation of inflammatory pathways in GCs was investigated. The results obtained showed that M1 and M2 macrophages were able to increase the expression of 12-LOX and COX-2, respectively. However, no significant difference was observed in the expression of 5-LOX and 15-LOX. Overall, it can be concluded that chalcones showed antioxidant activity against neutrophils and, the production of anti-inflammatory mediators by GCs, at least in part, was able to overturn the inflammation present in the ovaries.

Keywords: Inflammation, infertility, granulosa cells, macrophage, neutrophil, chalcone.

Resumo

A inflamação presente nos ovários pode desestabilizar o desenvolvimento normal dos folículos, levando a redução da qualidade dos ovócitos e infertilidade. Os neutrófilos e macrófagos são as primeiras células do sistema imunitário a serem recrutadas para o local da inflamação. Estas células têm como funções a produção de espécies reativas de oxigénio e nitrogénio, além de induzirem a expressão da ciclooxigenase-2 (COX-2) e 5-, 12-, e 15-lipoxigenases (LOXs) pelas células da granulosa (CGs). Em consequência, ocorre a formação de lípidos bioactivos que podem exercer ação pró-inflamatória, como os eicosanoides, ou anti-inflamatória, como os mediadores "pro-resolving". Uma vez que as CGs estão em contato direto com o ovócito, elas desempenham um papel fundamental em seu desenvolvimento. Entretanto, a produção excessiva de mediadores pró-inflamatórios pode impactar na qualidade dos ovócitos. A persistência do processo inflamatório nos ovários pode desencadear patologias associadas ao sistema reprodutor, como a endometriose e a síndrome dos ovários poliquísticos. Chalconas são uma família de compostos que apresentam propriedades antioxidante e anti-inflamatória. Desta forma, estes compostos podem ser úteis na suplementação nutricional de mulheres submetidas a técnicas de reprodução assistida, através da modulação do stress oxidativo/nitrosativo e processo inflamatório observados nos ovários. Neste sentido, o primeiro objetivo desta tese foi investigar a atividade antioxidante de um grupo de 34 chalconas, quanto à sua habilidade em captar e/ou inibir a produção de ácido hipocloroso (HOCl), espécie reativa com maior poder oxidante produzida durante o burst oxidativo nos neutrófilos. De acordo com os resultados obtidos, foi possível observar que a habilidade das chalconas em captar o HOCl depende da posição e do número de grupos hidroxilos nos anéis aromáticos A e B. No que diz respeito à inibição do burst oxidativo, a atividade das chalconas depende da presença de um grupo hidroxilo no C-2' do anel aromático A e de grupos/átomo, como metoxilo, hidroxilo, nitro e/ou cloro, no(s) C-2, -3 e/ou -4 do anel aromático B. A seguir, foi investigada a capacidade dos macrófagos M1 (pró-inflamatórios) e M2 (anti-inflamatórios) em modular a ativação de vias inflamatórias nas CGs. Os resultados obtidos demonstraram que os macrófagos M1 e M2 induziram a expressão da 12-LOX e COX-2, respectivamente. Porém, não foram observadas diferenças na expressão da 5- e 15-LOX. Portanto, pode-se concluir que as chalconas apresentaram atividade antioxidante perante aos neutrófilos e que a produção de mediadores anti-inflamatórios pelas CGs, pelo menos em parte, foi capaz de balancear o processo inflamatório presente nos ovários.

Palavras-chave: Inflamação, infertilidade, células da granulosa, macrófago, neutrófilo, chalcona.

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Abbreviation list

4CL	4-coumarin-CoA ligase
8-OHdG	8-hydroxy-2-deoxyguanosine
AA	Arachidonic acid
ALDH	Aldehyde dehydrogenase
ART	Assisted reproductive technology
BMP	Bone morphogenetic protein
C4H	Cinnamic acid 4-hydroxylase
CA	*Corpus albicans*
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CC	Cumulus cell
cGMP	Cyclic guanosine monophosphate
CHI	Chalcone isomerase
CHS	Chalcone synthase
CL	*Corpus luteum*
CM	Conditioned media
COC	Cumulus-oocyte complex
COX	Cyclooxygenase
CYP	Cytochrome
DHA	Docosahexaenoic acid
DHR	Dihydrorhodamine 123
DPA	Docosapentaenoic acid
EMC	Extracellular matrix component
eNOS	Endothelial nitric oxide synthase
EPA	Eicosapentaenoic acid
ETC	Electron transport chain
FF	Follicular fluid
FSH	Follicle-stimulating hormone
GC	Granulosa cell
GDF	Growth differentiation factor
GnRH	Gonadotropin-releasing hormone
GPx	Glutathione peroxidase
GSH	Glutathione

HA	Hyaluronic acid
hCG	Human chorionic gonadotropin
HO	Heme oxygenase
HETE	Hydroeicosatetraenoic acid
HPO	Hypothalamic-pituitary-ovarian
Hx	Hepoxilin
ICSI	Intracytoplasmic sperm injection
IFN- γ	Interferon-γ
IGF	Insulin-like growth factor
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IVF	*In vitro* fertilization
LH	Luteinizing hormone
LT	Leukotriene
LOX	Lipoxygenase
LX	Lipoxin
MaR	Maresin
MCP	Monocyte chemoattractant protein
MDM	Monocyte-derived macrophage
MMP	Matrix metalloproteinase
MGC	Mural granulosa cell
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa B
nNOS	Neuronal nitric oxide synthase
NOS	Nitric oxide synthase
NPD	Neuroprotectin
Nrf	Nuclear factor erythroid
NS	Nitrosative stress
NSAID	Non-steroidal anti-inflammatory drug
OS	Oxidative stress
OSF	Oocyte secreted factor
oxoETE	Oxoeicosatetraenoate
PAF	Platelet-activating factor
PAL	Phenylalanine ammonia lyase

PCOS	Polycystic ovary syndrome
PD	Protectin
PDGF	Platelet-derived growth factor
PG	Prostaglandin
PGHS	Prostaglandin H_2 synthase
PGI	Prostacyclin
PLA	Cytosolic phospholipase A
PMN	Polymorphonuclear leukocyte
POX	Peroxidase
PRDX	Peroxiredoxin
PUFA	Polyunsaturated fatty acid
RT-PCR	Reverse transcription polymerase chain reaction
Ref	Reduction-oxidation factor
RNS	Reactive nitrogen specie
ROS	Reactive oxygen specie
RS	Reactive specie
Rv	Resolvin
SAR	Structure-activity relationship
SOD	Superoxide dismutase
SPM	Specialized pro-resolving mediator
TAL	Tyrosine ammonia lyase
TC	Theca cell
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-alpha
TX	Thromboxane
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

Chapter I - Introduction

1 Inflammation

Inflammation is a nonspecific physiological defensive response of the innate immune system triggered by harmful stimuli, such as infection or tissue injury (trauma, allergens, irritants, and toxic compounds), that aims to restore homeostasis [1]. In the 1st century AD, the Roman doctor Cornelius Celsus defined the first symptoms of inflammation as the four classic signals: *rubor* (redness), *tumor* (swelling and oedema), *calore* (fever), and *dolore* (pain). Later, in 1858, Rudolph Virchow established the fifth classic signal of inflammation, *functio laesa* (loss of function), the only universal signal that accompanies all inflammatory processes [2].

In 2017, it was suggested that the typical definition of inflammation should be reconsidered to "*innate immune response to harmful stimuli, such as pathogens, injury, and metabolic stress*" [3]. The reason for this change considers the existence of pathologies that are not accompanied by the classic signals of inflammation, but that are responsible for its development, such as depression and obesity [3]. These conditions reflect a context of metabolic stress, sustaining the inflammatory response and causing an imbalance in body homeostasis [4].

The inflammatory response is a progression of coordinated events involving cellular, molecular, and physiological changes to restore affected tissues to their regular structural and functional state. Nonetheless, uncontrolled or unsuccessful immune responses can lead to continued recruitment of inflammatory cells. The persistence of tissue damage results in organ dysfunction and, consequently, in the establishment of a chronic inflammatory process, such as cardiovascular and respiratory diseases, cancer, rheumatoid arthritis, and atherosclerosis [5, 6].

1.1 The Inflammatory Response

The universal inflammatory response is initiated by inducers, which can be exogenous or endogenous signals, activating specialized sensors. These, in turn, promote the production of mediators, altering the functional states of tissues and organs, which are the effectors of inflammation [7].

The acute inflammatory response, the **onset phase**, begins with the recognition of initial stimuli by Toll-like receptors. Then, soluble mediators, such as cytokines, chemokines, reactive species, and bioactive lipids are released by tissue-resident cells, such as mast cells and macrophages, in injured or infected tissues [8-10]. Circulating neutrophils and endothelial cells up-regulate the expression of adhesion molecules, while

vascular changes occur, facilitating the protein discharge and influx of leukocytes from the blood into the tissues. Neutrophils, namely polymorphonuclear leukocytes (PMNs), function as phagocytic agents to remove tissue debris and pathogens through intra and/or extracellular mechanisms, such as the production of superoxide anion ($O_2^{\bullet -}$) radicals and the release of myeloperoxidase (MPO), neutrophil extracellular traps (NETs), proteases, and lactoferrin [10]. Thus, the onset of inflammation is accompanied by a sequence of events that aim to balance and control the inflammatory response. Inflammation 'stop signals' consist of negative feedback regulators, such as RNA gene regulators, transcription factors, anti-inflammatory cytokines, among others, which stop the production of pro-inflammatory mediators and can counter-regulate pro-inflammatory signalling pathways (**Figure 1**) [8].

The **resolution phase** consists of the interval between the peak of cellular influx and its clearance from the tissue site, restoring functional homeostasis. First, harmful agents are eliminated, the synthesis of the pro-inflammatory mediators is suspended, and any remaining mediators are catabolized, suspending neutrophil recruitment and oedema formation. Neutrophils then undergo apoptosis or local necrosis and are eliminated by efferocytosis by recruited monocyte-derived macrophages (MDMs) [10]. Successfully, efferocytosis stimulates the reprogramming of macrophages from inflammatory phenotype to resolving phenotype, also called classically (M1) and alternatively activated (M2) macrophages, respectively. During inflammation, the phenotypic change of macrophages is considered a key step in the resolution process. Anti-inflammatory mediators, such as interleukin (IL)-10, transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor-α (VEGF-α), are produced by polarized M2 macrophages, promoting cell proliferation and the development of new blood vessels. These factors also stimulate the differentiation of fibroblasts into myofibroblasts, remodelling of extracellular matrix component (EMC), stimulation of collagen synthesis, angiogenesis, in addition to restoring oxygen supply in wound healing, leading to complete tissue recovery. After the efferocytosis is over, macrophages may leave the inflamed site by lymphatic drainage or undergo local apoptosis [11] (**Figure 1**).

In the last phase of the inflammatory response, called the **post-resolution phase**, there is an influx of new immune cells into the tissues, persisting for weeks (T- and B-lymphocytes), and an association of tissue-resident macrophages and dendritic cells. Therefore, this phase is effective in handling adaptive immune responses and maintaining tolerance, regaining an "adapted homeostasis" status (**Figure 1**) [10].

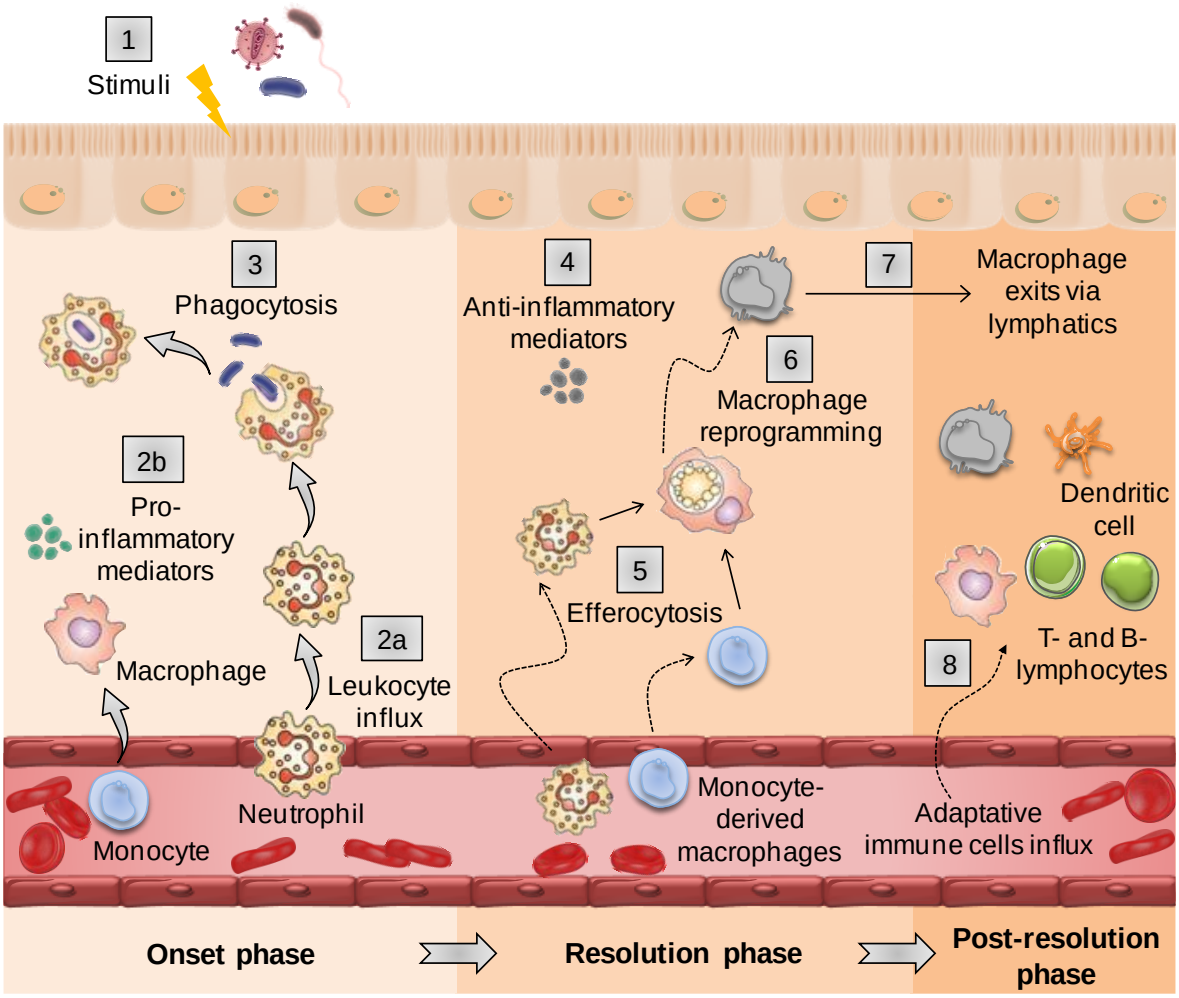


Figure 1: Phases and sequence of events that occur during the inflammatory response. The Inflammatory response is initiated by initial stimuli (**1**) that trigger the leukocyte influx (**2a**) from the blood to affected tissue and the release of pro-inflammatory mediators (**2b**). Recruited leukocytes phagocytize the pathogens and tissue debris (**3**). The resolution phase is characterized by the production of anti-inflammatory mediators (**4**), followed by neutrophil apoptosis and efferocytosis (**5**). The resolution cascade is amplified *via* further production of pro-resolving mediators by the reprogramming of macrophages from classically (M1) to alternatively activated (M2) phenotypes (**6**). Macrophages leave the inflammatory site *via* migration to the lymphatics (**7**). After resolution, the tissue is repopulated by adaptive and resident immune cells, such as dendritic cells, macrophages, and T- and B-lymphocytes, regaining a status of 'adapted homeostasis'.

1.2 Mediators of the Inflammatory Response

1.2.1 Reactive Species

Reactive species (RS) are molecules that contain unpaired electrons in their outer orbit, making them highly reactive compounds. The transfer of electrons to neighbouring molecules leads to the production of radical or non-radical species [12]. In living systems,

reactive oxygen species (ROS) are represented by oxygenated molecules, such as radicals, $O_2^{\bullet -}$ and hydroxyl ($HO^{\bullet}$) and; non-radicals, such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and singlet oxygen ($^1O_2$). Reactive nitrogen species (RNS) are represented by nitrogen molecules, such as radicals, nitric oxide ($^{\bullet}NO$) and nitrogen dioxide ($^{\bullet}NO_2$) and; non-radicals, such as peroxynitrite ($ONOO^-$), peroxynitrite acid (ONOOH), nitrate (NO_3^-), and nitrite (NO_2^-) [13].

Under physiological circumstances, the balance between pro-oxidant and antioxidant molecules moderately favours pro-oxidants, culminating in the establishment of mild oxidative stress (OS). This condition can be defined as an excessive amount of ROS as a consequence of an increase in its generation and/or a reduction in the physiological activity of the antioxidant defences [14].

Cells usually tolerate this mild OS, which can even up-regulate signalling pathways and redox regulation. Low levels of ROS can stimulate the production of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-1β, and interferon-γ (IFN-γ); apoptosis; proliferation and migration of vascular cells; activation of cancer pathways, and regulation of the expression of antioxidant genes, such as reduction-oxidation factor-1 (Ref-1), nuclear factor erythroid 2–related factor 2 (Nrf-2), and thioredoxin [14].

Cell redox homeostasis is maintained by its endogenous antioxidant defence systems including enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx); and non-enzymatic ones, such as glutathione (GSH), proteins, uric acid, coenzymeQ10, and lipoic acid. Exogenous antioxidants, such as vitamin C and E, carotenoids, and phenolic compounds present in fruits, vegetables, and whole grains, are also the counterpart of endogenous antioxidant defence activity [15].

The uncontrolled production or the inefficiency of endogenous antioxidant systems in removing the excessive amount of ROS/RNS leads to their accumulation and, consequently, to the disruption of redox signalling. This mechanism can be harmful to several cellular structures, such as lipid membranes, proteins, and DNA. In addition, the development of chronic inflammation pathologies, such as cancer, cardiovascular, neurological, respiratory and kidney diseases, rheumatoid arthritis, and immature sexuality, are strongly related to OS/nitrosative stresses (NS) conditions [16].

1.2.1.1 Reactive Species during an Inflammatory Response

In inflammatory processes, ROS/RNS can be produced through the electron transport chain (ETC) in mitochondria as by-products of cellular metabolism, as well as by cytochrome (CYP) P450. However, the main source of ROS/RNS during inflammation is the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a multi-subunit enzyme present in phagocytic cells, such as neutrophils and macrophages [17].

As mentioned earlier, neutrophils are well recognized as an important player during acute inflammatory response. They can phagocyte pathogens in vacuoles, called phagosomes, eliminating them through the production of ROS/RNS by activation of NADPH oxidase [18]. The prompt release of large amounts of ROS/RNS during phagocytosis or in response to an inflammatory stimulus is known as an oxidative burst [19].

NADPH oxidase comprises a family of seven homologs, NOX1-NOX5 and Duox1 and Duox2, differing in their structures, expression levels, and activation mechanisms. The catalytic core of NADPH oxidase consists of the membrane subunits $gp91^{phox}$ and $p22^{phox}$ forming the flavocytochrome b_{558} complex, along with the regulatory cytosolic subunits $p47^{phox}$, $p40^{phox}$, $p67^{phox}$, and GTPase RAC2. In resting neutrophils, NADPH oxidase is activated by pathogens and inflammatory mediators, triggering the phosphorylation of $gp91^{phox}$, activation of GTPase RAC2, and the translocation of cytosolic subunits to the flavocytochrome b_{558} complex. Assembly of all NAPDH oxidase subunits results in the transfer of electrons from the oxidation of NADPH (electron donor) to molecular oxygen (O_2), culminating in the formation of $O_2^{\bullet-}$ in the phagosome [20].

The half-life of $O_2^{\bullet-}$ is very tight (10^6 ns) and, therefore, it can produce H_2O_2 by spontaneous dismutation or by a SOD-catalyzed reaction. Under physiological conditions, H_2O_2 can be detoxified into O_2 and H_2O by the action of CAT; reacts with metal ions (Fe^{2+} and Fe^{3+}) to provide $HO^{\bullet}$ and hydroxide ion (HO^-); or consumed by MPO, an enzyme released in the phagosome. In the presence of Fe^{3+} and halides (Cl^-), MPO uses H_2O_2 to produce HOCl, the strongest bactericidal and oxidizing agent. HOCl, in turn, can react with amines ($R\text{-}NH_2$) to give chloramines (R-NH-Cl); with $O_2^{\bullet-}$ to re-supply $HO^{\bullet}$; and with H_2O_2 to produce $^1O_2$ (**Figure 2**) [21].

$^{\bullet}NO$, the main RNS mediator in the inflammatory response, is also generated by neutrophils, resulting from the metabolism of L-arginine by nitric oxide synthase (NOS).

This enzyme exists in different isoforms: inducible (iNOS), neuronal (nNOS), and endothelial (eNOS). The expression of iNOS is induced by pro-inflammatory cytokines, such as TNF-α and IFN-γ, and is determined by *de novo* iNOS mRNA and protein synthesis. ·NO and O_2·⁻ are poorly reactive but in the phagosome, they will readily react to each other, leading to additional production of $ONOO^-$ (**Figure 2**) [21].

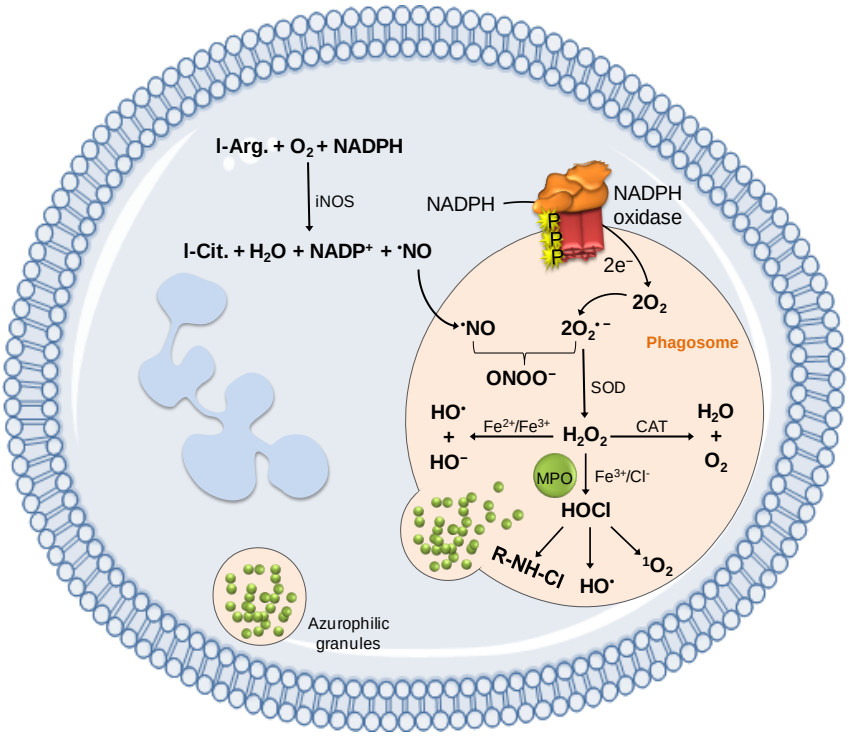


Figure 2: Schematic representation of ROS/RNS production. The production of ROS/RNS is initiated by the transfer of electrons from the NADPH to O_2. O_2·⁻ reacts with SOD, giving rise H_2O_2. This RS can be metabolized into H_2O and O_2 by CAT; reacts with $Fe^{2+/3+}$ to provide HO· and HO^-; or consumed by MPO, originating HOCl. This RS, in turn, can react with R-NH_2 to give R-NH-Cl; with O_2·⁻ to re-supply HO·; or with H_2O_2 to produce $^1O_2$. ·NO is generated from the metabolism of L-arginine and reacts with O_2·⁻, leading to the production of $ONOO^-$. CAT - catalase; iNOS - inducible nitric oxide synthase; L-Arg - L-arginine; L-Cit - L-citrulin; MPO - myeloperoxidase; NADPH - nicotinamide adenine dinucleotide phosphate; SOD - superoxide dismutase.

1.2.2 Bioactive Lipids

Bioactive lipids are mediators that are part of a complex network, modulating cellular and molecular mechanisms involved in health and inflammatory processes. According to their biochemical functions, these mediators can be divided in pro-inflammatory eicosanoids and specialized pro-resolving mediators (SPMs). During the

resolution phase, innate immune cells recruited to inflammatory sites begin to produce SPMs. These lipids control the end of inflammation, leading to the recovery of tissue homeostasis through the activation of resolution signals: removal, relief, restoration, regeneration, and remission. However, if the initiator of inflammation is not properly extinguished, it turns into chronic inflammation resulting in abnormal tissue remodelling and organ dysfunction. There is a set of enzymes that actively participate in the regulation of bioactive lipids production during the inflammatory response: cyclooxygenase-1 and -2 (COX-1 and -2) and 5-, 12- and 15-lipoxygenase (5-, 12-, and 15-LOX) [22].

COXs, also called prostaglandin H_2 synthase (PGHS), are bifunctional enzymes featuring cyclooxygenase and peroxidise (POX) active sites that give rise to eicosanoids with inflammatory action: prostaglandins (PGD_2, PGE_2, and $PGF_{2\alpha}$), prostacyclin (PGI_2), and thromboxane A_2 (TXA_2). There are two main isoforms associated with the metabolism of arachidonic acid (AA) released from lipid bilayer by phospholipase A_2 (PLA_2): COX-1 and -2 (**Figure 3**) [22]. These enzymes share nearly 60% of homology and exhibit three structurally distinct domains: *N*-terminal epidermal growth factor domain, membrane-binding domain, and C-terminal catalytic domain. The active sites of COX and POX are spatially distributed differently in the catalytic domain, with the prosthetic group heme located at the base of the POX site [6].

COX-1 is a membrane-bound enzyme found essentially in the endoplasmic reticulum, being constitutively expressed in most tissues. This isoform is intrinsically associated with the production of mediators involved in homeostatic functions, such as vascular homeostasis, platelet activity, generation of cytoprotective PGs, and reduction of gastric acid and increase of mucus and bicarbonate secretion in the stomach, kidney and uterus, where it helps the menstruation process and the onset of labour [23, 24].

COX-2, constitutively present in the kidney, brain, ovary, and uterus, has its expression induced by inflammatory triggers in monocytes, synovial cells, and fibroblasts. This enzyme can be found in the endoplasmic and nuclear reticulum. Although this isoform is mainly involved in the production of mediators responsible for the development of inflammation, it also plays some homeostatic functions, such as the blood flow regulation in the newborn, reproduction, and bronchoprotection function [24].

COX-3, a third isoform derived from COX-1 genes, was first described during the investigation of the mechanism of action of acetaminophen in dogs. After that, they

showed that COX-3 was also expressed in the cerebral cortex and heart of humans, being selectively inhibited by some non-steroidal anti-inflammatory drugs (NSAIDs) [25].

NSAIDs are a class of medications widely prescribed worldwide in pain and inflammation therapy, with 27 drugs approved on the market for clinical use in humans [26]. These anti-inflammatory agents can suppress the pro-inflammatory PGs biosynthesis through the inhibition of COX activity. Depending on which isoform will be affected, the therapeutic and side effects can be different. Most NSAIDs inhibit both COX isoforms in a range of selectivity degrees. Gastrointestinal side effects caused by COX-1 inhibition promoted the development of COX-2-selective inhibitors, "coxibs" [27]. Considering that all anti-inflammatory drugs available in the market exhibit diversified side effects, efforts are continually being made to develop safer and tolerable new agents that avoid potential side effects. Given this purpose, a diversity of compounds has been designed, synthesized, and evaluated for their potential analgesic and anti-inflammatory activities. Based on the chemical structures scaffold, the new compounds under development can be divided into groups: structural analogues of marketed drugs, synthetic compounds, and compounds based on natural products, which include chalcones that will be described below [6].

LOXs are non-heme iron-containing dioxygenases responsible to produce the most prominent eicosanoids: leukotrienes (LTA_4, LTB_4, LTC_4, LTD_4, and LTE_4), lipoxins (LXA_4, and LXB_4), oxoeicosatetraenoates (oxoETEs), and hepoxilins (HxA and HxB) (**Figure 3**) [23]. These lipid mediators are involved in physiological functions, such as skin barrier formation, cell differentiation and immunity, and in the inflammatory diseases, such as asthma, diabetes, Parkinson, and Alzheimer [28]. In humans, three main LOX isoforms were identified expressed in epithelial, endothelial, and immune cells, namely 5-, 12-, and 15-LOX [23]. It is important to emphasize that LOX enzymes require a latency period for the activation of the inactive ferrous form to an active ferric form by O_2 or lipid hydroperoxides [29]. Mammalian LOXs are proteins composed of two structurally distinct domains: an *N*-terminal domain and a C-terminal catalytic domain [23].

Since the participation of LOX isoforms in the pathogenesis of several human diseases was reported, LOX inhibitors became of interest from a pharmaceutical point of view. The unique 5-LOX inhibitor approved for use in humans is the zileuton, a leukotriene synthesis inhibitor indicated to the treatment of asthma. However, due to its pharmacokinetic disadvantages and the occurrence of hepatotoxicity, it has not gained wide acceptance [28].

COXs, LOXs, and CYP enzymes are also involved in the production of anti-inflammatory SPMs by macrophages, neutrophils, hypoxic endothelia, and platelets. These mediators are originated from the metabolism of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), and to a minor extent, AA. According to the polyunsaturated fatty acids (PUFA) that give rise to these mediators, they can be divided into classes: E-series resolvins (RvE_{1-3}) derived from the action of COX-2, CYP450 and 5-, 12-, and 15-LOX on EPA; D-series resolvins (RvD_{1-6}), maresins (MaR_{1-2}), and neuroprotectins/protectins (PD_1/NPD_1) derived from the action of 5-, 12-, and 15-LOX on DHA; and 13-series resolvins (RvT_{1-4}) derived from the action of COX-2 and 5-, 12-, and 15-LOX on DPA (**Figure 3**) [30].

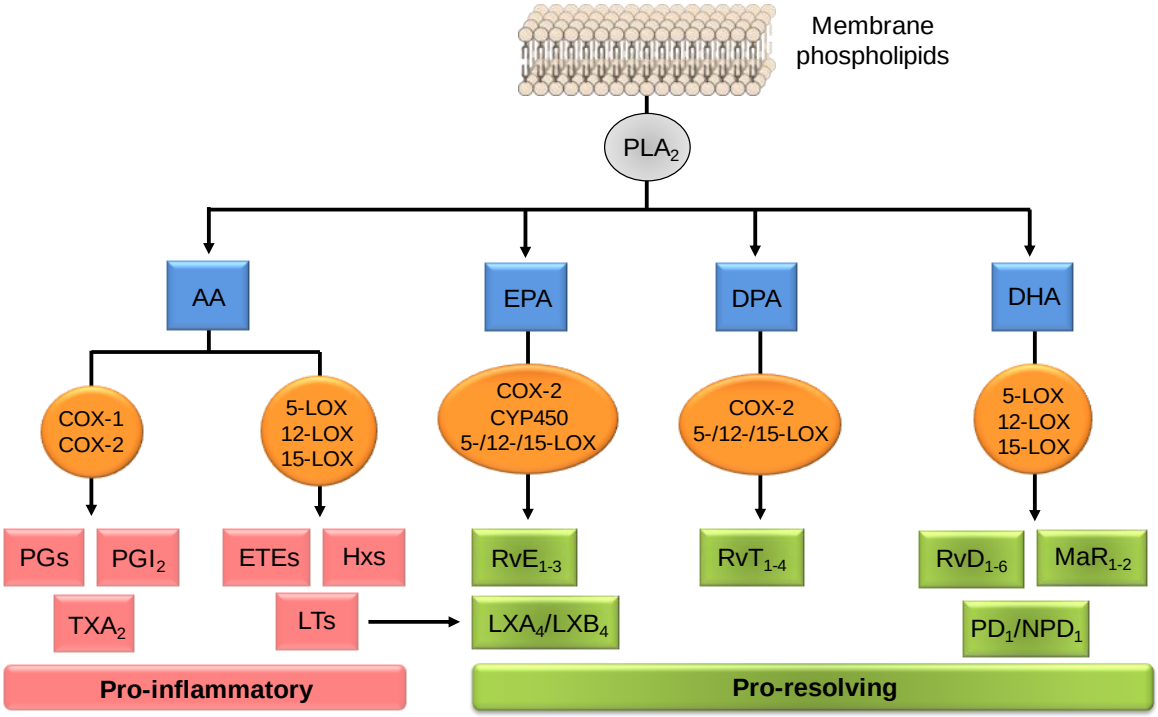


Figure 3: Metabolic pathways of eicosanoids and specialized pro-resolving mediators. Pro-inflammatory mediators are produced from the metabolism of AA by the action of COX-1 and -2 and 5-, 12-, and 15-LOX (in pink). Pro-resolving mediators are produced from the metabolism of different polyunsaturated fatty acids by the action of CYP450, COX-1 and -2, and 5-, 12-, and 15-LOX (in green). AA - arachidonic acid; COX - cyclooxygenase; CYP – cytochrome; DHA - docosahexaenoic acid; DPA - docosapentaenoic acid; EPA - eicosapentaenoic acid; ETEs – eicosatetraenoates; Hxs – hepoxilins; LOX – lipoxygenase; LTs – leukotrienes; LX - lipoxin; MaR – maresin; PD – protectin; PGs - prostaglandins; PGI_2 – prostaciclin; PLA_2 – phospholipase A_2; Rv – resolvin; TXA_2 – thromboxane A_2.

1.2.2.1 Bioactive Lipids during an Inflammatory Response

Eicosanoids and SPMs are recognized for their pleiotropic role in all phases of inflammation. During an inflammatory response, eicosanoids perform several functions, including leukocyte recruitment and activation, pain, fever, vascular permeability and tone, cytokine amplification, and platelet aggregation. These lipids are associated with the transition from the acute inflammatory response to chronic inflammation. In contrast, SPMs stimulate the reestablishment of physiological tissue functions after an acute inflammatory response, thus preventing collateral damage caused by a possible uncontrolled immune response. The resolution phase begins about 12 hours after the onset phase: SPMs are produced initiating its accumulation in the injured site. This event is accompanied by blocking of neutrophil influx and their apoptosis, promoting the input of pro-resolving macrophages responsible to guide the engulfment of dead neutrophils and tissue debris and activation of pro-inflammatory T-cells differentiation. Notably, it is becoming increasingly evident that the genesis of most chronic inflammatory diseases may be related in some way to the impairment, in whole or in part, of the molecular resolution system [22, 23].

2 Female Reproduction

Inflammation is associated with physiological reproductive processes, such as menstruation, ovulation, implantation, pregnancy, and parturition. These events are regulated by inflammatory mediators, like RS and bioactive lipid produced from the activation of specialized molecular pathways. The female reproductive tract exhibits the ability to self-eliminate, successfully, the inflammatory process established. However, its maintenance can be the initiator of many reproductive disorders, the most relevant cause of female infertility [31].

According to data published by World Health Organization (WHO), infertility is a condition that affects more than 15% of reproductive-aged couples worldwide and is characterized as the failure to achieve pregnancy after 12 months of unprotected intercourse. Infertility may be caused by disorders in the endocrine system, fallopian tubes, ovaries, and mostly, in the uterus with an inflammatory origin, such as endometriosis.

In women, the reproductive phase begins around age 11-16 with the onset of the menstrual cycle, which is coordinated by the hypothalamic-pituitary-ovarian (HPO) axis

in connection with the endometrium. The process starts with the release of gonadotropin-releasing hormone (GnRH) by the hypothalamus that stimulates the anterior pituitary gland to secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These gonadotropins stimulate ovarian steroidogenesis (production of estrogens, progesterone, and androgens), follicular growth (folliculogenesis), ovulation, and *corpus luteum* (CL) development [32].

2.1 Folliculogenesis

The main functions of the ovaries are the oocyte development for further fertilization and production and secretion of sex steroid hormones, which regulate physiological reproductive processes. These processes are controlled by steps that correspond to ovarian follicle formation (folliculogenesis) that is the functional unit of the ovaries [33].

In the presence of FSH, folliculogenesis occurs in the ovaries and can be grouped in follicular phase (before ovulation) and luteal phase (after ovulation). The follicular phase begins with the recruitment of primordial follicles, the most immature follicle type that consist of a single oocyte surrounded by a single layer of granulosa cells (GCs). These follicles develop into primary follicles in conjunction with the emergence of inner and outer membranes composed of theca cells (TCs). Oocytes that fail to develop into primary follicles are naturally eliminated by apoptosis, and many primordial follicles are also shed during fetal life, in a process known as *atresia*. During follicular maturation, there is multiplication and formation of several layers of GCs that function as nutritional support for oocyte growth. At this stage, it begins the production of follicular fluid (FF) containing hormones and proteins from the bloodstream and secreted by GCs e ovum. The accumulation of FF culminates in a formation of the central fluid cavity, named as *antrum* (antral follicle). These follicles continue to develop slowly and the FF in the *antrum* increases rapidly, giving rise to the mature Graafian follicle. The oocyte's growth is complete when it becomes able to store nutrients, mRNA, and a dispositive to synthesize proteins, which will be activated after fertilization. The Graafian follicle joins the ovary wall, disrupting it, releasing the oocyte into the fallopian tube and, consequently, ovulation occurs.

The luteal phase starts after ovulation with the transformation of the ruptured follicle into a functioning CL. Luteinization and CL formation are intrinsically correlated with the expression of different genes in GCs. Unless pregnancy develops, the CL is

converted into a non-vascular scar called the *corpus albicans* (CA), in a process known as luteolysis. This process is triggered by the uterine release of $PGF_{2\alpha}$, which activates numerous biochemical pathways, resulting in the blockage of progesterone production by luteinized GCs and apoptosis (**Figure 4**) [32].

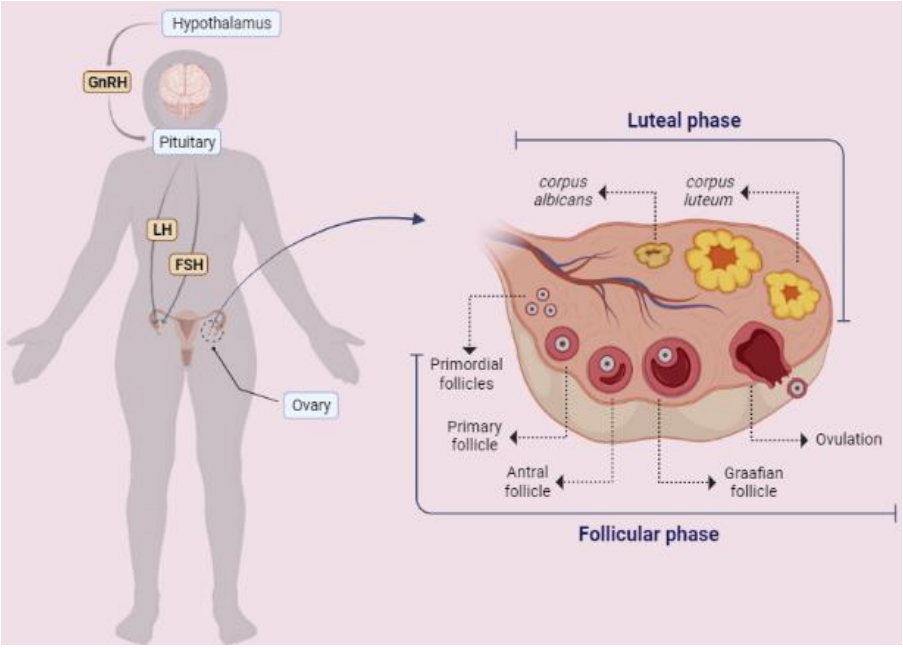


Figure 4: Ovarian cycle of the female reproduction system. The follicular phase begins with the release of gonadotropins by the hypothalamic-pituitary-ovarian axis. Then, the endometrium degenerates and primordial follicles develop until they reach the mature stage (Graafian follicle). At the end of follicular phase, the rupture of the follicle gives rise to ovulation. The luteal phase is characterized by the generation of the *corpus luteum*, preparing the endometrium for implantation. In case of a non-pregnancy cycle, *corpus luteum* degenerates and forms the *corpus albicans*.

When fertilization and implantation of the oocyte materialize in the endometrium, the placenta is stimulated to produce the human chorionic gonadotropin (hCG) to maintain the CL. However, if fertilization does not occur, the superficial layer of the endometrium degenerates, originating the menstruation [34].

2.2 Granulosa Cells

GCs play a remarkable role in the oocyte development, steroidogenesis, and cellular proliferation. These cells are interconnected with the plasma membrane of oocytes by gap junctions to sustain them in an arresting stage and to transport ions and small molecules, such as cyclic guanosine monophosphate (cGMP), cyclic adenosine monophosphate

(cAMP), metabolites, and amino acids. GCs are able to express receptors to respond to an extensive amount of factors produced by themselves, oocytes, and TCs or that access the follicular compartment from the bloodstream [35].

Depending on the anatomical location and growth stage of the follicle and the paracrine substances produced by TCs and oocytes, GCs can exhibit distinct phenotypes. At the stage of antral follicles, GCs are presented as **mural** cells (MGCs) and **cumulus** cells (CCs) (**Figure 5**). The **MGCs** cover the follicle wall around the *antrum* and play a central role in the physical barrier, estradiol production, and follicle growth. The MGCs formation is regulated by the anterior pituitary gland that secrets FSH. The **CCs** surround directly the oocytes providing nutrients and producing hyaluronic acid (HA), in additional to protect them from harmful signals that can trigger poor oocyte quality and infertility. The CCs formation is guided by oocyte secreted factors (OSFs) within the follicle, such as growth differentiation factor 9 (GDF9) that induces COX-2 expression and bone morphogenetic protein 15 (BMP15). Following ovulatory stimuli, such as a LH surge, CCs are activated to proliferate and produce an extracellular matrix, leading to the pre-ovulatory expansion of the cumulus-oocyte complex (COC) by accumulating HA crucial for ovulation [36]. Lastly, **luteinized GCs** give rise to the main cells of CL and have the competence to produce progesterone and androgens required for CL development [37].

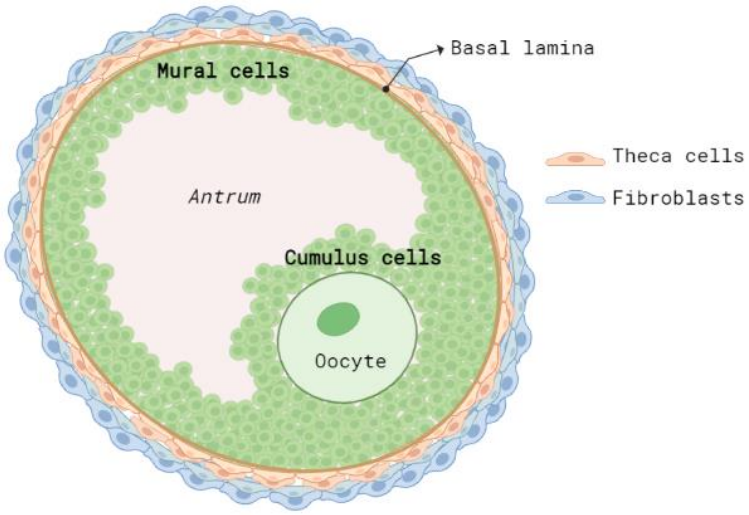


Figure 5: Schematic representation of antral follicle and its components. The antral follicles are comprised of an oocyte surrounded by the cumulus cells and by the antrum where the follicular fluid is found. The mural cells cover the entire inner surface of the follicle and are separated from the theca cells by the basal lamina.

GCs are considered by-products of follicular aspiration executed in women undergoing assisted reproductive technology (ART) as an alternative to infertility. In recent years, several studies have been published that aim to characterize potential biomarkers in GCs for oocyte competence with pregnancy outcome. Genes expressed by GCs related to the production of pro- and anti-inflammatory mediators, such as COX-2 and 5-, 12-, and 15-LOX, and also to apoptosis induced by OS/NS, such as survivin (anti-apoptotic gene) and caspase-3 and -7 (pro-apoptotic genes), may be useful as effective biomarkers to identify oocytes with high development potential, thus leading to increased implantation rates in women undergoing ART procedures [36].

2.2.1 Granulosa Cells *versus* Oxidative/Nitrosative Stresses

The ROS/RNS overproduction and/or the deregulation of endogenous antioxidant systems in the female reproductive system can lead to OS/NS. This imbalance, as well as causing damage and changes in cellular functions, plays a central role in the development of ovarian-associated pathologies, such as endometriosis, polycystic ovarian syndrome (PCOS), and unexplained infertility [38]. In women, the appearance of ROS/RNS can be due to the inflammatory response, ETC in mitochondria, lifestyle habits (smoking, alcohol, and drugs), environmental cause (heat, pollution, and radiation), and ovarian age. These species can affect stages in the reproductive processes, such as folliculogenesis, ovulation, luteolysis, and embryo development. Thus, women who are attempting to become pregnant, in addition to changing certain lifestyle habits, should make an antioxidant supplementation as a strategy to prevent infertility [39].

The relationship between the effects of ROS/RNS and reproductive processes has been investigated using a variety of approaches and methods. In women undergoing ART, the levels of ROS/RNS as biomarkers can be measured systemically, in serum and peritoneal fluid, and locally, in the ovary, FF, and GCs. The main methods applied are the quantification of secondary products derived from lipids oxidation, mitochondrial membrane potential, and the expression level of antioxidant enzymes involved in detoxification processes [38].

A large dataset published has demonstrated the relationship between ROS/RNS levels and ovarian pathologies. The oxidative environment in the ovaries of women diagnosed with endometriosis can lead to injury to ovarian tissue and affect the oocyte quality. Accordingly, human oocytes demonstrated that functional roles, such as cellular

oxidative detoxification, oxidative stress response, and the oxidation-reduction mechanisms, were markedly enriched in oocytes of women with endometriosis [40].

Seino *et al.* [41] explored the correlation between OS/NS and oocyte quality in women with endometriosis, using the biomarker 8-hydroxy-2-deoxyguanosine (8-OHdG) in GCs to measure the DNA damage and OS/NS. As a result, the authors showed an increase in 8-OHdG levels in GCs of women with endometriosis during the ovulation. This increase can interfere with cellular functionality, leading to a decrease in oocyte quality, fertilization rate, and embryo growth in women undergoing ART. Lin *et al.* [42] showed through the increased expression of iNOS and SOD1 that excess of ROS present in GCs of women with endometriosis resulted in a disturbance in the normal redox homeostasis in endoplasmic reticulum. Moreover, the results suggested that GCs exhibited an OS-induced senescence phenotype and mitochondrial dysfunction. Lastly, the treatment of the GCs with melatonin as an antioxidant was able to alleviate OS-induced decrease in fertility and restore the antioxidant functions of GCs *in vivo*.

The raised ROS/RNS levels are linked with PCOS and may have pathogenic involvement in the progression of insulin resistance, chronic inflammation, and hyperandrogenism [43]. Karuputhula *et al.* [44] investigated the morphological changes in GCs related to the intracellular ROS generation in women undergoing ART with endometriosis, PCOS, and tubal factor. Results indicated a considerable increase in ROS generation, DNA fragment levels, and apoptosis caused by the depolarization of mitochondrial membrane potential (MMP) in GCs of women with endometriosis and PCOS. In consequence, these women showed reduced ART success rates due to the poor oocyte quality. In a second study developed by Lai *et al.* [45], it was observed that the levels of ROS present in GCs of women with PCOS were higher than in women with tubal factor. These high levels of ROS involved with the stimulation of NADPH oxidase were responsible for the apoptosis of GCs, affecting the quality of oocytes and reducing ART success rates.

Tatone *et al.* [46] and Qian *et al.* [47] studied the effects of age-related OS/NS in GCs. They have demonstrated that women without ovarian pathologies showed a reduction in the expression of antioxidant enzymes with aging, such as peroxiredoxin 4 (PRDX4), SOD, and CAT. González-Fernández *et al.* [48] also demonstrated that the expression of aldehyde dehydrogenase 3 family member A2 (ALDH3A2), an antioxidant enzyme, in hGCs from women undergoing ART increases with age and high levels of ROS/RNS. The authors concluded that the raise of ROS/RNS levels as a result of age may

constitute one of the mechanisms that influence the decrease of FSH response and poor oocyte quality during ovarian stimulation.

Ovaries are strongly susceptible to the deleterious effects of tobacco smoking, being steroidogenesis and folliculogenesis the most ovarian processes impacted by this exposure. Moreover, cigarette smoke-induced OS/NS seem to be one of the major reasons for ovarian damage, together with unusual crosstalk between oocytes and GCs, cellular death, and DNA damage. The study carried out by Konstantinidou *et al.* [49] aimed to evaluate the influence of smoking in the expression of OS-related genes in hCCs of female smokers undergoing ART. Findings from this study provided evidences that hCCs of female smokers displayed down-regulation of genes related to antioxidant enzymes, which may contribute to an enhanced cellular oxidative damage in ovaries. In conclusion, data revealed that when harmful factors exceed the ability of hCCs to transform and/or scavenge ROS, they may have a marked deleterious impact on oocyte quality, with potential influences on female fertility.

Finally, it is unanimous that there must be a balance between the amounts of ROS/RNS produced and antioxidants that allow the functional development of biological systems. In this way, an inadequate balance can negatively influence folliculogenesis, oogenesis, and the establishment of a viable pregnancy [50].

2.2.2 Granulosa Cells *versus* Bioactive Lipids

In the ovaries, inflammatory cascades are triggered by several factors, including PGs, LTs, histamine, bradykinin, platelet aggregation factor (PAF), chemokines, and cytokines. During ovulation, the superficial epithelium of the ovary and vasculature rupture to release the oocyte and, concurrently, macrophages are recruited to the follicle theca layers. Macrophages migrate to the CCs, releasing inflammatory mediators, such as ·NO, IL-1β, IL-6, IL-8, nuclear factor kappa B (NF-κB), and TNF-α. Along with growth factors found in FF, these mediators induce the expression of COXs and LOXs enzymes, promoting the local production of bioactive lipids, such as eicosanoids and SPMs, which can directly influence the oocyte quality [31].

Feldam *et al.* [51] demonstrated for the first time the expression of 5-, 12-, and 15-LOXs in GCs isolated from women undergoing ART through the characterization of specific products derived from the metabolism of AA, such as 5-, 12-, and 15-hydroxyeicosatetraenoic acids (HETEs). Utilizing pre-ovulatory rat follicles, Wong *et al.* [52] showed that LH and FSH surges induced the COX expression through the increased

concentrations of PGE_2 and $PGF_{2\alpha}$ in ovarian follicles. In 2009, Thill *et al.* [53] reported increased levels of COX-2 mRNA expression in GCs isolated from women undergoing *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI). COX-2 expression in GCs from women undergoing IVF was also evidenced by Li *et al.* [54]. Treatment of cells with IL-1β and hCG for 24 h increased both mRNA expression and protein levels of COX-2 to a similar extent. *In vitro*-experiments conducted by Akinkuolie *et al.* [55] using GCs isolated from women with PCOS and healthy women was found that EPA treatment significantly reduced COX-2 expression in both groups. In a recent study, Zhang *et al.* [56] reported that RvE1 improved oocyte quality by decreasing apoptosis rate of CCs and increasing cell viability and proliferation.

Actually, there is still limited knowledge about the bioactive lipids effects, namely eicosanoids and SPMs, on the GCs function and their influence on oocyte quality. Moreover, until the present moment, there are not many studies published in the literature that describe the expression of 5-, 12-, and 15-LOXs enzymes by GCs. However, it is possible to deduce that, according to the pro- and anti-inflammatory properties of bioactive lipids, these may influence positively or negatively on oocyte quality. Therefore, a balance in the production of these mediators and expression of these enzymes is required.

3 Chalcones

Chalcones (1,3-diphenyl-2-propen-1-ones) are frequently found in natural sources, such as vegetables, fruits, and also some edible plants in our daily diet. They are the most important open-chain precursors in flavonoid biosynthetic pathways [57]. Given the simple scaffold and numerous biological activities already described, chalcones have been considered as privileged structures and applied as a molecular pattern in medicinal chemistry for drug discovery [58].

3.1 Structure, Nomenclature and Chemistry of Chalcones

The chalcone term originates from the Greek word *Chalcos*, which means “bronze”, explaining the colours of most natural chalcones. These compounds are constituted by “A” and “B” aromatic rings linked by an α,β-unsaturated electrophilic carbonyl system (**Figure 6**). This structural chemical configuration allows the occurrence of the trans (*Z*) and cis (*E*) isomers, being trans-isomer the most thermodynamically stable [58]. The nomenclature and numbering of chalcones adopted throughout this thesis are the same adopted by the British Chemical Abstract and Journal of Chemical Society. The “A” phenyl

ring connected to the carbonyl group is designated with ordinary numbers followed by a line (‘) and the “B” phenyl ring with only ordinary numbers [57].

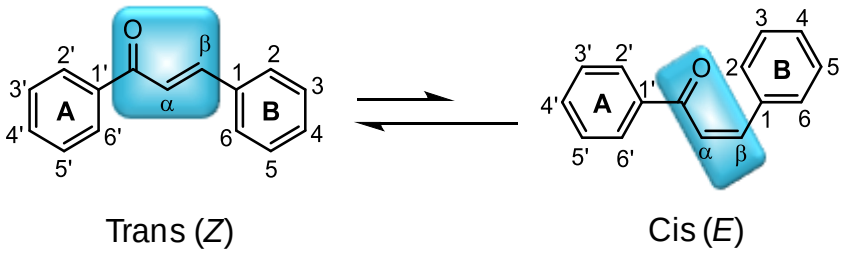


Figure 6: Structural and numerical representations of chalcones. The nomenclature and numbering of chalcones under discussion in this thesis was based on the rules established by British Chemical Abstract and Journal of Chemical Society.

The conjugation of the reactive keto-ethylenic system (-CO-CH=CH-) together with the electron-pulling and/or -pushing functional group(s) on the phenyl ring(s) induces chalcones to acquire fluorescent ability, making them potential chemical probes to be used in mechanistic and imaging/diagnostics investigations [58].

3.2 Biosynthesis of Chalcones

The initial precursors of chalcones biosynthesis are the amino acids L-tyrosine or L-phenylalanine produced by the shikimate pathway. L-phenylalanine is converted into cinnamic acid by phenylalanine ammonia-lyase (PAL) and then, it is oxidized by cinnamic acid 4-hydroxylase (C4H) to *p*-coumaric acid. The L-tyrosine conversion pathway is shorter and can be catalysed directly by tyrosine ammonia-lyase (TAL) to *p*-coumaric acid, which is converted into *p*-coumaroyl-CoA by 4-coumarin-CoA ligase (4CL). The key step in biosynthesis occurs when chalcone synthase (CHS) catalyses the condensation of three molecules of malonyl-CoA and one molecule of *p*-coumaroyl CoA, resulting in the naringenin chalcone (**1**) production. This chalcone is a biosynthetic precursor for numerous polyphenolic classes and can be converted into isoflavone by chalcone isomerase (CHI) and into flavone by CHS. Subsequently, these compounds are modified into various flavonoids by transferases, synthases, and reductases (**Figure 7**) [59].

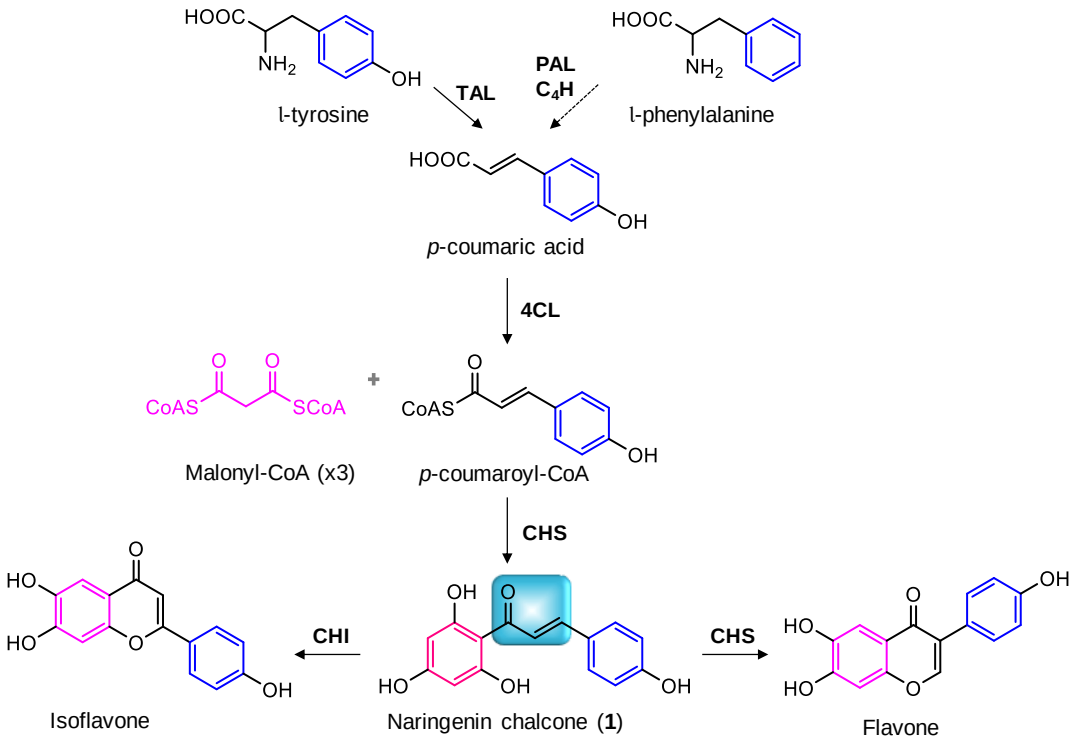


Figure 7: Biosynthetic pathways of chalcones. The biosynthesis of chalcones is initiated from the conversion of L-tyrosine or L-phenylalanine into *p*-coumaric acid. Then, it is transformed into *p*-coumaroyl-CoA that reacts with malonyl-CoA, giving rise the naringenin chalcone (**1**). Lastly, this chalcone can lead to the generation of isoflavones and flavones. 4CL - 4-coumarin-CoA ligase; CHI - chalcone isomerase; CHS - chalcone synthase; C_4H - cinnamic acid 4-hydroxylase; PAL - phenylalanine ammonia lyase; TAL - tyrosine ammonia lyase.

3.3 Bioactivities of Chalcones

Chalcone chemistry continues to be a subject of interest to the scientific society because of its synthetic facility and the presence of many replaceable hydrogens, allowing the production of plenty of derivatives [60]. Furthermore, the Michael acceptor (electrophilic centre) present in the chalcone scaffold is usually considered as a biological active site associated with the management of many cellular signalling pathways. Biologically, this acceptor can easily form covalent bonds with the sulfhydryl group of cysteine residues (nucleophilic centre) to form the Michael adduct, which plays an essential role in diverse biological activities (**Figure 8**) [58].

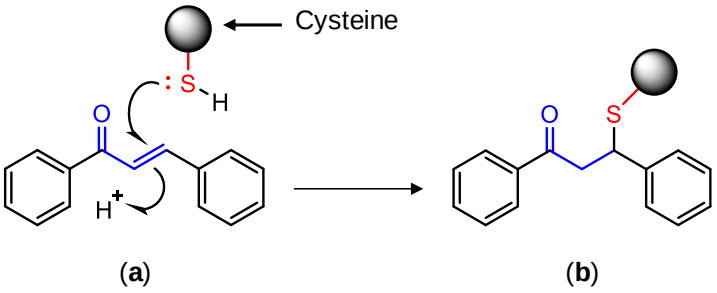


Figure 8: Reaction related to Michael acceptors. (**a**) Reaction between the Michael acceptor of chalcone (in blue) and the sulfhydryl group of cysteine (in red). (**b**) Michael adduct formation.

Srinivasan *et al.* [61] showed that chalcones with hydroxyl and methoxy substitutions, such as 3-hydroxy-4-3',4',5'-tetramethoxychalcone (**2**), can inhibit the kinase activity of inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ) through its covalent binding to the Michael acceptor of chalcones and, therefore, inhibiting the NF-κB pathway (**Figure 9**). These chalcones demonstrated promising anti-inflammatory activity [61].

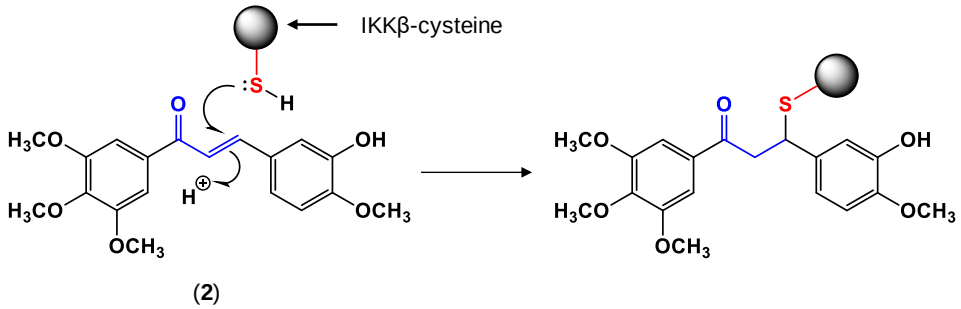


Figure 9: Reaction related to Michael acceptors. Reaction between the Michael acceptor of 3-hydroxy-4-3',4',5'-tetramethoxychalcone (**2**) (in blue) and the sulfhydryl group of IKKβ-cysteine (in red), with the Michael adduct formation.

Numerous published reports highlighted an extensive range of chalcones biological activities being these related to its structural diversity and the presence of Michael acceptor. The pharmacological activities of chalcones include anticancer, antifungal, anti-tubercular, anti-malarial, anti-leishmanial, anti-gout, anti-histaminic, anti-hypnotic, anti-spasmodic, anti-diabetic, antiviral, anti-microbial, anti-bacterial, and particularly, the antioxidant and anti-inflammatory [57, 58].

As previously mentioned, RS are generated during biological processes and chalcones showed their potential as an antioxidant agent in order to remove these species for the maintenance of cellular homeostasis. In an extensive review published by Martins *et al.* [21] covering the period from 1998 to 2020, the authors discussed the antioxidant activities of 113 chalcones during inflammatory processes. The literature review was based on the modulatory effects of chalcones against different targets, such as SOD, NADPH oxidase, CAT, MPO, and iNOS. There is no single determinant substitution pattern by which chalcones exert their antioxidant properties. However, some structural requirements seem to favour the modulation of mentioned therapeutic targets, such as the presence of hydroxyl, methoxyl and prenyl groups and/or halogen atoms [21].

Medications, such as NSAIDs, herbal supplements, and corticosteroids, are routinely used to decrease the pain caused by inflammatory conditions through COX activity inhibition. Gastric corrosion, renal toxicity, and ulcer are some common side effects associated with the use of NSAIDs. For those reasons, most current research has concentrated efforts on the design and development of new anti-inflammatory medicines in alternative to NSAIDs [62].

Regarding the anti-inflammatory activity of chalcones, it includes distinct therapeutic targets, such as COXs, LOXs, ILs, PGs, LTD_4, iNOS, monocyte chemoattractant protein-1 (MCP-1), heme oxygenase (HO-1), and expression of cellular adhesion molecules (CAM) [63-66].

In the most recent review published concerning the anti-inflammatory effects of chalcones, Rashid *et al.* [63] showed that it was mainly related to their inhibitory action against COX-2, PGE_2, iNOS, and NF-κB. The authors concluded some significant markers about the minimum structural requirements that seem to favour the modulation of the therapeutic targets mentioned above (**Figure 10**):

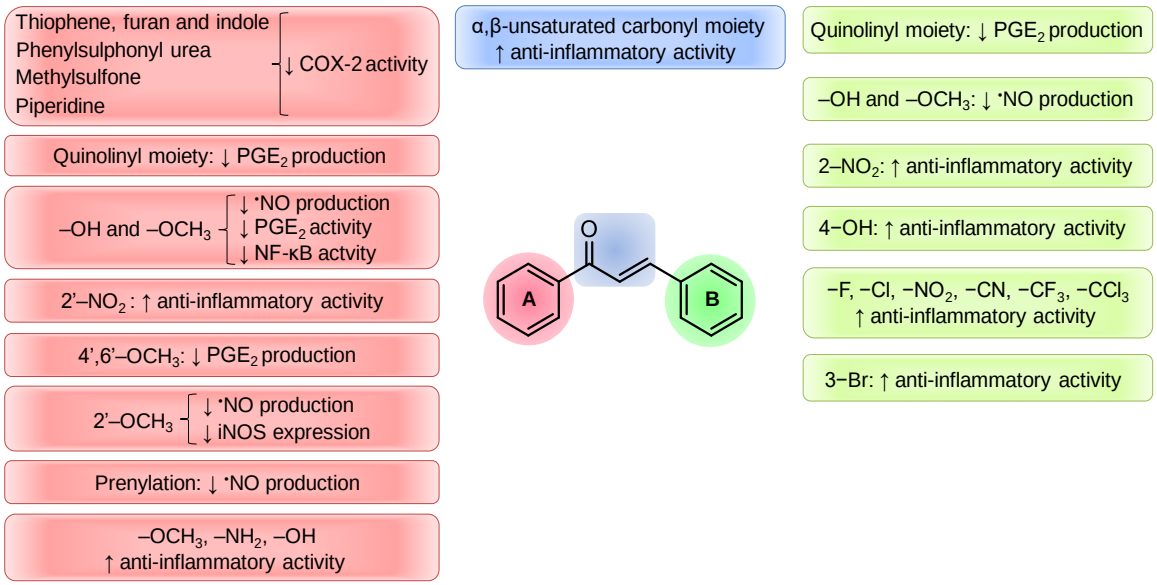


Figure 10: Overall structure activity relationship of chalcones. In pink and green boxes are represented the minimum structural requirements that must be present on A- and B-rings, respectively, for chalcones to exert their respective anti-inflammatory effects. The α,β-unsaturated carbonyl moiety represented in the blue box is a mandatory requirement that must be present in the chemical structure of chalcones for them to exert their anti-inflammatory activity, regardless of the biochemical pathway.

Importantly, the choleretic drug metochalcone (**3**) [67] and anti-ulcer agent sofalcone (**4**) [68], which increase the amount of PGs in the mucosa conferring a gastro-protective effect against *Helicobacter pylori*, were approved and have been used in the clinic. Furthermore, clinical trials showed that hesperidin methylchalcone (**5**) [69] tested for chronic venous lymphatic insufficiency and hesperidin trimethylchalcone (**6**) [70] evaluated for trunk or branch varicose led to the relief of symptoms, achieved acceptable plasma concentrations and were well tolerated (**Figure 11**).

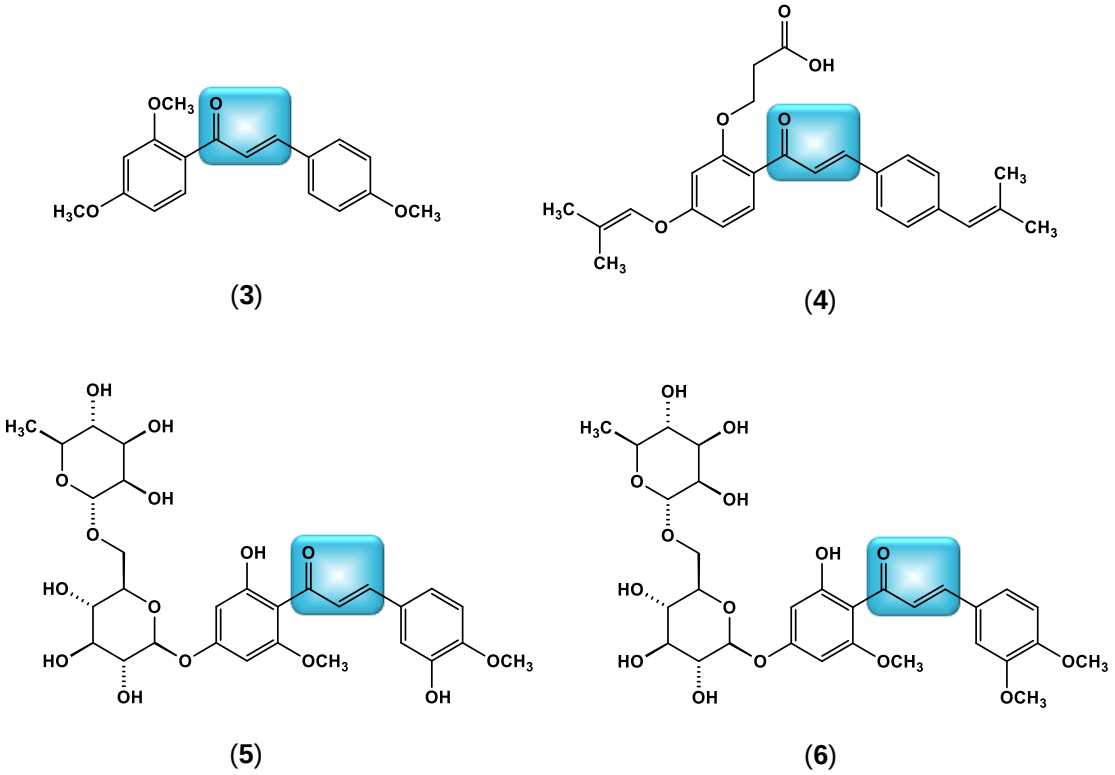
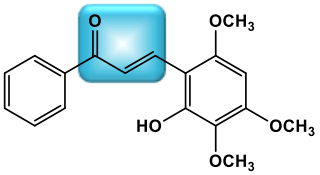
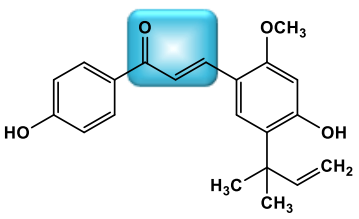


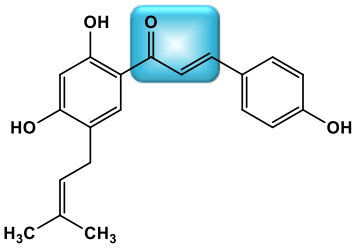
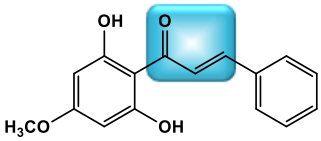
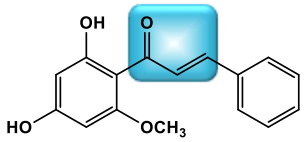
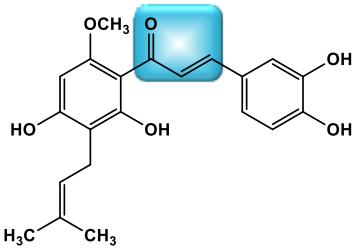
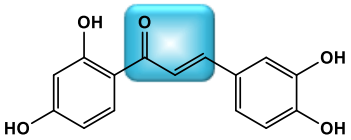
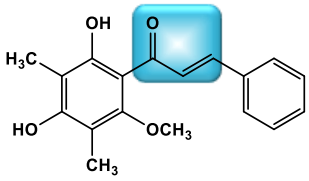
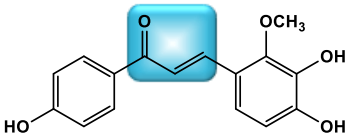
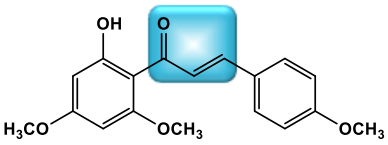
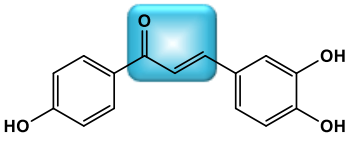
Figure 11: Chemical structures of clinically tested chalcones. Metochalcone (**3**), sofalcone (**4**), hesperidin methylchalcone (**5**), and hesperidin trimethylchalcone (**6**).

Table 1 shows examples of natural chalcones that demonstrate antioxidant or anti-inflammatory activities.

Table 1: Examples of natural chalcones and their biological activities.

Chalcone	Chemical Structure	Natural Source	Biological Activity	Ref.
Chalcone (**7**)		*Toussaintia orientalis*	COX-2 inhibition	[71]
Licochalcone A (**8**)		*Glycyrrhizae inflata*	COX-2 inhibition	[72]

(Table 1) contd...

Chalcone	Chemical Structure	Natural Source	Biological Activity	Ref.
Isobavachalcone (**9**)		*Cullen corylifolium*	·NO inhibition	[73]
Pinostrobin (**10**)		*Cajanus cajan*	IL-1β inhibition	[74]
Cardamonin (**11**)		*Alpinia conchigera*	NF-κB inhibition	[75]
		Alpinia rafflesiana	PGE_2 inhibition	[76]
Xanthohumol (**12**)		*Humulus lupulus*	IL-12 inhibition	[77]
Butein (**13**)		*Dalbergia odorifera*	iNOS inhibition	[78]
Dimethyl cardamonin (**14**)		*Cleistocalyx Operculatu*	↑ CAT activity	[79]
Xanthoangelol B (**15**)		*Glycyrrhiza Inflata*	$O_2^{\bullet-}$ scavenging	[80]
Flavokawain A (**16**)		*Piper methysticum forst*	ROS inhibition	[81]
Chalcone (**17**)		*Gynura segetum*	NADPH oxidase inhibition	[82]

4 Aims

Since ovulation exhibit hallmarks of inflammation, an excessive inflammatory state within the ovaries can disturb normal follicular dynamics, leading to a reduction in oocyte quality and, consequently, infertility. In the inflammatory response, pro-inflammatory mediators, such as ROS/RNS and eicosanoids, and anti-inflammatory mediators, such as SPMs, are produced and seem to be crucial at various stages of female reproduction. However, the exacerbated activation and maintenance of these inflammatory pathways can culminate in reproductive disorders, such as endometriosis and PCOS.

Considering that neutrophils are the first cells to be recruited during an inflammatory response to produce ROS/RNS, the initial aims of the present thesis were to investigate the ability of 34 structurally related chalcones to scavenge HOCl and/or suppress its production by inhibiting the human neutrophils' oxidative burst. To achieve these objectives, fluorimetric detection was used to measure the ability of the studied chalcones to scavenge HOCl and, consequently, inhibit the oxidation of dihydrorhodamine 123 (DHR). A chemiluminescent detection was used to measure the ability of the studied chalcones to inhibit the human neutrophils' oxidative burst and, consequently, inhibit the oxidation of luminol.

In a second phase, considering that the crosstalk between hGCs and macrophages is essential for the production of key factors during oocyte development, the following aim of this thesis was to investigate how the mediators produced by pro- and anti-inflammatory macrophages might activate inflammatory pathways in hGCs. To achieve these objectives, an indirect co-culture model was established between hGCs isolated from women with male factor undergoing ART procedures and conditioned media of macrophage phenotypes (M1 and M2) to simulate the ovarian microenvironment. Lastly, RT-PCR and Western Blotting were used to detect the expression of COX-2 and 5-, 12-, and 15-LOX as biomarkers of oocyte competence.

Chapter II - Experimental Results

Manuscript I

Chalcones as Scavengers of HOCl and Inhibitors of Oxidative Burst: Structure-Activity Relationship Studies

Thaise Martins, Vera L.M. Silva, Artur M.S. Silva, José L.F.C. Lima, Eduarda Fernandes, and Daniela Ribeiro

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RESEARCH ARTICLE

Chalcones as Scavengers of HOCl and Inhibitors of Oxidative Burst: Structure-Activity Relationship Studies

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Abstract: ***Aims*:** This study evaluates the ability of chalcones to scavenge hypochlorous acid (HOCl) and modulate oxidative burst.

***Background*:** The chemistry of chalcones has long been a matter of interest to the scientific community due to the phenolic groups often present and to the various replaceable hydrogens that allow the formation of a broad number of derivatives. Due to this chemical diversity, several biological activities have been attributed to chalcones, namely anti-diabetic, anti-inflammatory and antioxidant.

***Objectives*:** Evaluate the ability of a panel of 34 structurally related chalcones to scavenge HOCl and/or suppress its production through the inhibition of human neutrophils' oxidative burst, followed by the establishment of the respective structure-activity relationships.

***Methods*:** The ability of chalcones to scavenge HOCl was evaluated by fluorimetric detection of the inhibition of dihydrorhodamine 123 oxidation. The ability of chalcones to inhibit neutrophils' oxidative burst was evaluated by chemiluminometric detection of the inhibition of luminol oxidation.

***Results*:** It was observed that the ability to scavenge HOCl depends on the position and number of hydroxy groups on both aromatic rings. Chalcone **5b** was the most active with an IC_{50} value of 1.0 ± 0.1 μM. The ability to inhibit neutrophils' oxidative burst depends on the presence of a 2'-hydroxy group on A-ring and on other substituents groups, *e.g.* methoxy, hydroxy, nitro and/or chlorine atom(s) at C-2, C-3 and/or C-4 on B-ring, as in chalcones **2d**, **2f**, **2j**, **2i**, **4b**, **2n** and **1d**, which were the most actives with IC_{50} values ranging from 0.61 ± 0.02 μM to 1.7 ± 0.2 μM.

***Conclusion*:** The studied chalcones showed high activity at a low micromolar range, indicating their potential as antioxidant agents and to be used as a molecular structural scaffold for the design of new anti-inflammatory compounds.

Keywords: Chalcones, hypochlorous acid, scavenging activity, reactive species, human neutrophils, antioxidant activity.

1. INTRODUCTION

Neutrophils, also known as polymorphonuclear leukocytes, are the primary phagocytic cells to be recruited and actively act in the innate immune host defense [1]. Due to a chemical gradient of chemokines discharged by infectious agents or other inflammatory cells, neutrophils are enrolled to migrate to sites of injury or infection, to resolve the noxious stimuli and initiate the healing process [2]. These cells can act by multiple means, both intra- and extracellular: by releasing antibacterial proteins (*e.g.* defensins and lysozymes) and neutrophil extracellular traps (NETs), and by the production of reactive oxygen species (ROS) [3]. The rapid release of high levels of ROS during phagocytosis or in response to an inflammatory stimulus is known as oxidative burst. In neutrophils, the oxidative burst and the related production of ROS are mainly catalyzed by the activation of adenine dinucleotide phosphate (NADPH) oxidase, a multisubunit enzyme, with components found in the cytoplasm and membranes [4]. This process is initiated by the transfer of electrons from cellular NADPH to molecular oxygen (O_2), generating superoxide anion radical ($O_2^{\bullet-}$). This RS has a short life (10^6 ns) and can produce H_2O_2 by spontaneous dismutation or *via* a reaction catalyzed by superoxide dis-

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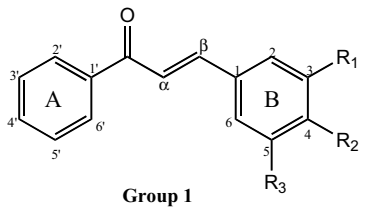
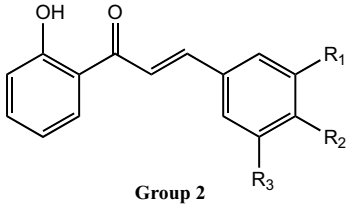
mutase (SOD). In biological conditions, H_2O_2 can react with metal ions (*e.g.* Fe^{2+} and Fe^{3+}) to yield $HO^{\bullet}$ and HO^{-}, through Haber-Weiss and Fenton reactions; or it is detoxified to O_2 and H_2O by the enzyme catalase (CAT) [5]. In the primary azurophilic granules existent in neutrophils, myeloperoxidase (MPO), a lysosomal heme-protein, is stored and, in the presence of H_2O_2 and halides (*e.g.* Cl^{-}), catalyzes the formation of hypochlorous acid (HOCl) [6]. HOCl is the most powerful microbicidal oxidant produced by neutrophils, showing high membrane permeability and strong ability to interact with a wide range of biological molecules [7]. However, this non-radical ROS can react with several molecules to generate other compounds: with nitrogen dioxide radical ($^{\bullet}NO_2$) to produce nitryl chloride (NO_2Cl), a strong nitrating and chlorinating oxidant; with amines ($R\text{-}NH_2$) to yield chloramines (R-NH-Cl); with $O_2^{\bullet-}$ to form once again $HO^{\bullet}$; and with H_2O_2 to originate oxygen singlet ($^1O_2$). All these oxidants can react with membrane lipids to initiate peroxidation, therefore extending the oxidative burst and the inflammatory response [7, 8]. When this overproduction of ROS occurs, and it is not compensated by the endogenous antioxidant defences, it may lead to oxidative stress [9]. Oxidative stress is strongly implicated in the initiation and aggravation of chronic inflammatory pathologies, such as neurodegenerative, cardiovascular and metabolic diseases, cancer and rheumatoid arthritis [8]. In this context, the development of new pharmacological therapies able to modulate the exacerbated production of ROS may be a determining factor for the management of these diseases.

The chalcone scaffold (1,3-diaryl-2-propen-1-one) is commonly encountered in many natural compounds and is one of the most important classes of flavonoids across the whole plant kingdom. The chemical structure of chalcones comprises two aromatic rings interconnected by an α,β-unsaturated carbonyl bridge [10] (Fig. **1**). Their chemistry remains a matter of interest to the scientific community due to their several mechanisms of action as well as biological targets [10]. Chalcones display radical scavenging properties conferred by the phenolic groups and/or by the various replaceable hydrogens that allow the formation of a broad number of derivatives [11, 12]. This chemical diversity leads to a range of biological activities, such as anticancer [13], anti-diabetic [14], antibacterial [15] and, particularly, anti-



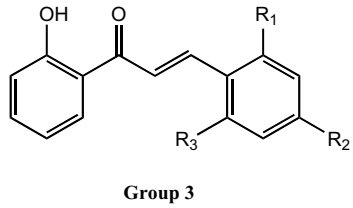
Group 1

1a) $R_1 = R_2 = R_3 = H$ (chalcone)
1b) $R_1 = R_2 = OCH_3$; $R_3 = H$
1c) $R_1 = R_3 = H$; $R_2 = Cl$
1d) $R_1 = R_3 = H$; $R_2 = NO_2$
1e) $R_1 = R_2 = H$; $R_3 = OH$
1f) $R_1 = R_3 = H$; $R_2 = OH$



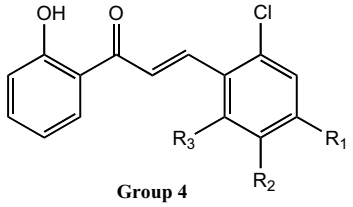
Group 2

2a) $R_1 = R_2 = R_3 = H$
2b) $R_1 = R_2 = R_3 = OCH_3$
2c) $R_1 = OCH_3$; $R_2 = R_3 = H$
2d) $R_1 =$ Cl; $R_2 = R_3 = H$
2e) $R_1 = R_2 = OCH_3$; $R_3 = H$
2f) $R_1 = R_2 =$ Cl; $R_3 = H$
2g) $R_1 = R_2 = H$; $R_3 = CH_3$
2h) $R_1 = R_3 = H$; $R_2 = OCH_3$
2i) $R_1 = R_3 = H$; $R_2 = NO_2$
2j) $R_1 = OCH_3$; $R_2 = OH$; $R_3 = H$
2l) $R_1 = R_3 = H$; $R_2 = Cl$
2m) $R_1 = R_2 = H$; $R_3 = OH$
2n) $R_1 = R_3 = H$; $R_2 = OH$
2o) $R_1 = R_2 = R_3 = OH$



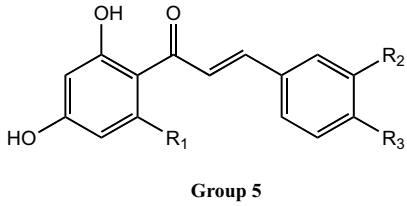
Group 3

3a) $R_1 = R_2 = R_3 = CH_3$
3b) $R_1 = OCH_3$; $R_2 = R_3 = H$
3c) $R_1 = OH$; $R_2 = R_3 = H$



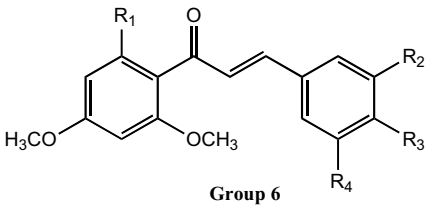
Group 4

4a) $R_1 = R_2 = R_3 = H$
4b) $R_1 = R_2 = H$; $R_3 = Cl$
4c) $R_1 = Cl$; $R_2 = R_3 = H$



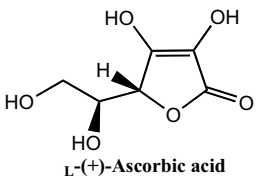
Group 5

5a) $R_1 = R_2 = R_3 = H$
5b) $R_1 = R_2 = R_3 = OH$
5c) $R_1 =$ H; $R_2 = R_3 = OCH_3$
5d) $R_1 =$ H; $R_2 = R_3 = OH$ (butein)
5e) $R_1 = R_2 = H$; $R_3 = OCH_3$

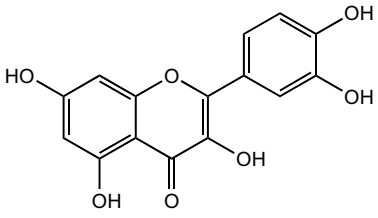


Group 6

6a) $R_1 = OH$; $R_2 = R_3 = R_4 = OCH_3$
6b) $R_1 = OH$; $R_2 = R_4 = H$; $R_3 = OCH_3$
6c) $R_1 = R_2 = R_3 = OCH_3$; $R_4 = H$



L-(+)-Ascorbic acid



Quercetin

Fig. (1). Chemical structures of the studied chalcones (group 1 to 6) and of the positive controls used, L-(+)-ascorbic acid and quercetin.

inflammatory [16] and antioxidant [17] activities. Several works have been published in the literature regarding these last two activities. Araico *et al*. [18] showed that phenylsulphonyl urenyl chalcones were able to inhibit the release of MPO following the stimulation of human neutrophils by cytochalasin B and *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine. Zeraik *et al.* [19] demonstrated that 4'-aminochalcones were inhibitors of MPO activity in human neutrophils activated by phorbol 12-myristate 13-acetate (PMA). Bukhari *et al.* [20] described that the 2',4',6'-trimethoxy-4-methylaminoethanol-chalcone was able to prevent the intra- and extracellular ROS production in human neutrophils activated by zymosan. In the study performed by Yuandani *et al.* [21], a trihydroxylated chalcone was able to inhibit ROS production in human neutrophils stimulated by zymosan and PMA.

In this sense, the aim of this work was to evaluate the ability of a panel of 34 chalcones, replaced at distinct positions of both aromatic rings, by different chemical groups (*e.g.* hydroxy, methoxy, methyl and nitro) and/or by chlorine atom(s) (Fig. **1**), to scavenge HOCl and to modulate neutrophils' oxidative burst. From the obtained results, a structure-activity relationship (SAR) was established towards the finding of the best chalcone substitution pattern to act as a useful antioxidant and to develop potential anti-inflammatory agents.

2. MATERIALS AND METHOD

2.1. Materials

All reagents and solvents used were of analytical grade. L-(+)-Ascorbic acid was obtained from Alfa Aesar (Kandel, Germany). Acetone, calcium chloride ($CaCl_2$), dihydrorhodamine (DHR), dimethylformamide (DMF), dimethylsulfoxide (DMSO), D-(+)-glucose, Dulbeccos' phosphate buffered saline (DPBS) without calcium (Ca^{2+}) and magnesium (Mg^{2+}), histopaque 1077, histopaque 1119, PMA, potassium chloride (KCl), quercetin, sodium chloride (NaCl), sodium phosphate dibasic (Na_2HPO_4), sulfuric acid (H_2SO_4), trizma® base and trypan blue solution 0.4% were obtained from Sigma-Aldrich (Steinheim, Germany). Luminol was obtained from Fluka BioChemika (Buchs, Slovakia), magnesium sulfate anhydrous ($MgSO_4$) was obtained from Merck (Darmstadt, Germany) and sodium hypochlorite (NaOCl) was obtained from Acros Organics (Geel, Belgium). Chalcones **1a-b**, **2a-c**, **2e**, **2h**, **2j**, **5a**, **5c**, **5e** and **6c** were obtained from Indofine Chemical Company, Inc. (Stryker Lane Hillsborough, USA) and chalcones **5b** and **5d** were obtained from Extrasynthese (Genay, France). Chalcones **1c-f**, **2d**, **2f-g**, **2i**, **2l-o**, **3a-c**, **4a-c** and **6a-b** were synthesized according to established methods [22-25].

2.2. Hypochlorous Acid Scavenging Assay

The HOCl scavenging activity was determined by following the ability of HOCl to oxidize the non-fluorescent DHR to the fluorescent rhodamine 123, as described before [26]. Briefly, HOCl was routinely prepared by adjusting the pH of a NaOCl solution (1%, v/v) to 6.2, with a solution of H_2SO_4. The concentration of the prepared HOCl solution was subsequently determined spectrophotometrically [Spectrophotometer Jasco V-660), at 235 nm, applying the molar absorption coefficient (ε = 100 $M^{-1}cm^{-1}$)]. The working HOCl dilution was prepared using Na_2HPO_4 buffer (100 mM), at pH 7.4, and the stock solution of DHR (2.89 mM) was prepared using DMF as solvent.

In a 96 wells black-walled microplate, the reaction mixture contained the following reagents, at the indicated final concentrations (in a final volume of 300 µL): Na_2HPO_4 buffer solution (100 mM), at pH 7.4; tested compounds (0.08 - 500 µM), dissolved in acetone; DHR (5 µM) and HOCl (5 µM), both dissolved in Na_2HPO_4 buffer solution (100 mM), at pH 7.4. The fluorimetric assays were carried out at 37 °C, in a microplate reader (Synergy HT, BIO-TEK), at the emission wavelength of 528 ± 20 nm, with excitation at 485 ± 20 nm. L-(+)-Ascorbic acid (1.56 - 100 µM) was used as positive control. In each assay, at least three individual and independent experiments were performed in triplicate. Results are expressed as the percentage of inhibition of DHR oxidation (mean ± standard error of the mean, SEM) and the half maximal inhibitory concentration (IC_{50}, mean ± SEM).

2.3. Assessment of Human Neutrophils' Oxidative Burst

2.3.1. Isolation of Neutrophils

After obtaining signed informed consent by healthy human blood donors, from the Centro Hospitalar do Porto - Hospital de Santo António (Porto, Portugal), venous blood was collected into K_3EDTA vacuum tubes through antecubital venipuncture. The isolation of neutrophils from the whole blood was achieved by the gradient density centrifugation method, as previously described [27], using two solutions with different densities, histopaque 1119 (density = 1.119) and histopaque 1077 (density = 1.077). After a sequence of centrifugations (890*g*, for 30 minutes, at 20ºC and 870*g*, for 5 minutes, at 4ºC), the cell suspension was washed and resuspended with DPBS, the remaining erythrocytes were lysed with sterile distilled water and the isotonicity was re-established with a solution of NaCl 3%. Finally, isolated neutrophils were resuspended in Tris-glucose buffer (5.5 mM) [$CaCl_2$, KCl, $MgSO_4$, NaCl, D-(+)-glucose and trizma® base], at pH 7.4, and were kept on ice, under gentle soft shaking until the determination of their viability and their use in the oxidation of luminol assay.

2.3.2. Cell Viability

Neutrophils viability was estimated by the trypan blue exclusion assay. Neutrophils, suspended in Tris-glucose buffer solution (5.5 mM) at pH 7.4, were incubated with chalcones (at the highest tested concentrations, ranging from 3.125 µM to 100 µM) for 30 minutes, at 37 ºC. After incubation, in a microtube, neutrophils suspension was gently mixed with the trypan blue solution. Subsequently, neutrophils viability was established by counting the viable and non-viable neutrophils. All the assays were performed in duplicate. Cell viability over 90 % was considered acceptable.

2.3.3. Oxidation of Luminol

The luminol-amplified chemiluminometric technique was used to evaluate neutrophils' oxidative burst. Luminol reacts with ROS ($O_2^{\bullet-}$, $HO^{\bullet}$, H_2O_2 and HOCl), produced by the

stimulated neutrophils, through an oxidation reaction to generate an exciting aminophthalate anion that emits light when returning to the ground state. The chemiluminescence measurement was undertaken according to a previously described procedure [28]. Briefly, the isolated human neutrophils suspension (1×10^6/mL) was firstly placed in a 96 wells white-walled microplate; followed by the reagents, at the indicated final concentrations (in a final volume of 250 µL): tested compounds (0.1 - 100 µM), dissolved in DMSO; luminol (500 µM) and PMA (160 nM), both diluted in Tris-glucose buffer solution (5.5 mM), at pH 7.4. Before the addition of PMA, the microplate containing neutrophils, chalcones and luminol was pre-incubated for 5 minutes, at 37 ºC, in the microplate reader (Synergy HT, BIO-TEK) under gentle shaking. Kinetic readings were initiated promptly after the cells' stimulation with PMA and lasted 30 minutes. Measurements were taken at the maximum peak of the relative luminescence unit *versus* time curve, which was observed at around 10 minutes. Quercetin (0.39 – 50 µM) was used as positive control. In each assay, at least four individual and independent experiments were performed in duplicate. Results are expressed as the percentage of inhibition of luminol oxidation (mean ± SEM) and the half maximal inhibitory concentration (IC_{50}, mean ± SEM).

2.4. Statistical Analysis

GraphPad Prism™ (version 8.0; GraphPad Software, San Diego, CA, USA) was used to calculate the percentage of inhibition of DHR and luminol oxidation and to determine the IC_{50} values of the compounds under study, displayed as mean ± SEM. Statistical comparison between groups was estimated using the one-way analysis of variance (one-way ANOVA), followed by Bonferroni's multiple comparisons test with a confidence level of 95%.

3. RESULTS AND DISCUSSION

In the present study, a series of structurally related chalcones were studied: with the A- and/or B-rings unsubstituted or bearing the following substituents: hydroxy, methoxy, methyl, nitro groups and/or chlorine atom(s), in various positions on both rings (Fig. **1**). In terms of antioxidant activity, the tested chalcones may act through different mechanisms, namely by scavenging of reactive prooxidant species or by the inhibition of the expression or activity of several enzymes involved in their production (*e.g*. NADPH oxidase, SOD, CAT or MPO). Physiologically, the production of HOCl by human neutrophils has an effective microbicidal activity [29]. However, its overproduction, and of other ROS, may be harmful, where the chlorinative stress is strongly correlated with inflammatory diseases and aging conditions [30]. In this context, and due to the above described importance of HOCl, this work aimed, to specifically study the HOCl scavenging and the modulation of neutrophils' oxidative burst by a panel of chalcones. The chalcones tested in the present work, **1a-f**, **2a-o**, **3a-c**, **4a-c**, **5a-e** and **6a-c**, allowed the establishment of a SAR concerning HOCl scavenging and modulation of neutrophils' oxidative burst. Analysing the results obtained for the inhibition of DHR oxidation (Table **1**), *i.e.*, HOCl scavenging, the most active chalcone was found to be 3-(3,4-dihydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)prop-2-en-1-one (**5b**) with an IC_{50} value of 1.0 ± 0.1 µM; followed by chalcones 1-(2,4-dihydroxyphenyl)-3-(3,4-dihydroxyphenyl)prop-2-en-1-one (**5d**) (IC_{50} = 15 ± 2 µM); 1-(2-hydroxy-4,6-dimethoxy-phenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (**6b**) (IC_{50} = 18 ± 1 µM); 1-(2-hydroxyphenyl)-3-(3,4,5-trihydroxy-phenyl)prop-2-en-1-one (**2o**) (IC_{50} = 18.5 ± 0.5 µM) and 1-(2,4-dihydroxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (**5e**) (IC_{50} = 19 ± 2 µM). Of note, these chalcones have in common a hydroxy group at C-2' on A-ring. Chalcones **5b**, **5d** and **2o** also have in common hydroxy groups at C-3 and C-4; and chalcones **6b** and **5e** also have in common a methoxy group at C-4, both on B-ring. Overall, what seems to turn chalcones into better HOCl scavengers is a high number of hydroxy groups distributed on both rings (**5b**).

Analysing the obtained results by group, and as shown in Table **1**, chalcones unsubstituted on A-ring, belonging to group 1 (**1a-f**), did not show an expressive activity. These chalcones showed maximal inhibition percentages of DHR oxidation ranging from 17 ± 1 % to 41 ± 1 %, up to the highest tested concentrations.

Group 2 comprises 2'-hydroxychalcones. Chalcone **2o**, bearing a hydroxy group at C-2' on A-ring and also at C-3, C-4 and C-5 on B-ring, was the most active of this group, with an IC_{50} value of 18.5 ± 0.5 µM; followed by chalcones **2j** (IC_{50} = 24 ± 2 µM) and **2n** (IC_{50} = 57 ± 2 µM). Comparing the chemical structures of these three most active chalcones, it can be observed that all have in common hydroxy groups at C-2' on A-ring and at C-4 on B-ring. Chalcones **2e** and **2m** showed the highest IC_{50} values of group 2, 290 ± 16 µM and 99 ± 2 µM, respectively. These results may be justified by the fact that these chalcones did not present a hydroxy group at C-4 on B-ring; **2e** has a methoxy group at C-4 and **2m** has no substituent at C-4. For all other chalcones from group 2 (**2a-d**, **2f-i** and **2l**), it was not possible to determine the IC_{50} values (Table **1**). Perjési *et al*. [31], in a non-cellular assay, demonstrated that 4-hydroxychalcone also showed a pronounced antioxidant effect through $HO^{\bullet}$ scavenging. Thus, it is possible to infer that the presence of a hydroxy group at C-2' on A-ring and, more importantly, at C-4 on B-ring, seems to be essential for scavenging activity. Moreover, Xue *et al*. [32], in a theoretical study for the evaluation of the antioxidant properties of 2′-hydroxychalcones, concluded that chalcones with hydroxy groups at C-4 on B-ring, as in C-2, have higher H-atom donation ability, compared to hydroxy groups at C-3.

From chalcones belonging to group 3 (**3a-c**), chalcone **3c**, bearing hydroxy groups at C-2' on A-ring and at C-2 on B-ring, was the only one that was able to achieve an IC_{50} value (55 ± 7 µM) (Table **1**). In the face of this observation and comparing the chemical structures of chalcones **3c** and **2n**, it can be seen that, in addition to the hydroxy group at C-2' on A-ring, the presence of an extra hydroxy group at C-2 or C-4 on B-ring seems to favour the scavenging activity. Indeed, the results obtained by Xue *et al*. [32] also confirmed chalcones **2n** and **3c** as potential radical scavengers.

Concerning group 4, composed of 2'-hydroxychalcones chlorinated at C-2 on B-ring (**4a-c**), none of them demonstrated a promising activity (Table **1**). Therefore, it can be concluded, under the current experimental conditions, that

Table 1. HOCl scavenging activity and inhibition of neutrophils' oxidative burst by the tested compounds.

Chalcone	Inhibition of DHR oxidation		Inhibition of luminol oxidation	
	Tested Concentrations (μM)[#]	IC_{50} (μM) (mean ± SEM)[*]	Tested Concentrations (μM)[#]	IC_{50} (μM) (mean ± SEM)[*]
Group 1				
1a (chalcone)	2.5 - 150	N.A.	0.39 - 50	13 ± 2
1b	25 - 500	22 ± 2 %[*]	1.56 - 100	15 ± 2
1c	12.5 - 125	31 ± 5 %[*]	0.39 - 12.5	5.4 ± 0.5
1d	12.5 - 200	34 ± 1 %[*]		1.7 ± 0.2
1e	1.56 - 125	17 ± 1 %[*]	1.56 - 100	27 ± 3
1f		41 ± 1 %[*]	1.56 - 50	8.8 ± 0.3
Group 2				
2a	25 - 500	40 ± 2 %[*]	0.39 - 25	6 ± 1
2b	2.5 - 50	27 ± 3 %[*]	0.19 - 50	3.4 ± 0.4
2c	25 - 50	20 ± 8 %[*]	0.19 - 25	3.0 ± 0.4
2d	12.5 - 125	34 ± 1 %[*]		0.61 ± 0.02
2e	25 - 500	290 ± 16	0.78 - 25	7 ± 1
2f	25 - 50	33 ± 2 %[*]	0.1 - 25	0.8 ± 0.2
2g	12.5 - 125	27.8 ± 0.4 %[*]	0.78 - 12.5	2.8 ± 0.3
2h	25 - 50	34 ± 10 %[*]	0.19 - 25	50 ± 3 %[*]
2i	6.25 - 200	42 ± 3 %[*]		1.3 ± 0.1
2j	2.5 - 200	24 ± 2	0.19 - 6.25	1.0 ± 0.2
2l	25 - 125	37 ± 3 %[*]	0.19 - 25	5 ± 1
2m	25 - 500	99 ± 2	0.78 - 50	6.4 ± 0.3
2n	1.56 - 125	57 ± 2	0.39 - 12.5	1.6 ± 0.2
2o	3.125 - 125	18.5 ± 0.5	0.78 - 12.5	7 ± 1
Group 3				
3a	12.5 - 125	32 ± 1 %[*]	1.56 - 6.25	N.A.
3b	2.5 - 50	35 ± 2 %[*]		50 ± 2 %[*]
3c	1.56 - 417	55 ± 7	1.56 - 50	6 ± 1
Group 4				
4a	25 - 125	N.A.	0.19 - 25	2.5 ± 0.3
4b	25 - 150	39 ± 3 %[*]	0.19 - 3.125	1.4 ± 0.3
4c	12.5 - 50	25 ± 1 %[*]	0.19 - 12.5	30 ± 4 %[*]
Group 5				
5a	6.25 - 500	37 ± 4	0.19 - 25	13 ± 1
5b	0.08 - 12.5	1.0 ± 0.1	0.01 - 25	4.4 ± 0.6
5c	6.25 - 500	25 ± 2	0.39 - 12.5	6 ± 1

(Table 1) contd….

Chalcone	Inhibition of DHR oxidation		Inhibition of luminol oxidation	
	Tested Concentrations (μM)[#]	IC_{50} (μM) (mean ± SEM)[*]	Tested Concentrations (μM)[#]	IC_{50} (μM) (mean ± SEM)[*]
5d (butein)	6.25 - 250	15 ± 2	0.19 - 12.5	2.9 ± 0.5
5e	2.5 - 500	19 ± 2	0.78 - 12.5	46 ± 4 %[*]
Group 6				
6a	1.25 - 12.5	36 ± 2 %[*]	0.19 - 50	43 ± 5 %[*]
6b	0.64 - 50	18 ± 1	0.78 - 3.125	N.A.
6c	25 - 500	24 ± 5 %[*]	1.56 - 100	78 ± 4
Positive controls				
L-Ascorbic acid	1.56 - 100	11 ± 1	-	-
Quercetin	-	-	0.39 - 50	3.3 ± 0.6

***** Percentage of inhibition (%) of DHR/luminol oxidation, at the indicated highest tested concentration.
# Range of tested concentrations, considering chalcones' solubility and/or cell viability under the present experimental conditions.
N.A., no activity found when the inhibition of DHR/luminol oxidation was below 10%, up to the highest tested concentration.

the presence of chlorine atom(s) on B-ring in chalcone moiety does not appear to favour the scavenging of HOCl.

In general, chalcones from group 5 (**5a-e**) were good scavengers of HOCl, in this order of potencies **5b** > **5d** > **5e** > **5c** > **5a**. These results may be related to the free radical scavenging ability of the two hydroxy groups present at C-2' and C-4' on A-ring. The most active chalcone **5b** has three hydroxy groups at C-2', C-4' and C-6' on A-ring and two hydroxy groups at C-3 and C-4 on B-ring. This chalcone was almost 10x more potent (IC_{50} = 1.0 ± 0.1 μM) than the positive control used, L-(+)-ascorbic acid (IC_{50} = 11 ± 1 μM). In a direct comparison between chalcones **5b** and **5d** (butein), they only differ on the hydroxy group at C-6' on A-ring. This replacement seems to be a very important factor for the HOCl scavenging, since chalcone **5d** (butein), without this substitution, showed an IC_{50} value almost 15x higher (IC_{50} = 15 ± 2 μM) than chalcone **5b** (IC_{50} = 1.0 ± 0.1 μM). In addition, the HOCl scavenging activity is also not so favoured when B-ring is not replaced by any hydroxy group (**5a**) or is replaced by methoxy groups (**5c** and **5e**) (Table **1**). Interestingly, Kozlowski *et al*. [33] from their results of the quantum chemical calculations, demonstrated the importance of the H atom transfer mechanism to explain the chalcones ability to scavenge free radicals. They confirmed that the catechol moiety on B-ring explains the redox properties of chalcones; and the hydroxy group on C-6' enables H transfer to a group with a relatively high H atom affinity (*e.g*. peroxyl radical), but with a weaker redox capacity when compared to the catechol. In conclusion, in this group of chalcones, the number and position of hydroxy groups present are the most important contributing factor to the scavenging activity.

Finally, concerning chalcones from group 6 (**6a-c**), chalcone **6b,** bearing a hydroxy group at C-2' and methoxy groups at C-4', C-6' and C-4 on A- and B-rings, was the most active, with an IC_{50} value of 18 ± 1 μM. It should be noted that the presence of two or three methoxy groups on B-ring (**6c** and **6a**, respectively) led to a decrease in the HOCl scavenging activity (Table **1**). However, studies published by Perjési *et al*. [31], Detsi *et al*. [34] and Sivakumar *et al*. [35] have shown that chalcones bearing only one methoxy group at C-4 on B-ring also displayed antioxidant activity through the scavenging of several RS ($O_2^{\bullet-}$, H_2O_2 and $HO^{\bullet}$).

The study of neutrophils' oxidative burst modulation was carried out using the probe luminol. This probe not only detects the production of ROS extracellularly, but also intracellularly due to its small size and the intrinsic lipophilicity which allow this molecule to cross biological membranes and achieve the inner of cells [28]. The spatial conformation and lipophilicity of the chalcones have been considered in the analyses of the SAR studies. The cytotoxic effect of all the tested compounds was evaluated at the correspondent highest tested concentration for each compound (3.125 - 100 μM). The cell viability was maintained over 90 % under the experimental conditions of the luminol oxidation assay (for 30 minutes, at 37ºC, under gentle shaking). Therefore, these were the highest concentrations tested in neutrophils' oxidative burst assays.

Comparing all the results obtained for the inhibition of luminol oxidation assay (Table **1**), *i.e.*, inhibition of neutrophils' oxidative burst, the most active chalcones found were 3-(3-chlorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one (**2d**), 3-(3,4-dichlorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one (**2f**), 3-(4-hydroxy-3-methoxyphenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one (**2j**), 1-(2-hydroxyphenyl)-3-(4-nitrophenyl)prop-2-en-1-one (**2i**), 3-(2,6-dichlorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one (**4b**), 1-(2-hydroxyphenyl)-3-(4-hydroxyphenyl)prop-2-en-1-one (**2n**) and 3-(4-nitrophenyl)-1-phenylprop-2-en-1-one (**1d**), which showed IC_{50} values below 2 μM: 0.61 ± 0.02 μM; 0.8 ± 0.2 μM; 1.0 ± 0.2 μM; 1.3 ± 0.1 μM; 1.4 ± 0.3 μM; 1.6 ± 0.2 μM and 1.7 ± 0.2 μM, respectively. Analyzing the structures of these chalcones, it can be seen that all of them have in common a hydroxy group at C-2' on A-ring, besides chalcone **1d**. Analyzing the chemical structures of chalcones **2d** and **2f**, it is possible to note that both have in common a chlorine atom at C-3 on B-ring. Chalcones **1d** and **2i** have in common a nitro

group at C-4 on B-ring and chalcones **2j** and **2n** have in common a hydroxy group at C-4 on B-ring. Lastly, chalcones **2f** and **4b** have in common a chlorine atom at C-4 on B-ring.

Regarding group 1 (**1a-f**), and as shown in Table **1**, chalcone **1d** substituted by a nitro group (a strong electron-withdrawing group) at C-4 on B-ring was the most active, with an IC_{50} value of 1.7 ± 0.2 μM. Chalcone **1c** which has a chlorine atom (a weak electron-withdrawing substituent) at C-4 on B-ring showed an IC_{50} value of 5.4 ± 0.5 μM. Chalcone **1f**, bearing a hydroxy group (an electron donor group) at C-4 on B-ring, showed an IC_{50} value of 8.8 ± 0.3 μM. In view of these results, it can be concluded that the presence of a strong electron-withdrawing group (*e.g*. nitro group) at C-4 on B-ring favours the studied activity.

Group 2 comprises hydroxylated chalcones at C-2' on A-ring, being chalcones **2d**, **2f**, **2j**, **2i** and **2n** some of the most active among all the studied chalcones (IC_{50} values of 0.61 ± 0.02 μM; 0.8 ± 0.2 μM; 1.0 ± 0.2 μM; 1.3 ± 0.1 μM and 1.6 ± 0.2 μM, respectively). Analyzing their chemical structures, it is possible to note that all present substituents at C-3 and/or C-4 on B-ring. However, the presence of chlorine atom(s) in that position(s), as in chalcones **2d** and **2f**, seems to slightly favour the activity. Chalcones **2a-c**, **2e**, **2g**, **2l-m** and **2o** also demonstrated good activities, with IC_{50} values ranging from 2.8 ± 0.3 μM to 7 ± 1 μM. These chalcones are substituted by various groups, such as methoxy, methyl and/or hydroxy or by chlorine atom at C-3, C-4 and/or C-5 on B-ring. The least active chalcone in this group was chalcone **2h** (50 ± 3 %, at 25 μM), bearing a 4-methoxy group on B-ring. Therefore, it can be concluded that the presence of chlorine atom(s) at C-3 and/or C-4 on B-ring seem(s) to favour the inhibitory activity. The presence of a chlorine atom in an aromatic ring of a molecule causes, overall, its protection against metabolic hydroxylation; an increase in its electrophilic reactivity, acidity and lipophilicity and a decrease in the basicity of the neighbouring hydrogen atoms. These effects can facilitate the transport of a compound through the biological membranes and its interaction with amino acids in the binding pocket of a protein, hence increasing the biologic activity [36].

With respect to chalcones from group 3 (**3a-c**), chalcone **3c** bearing hydroxy groups at C-2' and C-2 on A- and B-rings, respectively, was the most active, with an IC_{50} value of 5.8 ± 0.7 μM. The methoxy group at C-2 on B-ring (**3b**) leads to a considerable decrease in the activity (50 ± 2 %, at 50 μM) (Table **1**). Thus, in this group of chalcones, it is possible to observe that the presence of two hydroxy groups benefits the inhibition of neutrophils' oxidative burst.

Chalcones from group 4 (**4a-c**) are also 2'-hydroxychalcones. Chalcone **4b** bearing chlorine atoms at C-2 and C-6 on B-ring was the most active (IC_{50} = 1.4 ± 0.3 μM). Chalcone **4c**, also having chlorine atoms on B-ring but, at C-2 and C-4, showed a decrease in activity (30 ± 4 %, at 12.5 μM) (Table **1**). However, comparing chalcones **4b** and **2f**, it is possible to note that di-substitution on B-ring by chlorine atoms is more favourable to the activity when it occurs at C-3 and C-4 or at C-2 and C-6.

Group 5 comprises chalcones which are hydroxylated at C-2' and C-4' on A-ring (**5a-e**). Chalcone **5d** (butein), also hydroxylated at C-3 and C-4 on B-ring, was the most active, with an IC_{50} value of 2.9 ± 0.5 μM. When an additional hydroxy group is present at C-6' on A-ring, as in chalcone **5b**, it is possible to note a slight decrease in activity (IC_{50} = 4.4 ± 0.6 μM). Moreover, a drastic decrease in the activity is noted when methoxy group(s) was/were added on B-ring, as in chalcones **5c** and **5e** (Table **1**). In conclusion, the presence of four hydroxy groups distributed in the aromatic rings seems to favour the studied activity.

Lastly, chalcones from group 6 (**6a-c**) bearing methoxy groups at C-2' and C-4' on A-ring did not exhibit favourable activities. Chalcone **6c**, having only methoxy groups at C-2', C-4' and C-6' on A-ring and at C-3 and C-4 on B-ring, showed a high IC_{50} value of 78 ± 4 μM, but was the most active of this group. From these results, it is possible to deduce that the presence of methoxy groups on A-ring does not favour the activity under study.

Finally, the most active chalcone in HOCl scavenging assay (**5b**) was not the most active one in the modulation of neutrophils' oxidative burst (**1d**, **2d**, **2f**, **2i**, **2j**, **2n** and **4b**). The possible reasons that may explain these results were described in the studies performed by Leopoldini *et al*. [11] and Constantinescu *et al*. [37]. In the first study, the authors showed that polyphenolic compounds are scavengers of free radicals through hydrogen atom transfer, single electron transfer and metals chelation. In the second study, the authors demonstrated that the lipophilicity is a central component of drug-like properties of chalcones, which influences the membrane transportation and the binding of these compounds to intracellular targets. Once chalcones may act through the scavenging

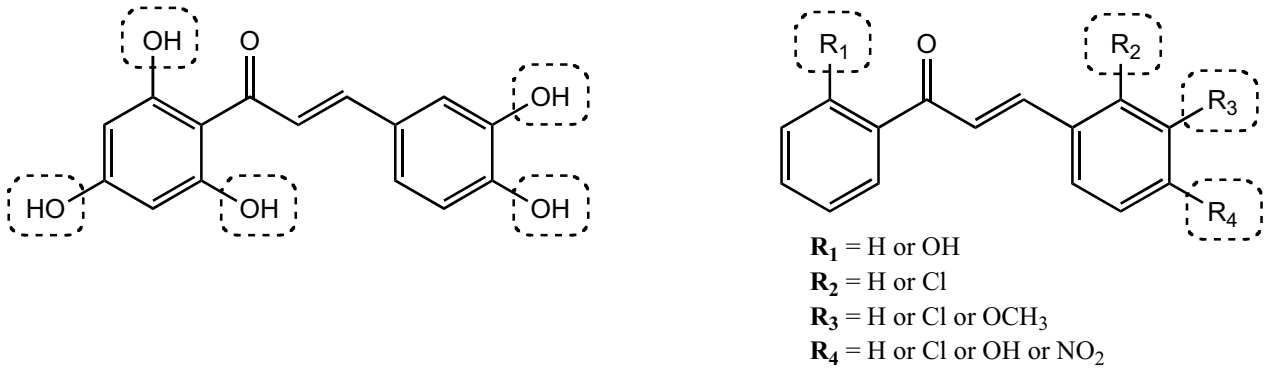


Fig. (2). Chemical structural features of the most active chalcones for: (**a**) HOCl scavenging and (**b**) neutrophils' oxidative burst inhibition.

of ROS or the inhibition of a certain targets inside the cells, the balance between the degrees of hydrophilicity and lipophilicity of these molecules, as well as the possible presence of specific membrane transporters should be considered.

CONCLUSION

In this work, chalcone **5b**, bearing hydroxy groups at C-2', C-4' and C-6' on A-ring and at C-3 and C-4 on B-ring, was highly effective in HOCl scavenging. In the neutrophils' oxidative burst assay, chalcones **1d**, **2d**, **2f**, **2i**, **2j**, **2n** and **4b**, unsubstituted or bearing a hydroxy group at C-2' on A-ring, and methoxy, hydroxy and/or nitro groups or chlorine atom(s) at C-2, C-3 and/or C-4 on B-ring, were the most active. As far as we know, the most active chalcones found in this work had not been investigated for both studied activities, which enabled us to contribute with new SAR findings. The present study highlights not only the ideal structural features for HOCl scavenging, but also the importance of the balance between lipophilicity and hydrophilicity for an excellent ability to inhibit neutrophils' oxidative burst. The SAR discussed throughout this work provide a platform for the structural requirements of chalcones (Fig. **2**) to act as antioxidant agents and that may allow the design and synthesis of new and promising anti-inflammatory chalcones.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Ethics Committee of Centro Hospitalar Universitário do Porto / Instituto de Ciências Biomédicas Abel Salazar, Oporto, Portugal.

HUMAN AND ANIMAL RIGHTS

No Animals were used in this research. All human procedures were in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013.

CONSENT FOR PUBLICATION

Consent has been obtained from all the participants of the study.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Manuscript II

The role of polarized macrophages in the activation of resolution pathways within human granulosa cells

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RESEARCH Open Access

The role of macrophages phenotypes in the activation of resolution pathways within human granulosa cells

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Abstract

Background: Inflammatory state within the ovaries can disrupt normal follicular dynamics, leading to reduced oocyte quality and infertility. How the production of inflammatory mediators generated by macrophages with different gene expression profile (M1 and M2) might activate inflammatory pathways, such as cyclooxygenase-2 (COX-2) and 5-, 12-, and 15-lipoxygenase (LOX), in human granulosa cells (hGCs) remains unclear.

Methods: In this study, we evaluated how M1 and M2 macrophages found in the ovaries affect the functions of hGCs isolated from women undergoing assisted reproductive technology (ART) and human ovarian granulosa COV434 cells. For this purpose, a model of interaction between hGCs and COV434 cells and conditioned media (CMs) obtained from culture of M0, M1 and M2 macrophages was established. We used real-time PCR and western blotting to detect the expression of COX-2 and 5-, 12-, and 15-LOX as biomarkers of oocyte competence.

Results: Our data showed that M2 macrophages with anti-inflammatory characteristics were able to significantly increase the expression of COX-2 in hGCs. We also demonstrated that M1 macrophages with pro-inflammatory characteristics were able to significantly increase the expression of 12-LOX in hGCs. However, there was no observed expression of 5-LOX and no significant alteration in the expression of 15-LOX in hGCs. Regarding COV434 cells, we found that CM from M2 macrophage resulted in an increase in COX-2, 5-LOX and 15-LOX mRNA and protein levels. No expression of 12-LOX by COV434 cells was observed when exposed to CMs from M1 and M2 macrophages.

Conclusions: Our research indicated that the production of pro-resolving mediators by hGCs can, at least in part, reverse the physiological inflammation present in the ovaries.

Keywords: Inflammation, Infertility, Macrophage, Granulosa cells, Cyclooxygenase, Lipoxygenase

Introduction

Ovulation displays features that resemble inflammation, which can disrupt the normal follicular dynamics and lead to reduced oocyte quality and infertility [1]. According to the World Health Organization (WHO), infertility is a condition that affects 15% of reproductive-aged couples worldwide, with many requiring the support of assisted reproductive techniques (ART) [2, 3]. Several studies aimed to characterize potential biomarkers of oocyte competence with pregnancy outcomes. Genes expressed by human granulosa cells (hGCs), such as cyclooxygenase-2 (COX-2) and 5-, 12-, and 15-lipoxygenase (LOX), may be useful as effective biomarkers to identify oocytes with high development potential [4, 5].

Ovulation is initiated by the onset of a luteinizing hormone (LH) surge that is accompanied by inflammatory signals, such as inflammatory mediator's production, blood flow increase, leukocyte infiltration, swelling, and

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tissue degradation and repair. Pre-ovulatory follicles are comprised of an oocyte surrounded by hGCs, follicular fluid (FF), and a highly vascularized theca cells (TCs) compartment, in which resident leukocytes are found [6].

GCs play a remarkable role in oocyte development, being interconnected with their plasma membrane by gap junctions that sustain them at an arresting stage and transport nutrients. These cells can exhibit distinct phenotypes: mural GCs (MGCs) covering the follicle wall and participating in the physical barrier, estradiol production, and follicle growth; and cumulus cells (CCs) that directly surround the oocyte, providing them with nutrients and protection from harmful signals that can influence their quality [7].

The LH surge activates signalling pathways in GCs of pre-ovulatory follicles associated with inflammation, such as the expression of COX-2 and 5-, 12-, and 15-LOX. These activation leads to the production of inflammatory mediators, such as eicosanoids and specialized pro-resolving mediators (SPMs), which can be found at high levels in human FF impacting on oocyte growth and quality [6–8]. In the ovaries, PGE_2 contributes to tissue remodelling, follicular wall proteolysis, corpus luteum formation, and regulating vascular changes. All these events are considered as final players of ovulation that coordinate the overall response, which results in the release of the cumulus-enclosed oocyte [6].

In general, inflammation is a defensive response triggered by stimuli that aims to restore homeostasis. The initial response consists of a progression of events from leukocytes recruitment to pro-inflammatory lipids production. A successful acute inflammatory response leads to the elimination of the stimuli and suspension of pro-inflammatory lipids production, initiating the resolution and repair phases with the production of SPMs [9]. The influx of neutrophils is replaced by macrophages that undergo reprogramming from classically activated M1 macrophages to alternatively activated M2 macrophages [10].

Macrophages are among the predominant immune cells in the acute inflammatory response and ovulation, being found at high levels in FF [11]. The molecular pathways in which these cells are involved in regulation ovulation are not yet fully elucidated. However, evidence suggests that macrophages are implicated in leukocyte recruitment, secretion of immune mediators, breakdown of the extracellular matrix component, and regulation of tissue remodelling after ovulation [12]. Since macrophages act as drivers and regulators of diseases, therapeutic strategies that either decrease the amount and function of M1 or increase M2 activity are undergoing extensive preclinical and clinical research [13].

However, uncontrolled or unsuccessful immune responses can lead to continued recruitment of inflammatory cells. The persistence of tissue damage results in organ dysfunction and, consequently, the establishment of chronic inflammatory diseases [14]. An enhanced understanding of the role of inflammation in ovulation and the control of this process may lead to exploiting this knowledge to treat anovulatory infertility.

Since the crosstalk between hGCs and macrophages shows issues relevant to the production of key factors during oocyte development, this relationship requires further investigation. Therefore, the present study aimed to simulate the ovarian microenvironment and investigate how the production of pro- and anti-inflammatory mediators generated by M1 and M2 macrophages, respectively, might activate inflammatory pathways in GCs isolated from women undergoing in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI) techniques.

Materials and methods

Materials

All reagents and solvents were used of analytical grade. Dulbecco's modified eagle medium F-12 (DMEM/F-12), Roswell park memorial institute 1640 (RPMI-1640), methylthiazolyldiphenyl-tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS), were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Phorbol 12-myristate 13-acetate (PMA) was obtained from Santa Cruz Biotechnology. Penicilin and streptomycin (AB-AM) were bought from Grisp (Porto, Portugal). Trypsin and ethylenediaminetetraacetic acid (EDTA) were obtained from Gibco/Invitrogen Co. (Carlsbad, CA, USA). Fetal bovine serum (FBS) came from Biochrom and percoll from GE Healthcare (Buckinghamshire, UK). Recombinant human interferon-γ (IFN-γ) was purchased from R&D Systems and recombinant human interleukin-13 (IL-13) from Biotechne. Dibutylphthalate polystyrene xylene (DPX) was bought from VWR-Prolabo (Radnor, PA, USA). THP-1 cells (#88,081,201) and COV434 cells (#07,071,909) were purchased from the European Collection of Authenticated Cell Cultures (ECACC). Cell culture flasks were obtained from Sarstedt (Nümbrecht, Germany) and all other plastic materials used in cell culture were from Falcon (Tewksbury, MA, USA) and Nerbe plus (Winsen, Germany).

Methods

Study design

FF samples containing hGCs retrieved from women undergoing ovarian stimulation for ART were harvested with their agreement at Unidade de Medicina da

Reprodução Dra. Ingeborg Chaves-Centro Hospitalar de Vila Nova de Gaia/Espinho, from January 2021 to April 2022. A group of 32 patients was enrolled in this study, with a mean of 32 years of age and 21.5 kg/m^2 of body mass index (BMI). Inclusion criteria were women presenting male factor associated to infertility. Women with endometriosis, polycystic ovary syndrome (PCOS), tubal factor, and idiopathic factor, as well as women with infertility associated to hormonal factors, were excluded. Data regarding the oocyte number, maturity, fertilization rate, and clinical pregnancy were collected. All procedures were conducted in accordance with the Declaration of Helsinki, endorsed by the local ethics committee and approved by Comissão de Proteção de Dados (Proc. no. 764/2017).

Collection and isolation of primary human granulosa cells

Ovarian stimulation was triggered with different dosages of gonadotropins, follicle stimulating hormone (FSH) and/or LH, for multifollicular development. Then, the final stimulus was performed by a single administration of human chorionic gonadotropin (hCG) or gonadotropin-releasing hormone (GnRH) agonist 36 h before the oocyte retrieval. For ART performance, the FF consisting of oocyte and hGCs was aspirated and the oocyte was isolated for further fertilization. The remaining FF was transferred to 100 mL polypropylene tubes and stored at 37ºC for a maximum of 2 h until sample processing. hGCs were isolated and cultured independently according to the protocol previously published by Moreira-Pinto et al. [15], with some modifications. Briefly, FF samples were centrifuged at 350 g at 10 °C for 10 min. The cell pellet was collected and slowly added to a Percoll:PBS (1:1) density gradient. The samples were then centrifuged at 900 g at 10 °C for 20 min. hGCs present at the interface of Percoll:PBS and FF gradient were collected into a new tube, in which DMEM/F12 10% FBS was added and centrifuged at 350 g at 10 °C for 5 min to wash cells. The supernatant was discarded and the hGCs were resuspended in DMEM/F12 10% FBS.

Human granulosa cell line COV434 culture conditions

Human ovarian granulosa COV434 cells originally derived from a solid primary tumor were used as model of GCs and cultured according to specific indications from ECACC [16]. COV434 cells were cultured in DMEM/F12 10% FBS medium supplemented with l-glutamine (2 mM), 1% AB-AM, and 10% FBS at 37 °C with 5% CO_2 atmosphere. COV434 cells were maintained at a minimum density of 2.0×10^4 cells/mL and were trypsinized using 0.25% trypsin/EDTA (1 mM) upon reaching 4.0×10^4 cells/mL.

Human monocyte cell line THP-1 culture conditions

Human acute monocytic leukemia THP-1 cells derived from the peripheral blood were used as model of human monocytic cells and cultured according specific indications from ECACC [17]. THP-1 cells were cultured in RPMI 1640 medium supplemented with l-glutamine (2 mM), 1% AB-AM, and 10% FBS at 37 °C with 5% CO_2 atmosphere. THP-1 cells were maintained at a minimum density of 3.0×10^5 cells/mL and were passaged upon reaching 8.0×10^5 cells/mL.

THP-1 derived macrophages culture conditions

THP-1-derived macrophages were obtained following a previous procedure described by Fonseca et al. [18], with some modifications. Firstly, THP-1 cells were seeded at a density of 5.0×10^5 cells/well in a 24-well plate and stimulated with PMA (25 ng/mL) for 48 h. After PMA exposure, the medium was discarded and the cells were washed with PBS, followed by a further incubation in fresh medium for 24 h as a resting period. Then, macrophages were differentiated into M1 and M2 phenotypes by incubation with IFN-γ (20 ng/mL) + LPS (10 pg/mL) and IL-13 (20 ng/mL), respectively, for 24 h. After macrophages polarization, the differentiation factors were removed and the cells were washed with PBS, followed by a new incubation in fresh medium for 72 h, initiating cytokines production. After 72 h, macrophage supernatants were collected, centrifuged at 300 g at 10ºC for 5 min to remove cell debris and then stored at -80ºC until use.

Interaction model between cell cultures

To evaluate how macrophage phenotypes found in the ovaries affect the GCs functions, an interaction model was established between hGCs and COV434 cells and conditioned media (CMs) obtained from culture of M0, M1 and M2 macrophages [18]. hGCs were seeded in a 6-well plate (1.5×10^6 cells/well) and 24-well plate (7.0×10^5 cells/well) and COV434 cells were also seeded in a 6-well plate (8.0×10^5 cells/well) and 24-well plate (3.5×10^5 cells/well) in DMEM/F12 10% FBS, and incubated for 24 h for cell adhesion. After incubation, the medium was removed, the CMs obtained from culture of M0, M1 and M2 macrophages were added in a 6-well plate (2000 µL) and 24-well plate (500 µL), and incubated for 72 h at 37 °C with 5% CO_2 atmosphere.

Cell viability

hGCs and COV434 cells were seeded at densities of 7.5×10^4 and 5.0×10^4 cells/well, respectively, in a transparent 96-well plate. After 24 h, the medium was removed, CMs obtained from culture of M0, M1 and M2

macrophages were added to a final volume of 200 µL, and incubated for 72 h at 37 °C with 5% CO_2 atmosphere. MTT assay conditions were adapted from the work by Castelôa et al. [19]. Briefly, at the end of incubation time, an MTT solution was added (0.5 mg/mL) and the plate was incubated for another 3 h at 37°C with 5% CO_2 atmosphere. After incubation, the medium was removed and a solution of DMSO/isopropanol (3:1) was added to dissolve the purple MTT formazan crystals. The plate was gently shaken at 70 rpm for 15 min to dissolve the crystals, which were quantified spectrophotometrically at 540 nm using a microplate reader (BioTek Synergy HTX Multi-Mode).

Cell morphology analysis

After 72 h of interaction between CMs obtained from culture of M0, M1 and M2 macrophages, morphological abnormalities in hGCs and COV434 cells were evaluated by Giemsa stain [19]. hGCs and COV434 cells were seeded in a 24-well plate with round coverslips at densities of 7.0×10^5 and 3.5×10^5 cells/well, respectively. After adherence, 500 µL of CMs of M0, M1 and M2 were added to the cells and incubated for 72 h at 37 °C with 5% CO_2 atmosphere. After incubation, the CMs were removed, cells were washed twice with PBS, and fixed with cool methanol for 10 min at room temperature (RT). Then, the methanol was removed, cells were washed again twice with PBS, Giemsa solution in H_20 (1:10) was added, and the cells were incubated for a further 30 min. After incubation, the cells were washed several times with tap water and the coverslips were removed, dried, and mounted with DPX medium. Cells were observed under the Eclipse CI microscope (Nikon, Japan).

RNA extraction, cDNA synthesis and Real-Time Polymerase Chain Reaction (RT-PCR)

For macrophages derived from THP-1 differentiation ($n = 3$, in triplicate) and hGCs and COV434 cells experiments ($n = 3$, in duplicate), cells were lysed with TripleXtractor reagent (#GB23.0050; GRiSP Research Solution, Porto, Portugal) to isolate total RNA according to the manufacturer's protocol. The concentration and purity of the isolated RNA were measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and samples were stored at -20°C prior to cDNA synthesis. The isolated RNA was converted to cDNA using the Xpert cDNA Synthesis Mastermix (#GK81.0100; GRiSP Research Solution, Porto, Portugal) according to the manufacturer's protocol. Samples were diluted in H_2O treated with diethyl pyrocarbonate to a final volume of 40 µL and then stored at -20°C or immediately used as a template in RT-PCR. The cDNA was amplified with specific primers (Table 1), using Xpert Fast SYBR Mastermix (#GE20.2501; GRiSP Research Solution, Porto, Portugal) in Bio-Rad Real-Time PCR Detection System (Bio-Rad Laboratories, USA), according to the manufacturer's protocol. RT-PCR conditions were,

Table 1 Sequences of transcript primers and annealing temperatures for RT-PCR

mRNA Target	Primer Sequence: 5'-3'	Annealing temperature	Acession Number[a]	Ref
GADPH	Forward-GGATGATGTTCTGGAAGAGCC	55°C	-	[15]
	Reverse-AACAGCCTCAAGATCATCAGC			
IL-1β	Forward-ATGATGGCTTATTACAGTGGCAA	60°C	-	[20]
	Reverse-GTCGGAGATTCGTAGCTGGA			
TNF-α	Forward-GCTGCACTTTGGAGTGATCG	60°C		
	Reverse-GTGTGCCAGACACCCTATCT			
CCL18	Forward-TGCATTGCAGCGTCATCTTG	57°C	NM_002988.4	-
	Reverse-GAGTCCCATCTGCTATGCCC			
CCL22	Forward-AGGGCCAGGGGACATCTAAT	57°C	NM_002990.5	-
	Reverse-GAGATCTGTGCCGATCCCAG			
COX-2	Forward-GTTCCACCCGCAGTACAGAA	58°C	NM_000963.4	-
	Reverse-AGGGCTTCAGCATAAAGCGT			
5-LOX	Forward-CATGCCCAGGAACAGCTCGTT	57°C	-	[21]
	Reverse-AGTCCTCAGGCTTCCCCAAGT			
12-LOX	Forward-GCCCAAAGCTGTGCTAAACC	58°C	NM_000697.3	-
	Reverse-TGGGGGAGGAAATAGAGCCT			
15-LOX	Forward-CCTTCGTCCTCCAAACCTGT	57°C	NC_000017.11	-
	Reverse-GCTCGGATGTGGGTAGTGAC			

[a] Primers have been designed in-house by using Primer Blast from NIH

in all cases, initiated with a denaturation step at 95 °C for 3 min, followed by up to 40 cycles of denaturation, annealing, and primer extension. The fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized with the housekeeping gene glyceraldehydes 3-phosphate dehydrogenase (GAPDH).

Western blotting

For hGCs and COV434 cells experiments ($n=3$ for each target), CMs obtained from culture of M0, M1 and M2 macrophages were removed, cells were washed twice with PBS, lysed with lysis buffer (Tris–HCl 20 mM, NaCl 100 mM, EDTA 1 mM, and Triton X-100 1%) containing a protease inhibitor cocktail (#P8340; Sigma–Aldrich Co., St. Louis, MO, USA), and incubated on ice for 30 min. After incubation, cell extracts were harvested, frozen/thawed three times and centrifuged at 14000 g at 4°C for 10 min. The supernatant was collected and the protein concentration was quantified by Bradford assay. Samples (5–40 µg) were subjected to 10% SDS–PAGE and proteins were transferred to nitrocellulose membranes in a turbo transfer system (Trans-Blot®, Bio-Rad). After blocking in 5% non-fat milk for 1 h at RT, the membranes were incubated with the primary antibodies, COX-2 (#sc-23984, Santa Cruz Biotechnology), ALOX5 (#MA5-26,829, ThermoFisher Scientific), and ALOX12 (#MA5-26,911, ThermoFisher Scientific) diluted at 1:500, and 15-LOX (#sc-133085, Santa Cruz Biotechnology) diluted at 1:250. After incubation, primary antibodies were removed, membranes washed with PBS-Triton, and incubated with the secondary antibodies, peroxidise-linked anti-goat (#sc-2354, Santa Cruz Biotechnology) and anti-mouse (#sc-516102, Santa Cruz Biotechnology) both diluted at 1:2500 for 1 h at RT. Then, secondary antibodies were removed, membranes were washed again with PBS and PBS-Triton, and exposed to the chemiluminescent substrate. Immunoreactive bands were visualized by the ChemiDocTM Touch Imaging System (BioRad, Laboratories Melville, NY, USA). Membranes were then stripped and incubated with β-Actin (sc-47778, Santa Cruz Biotechnology) diluted at 1:500 to control loading variation.

Statistical analysis

Statistical analysis was performed employing One-Way ANOVA, followed by post hoc Bonferroni's test to compare the individual means. Results are displayed graphically as means ± SEM (standard error mean). A p value < 0.05 was considered statistically significant. Statistical analysis was performed using GraphPad PRISM (version 8.0; GraphPad Software, Inc., San Diego, CA).

Results

M0, M1 and M2 macrophages derived from THP-1 differentiation

Following THP-1 monocytes stimulation with PMA (25 ng/mL) for 48 h, it was possible to confirm the differentiation of monocytes into resting macrophages (M0) by checking cell morphology under phase contrast microscopy.

After obtaining resting macrophages (M0), the polarization of M1 with IFN-γ (20 ng/mL) + LPS (10 pg/mL) and M2 with IL-13 (20 ng/mL) for 24 h, was confirmed in mRNA levels by RT-PCR measuring the expression of specific marker genes for M1 (IL-1β and TNF-α) and M2 (CCL18 and CCL22). No expression of any M2 marker was highlighted in M1 and vice versa (Fig. 1).

Effects of conditioned media obtained from culture of M1 and M2 macrophages in hGCs and COV434 cells viability

After 72 h of interaction, it was possible to observe that in both hGCs and COV434 cells, the CMs obtained from culture of M1 and M2 macrophages affected significantly the cell viability when compared to the resting macrophages (M0) considered as control (Fig. 2).

Effects of conditioned media obtained from culture of M1 and M2 macrophages in hGCs and COV434 cells morphology

No morphological changes could be noted in hGCs and COV434 cells after 72 h exposed to CMs obtained from culture of M1 and M2 macrophages (Fig. 3).

Effects of conditioned media obtained from culture of M1 and M2 macrophages on COX-2 and 5-, 12-, and 15-LOX expression in hGCs and COV434 cells

After confirmation of THP-1 differentiation, an interaction model between CMs obtained from culture of M1 and M2 macrophages and hGCs and COV434 cells was established. After 72 h of incubation, cells were lysed for mRNA and protein extraction and checking the COX-2 and 5-, 12-, and 15-LOX expression in hGCs (Fig. 4) and COV434 cells (Fig. 5), by mRNA levels using RT-PCR and protein levels using western blotting.

Discussion

Macrophages are the most dominant ovarian leukocytes that can be found in the theca layers of growing follicles and in human FF [22]. Over the last few decades of investigation, it is becoming increasingly evident that macrophages are phenotypically and functionally heterogeneous. In fact, macrophages display high degrees of plasticity in their roles when exposed to various environments. M1 macrophages are differentiated by Th1 cytokines, such as IFN-γ, or by recognition of LPS,

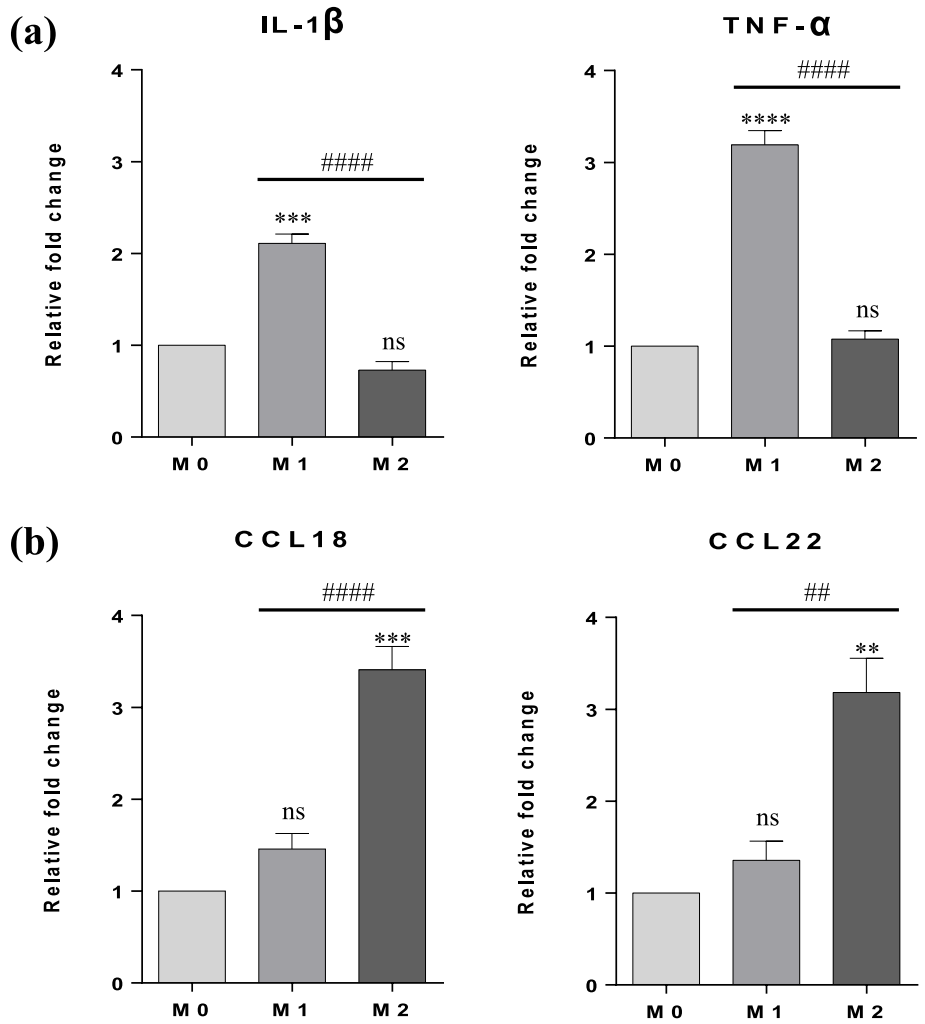

Fig. 1 Expression of M1 and M2 markers after 24 h of polarization with differentiation factors. (**a**) mRNA expression levels of M1 markers (IL-1β and TNF-α). (**b**) mRNA expression levels of M2 markers (CCL18 and CCL22). The relative fold change in gene expression was calculated using $2^{-\Delta\Delta Ct}$ method and normalized with the housekeeping gene GAPDH. Results are expressed as means ± SEM ($n = 3$, in triplicate). Statistical analysis was based on one-way ANOVA, followed by a post hoc Bonferroni's multiple comparisons test. ns: not significant. ** $p < 0.01$, *** $p < 0.001$ or $p <$ **** 0.0001: significantly different from the control (M0). ## $p < 0.01$, ### $p < 0.001$ or $p <$ #### 0.0001: significantly different between M1 versus M2

producing high levels of pro-inflammatory cytokines (TNF-α and IL-1β). Functionally, the pro-inflammatory M1 macrophages are implicated in the clearance of pathogens in the course of infection by generating reactive oxygen/nitrogen species (ROS/RNS), which can induce tissue damage. In contrast, M2 macrophages are differentiated by Th2 cytokines, such as IL-4 and/or IL-13, secreting pro-resolving mediators (CCL18, CCL22, and SPMs). Functionally, pro-resolving M2 macrophages show the ability to phagocytose and promote tissue remodelling and wound healing. The heterogeneous and dynamic nature of macrophages in the ovaries implies their proactive involvement in ovarian homeostasis and hormonal control [23].

Considering that M1 and M2 macrophages are associated with inflammatory processes, macrophages polarization may be an important aspect of their interaction with hGCs. Thus, the first aim of this study was to obtain M1 and M2 macrophages phenotypes from the THP-1 human monocyte cell line. The THP-1 is an immortalized cell line isolated from the peripheral blood of a 1-year-old male patient with monocytic leukemia. Resting THP-1 cells retain most of the inflammatory monocytes signalling pathways and exhibit the ability to differentiate into macrophages when stimulated with PMA. For these reasons, this cell line was chosen as an appropriate cell model for obtaining macrophages derived from human monocyte [24].

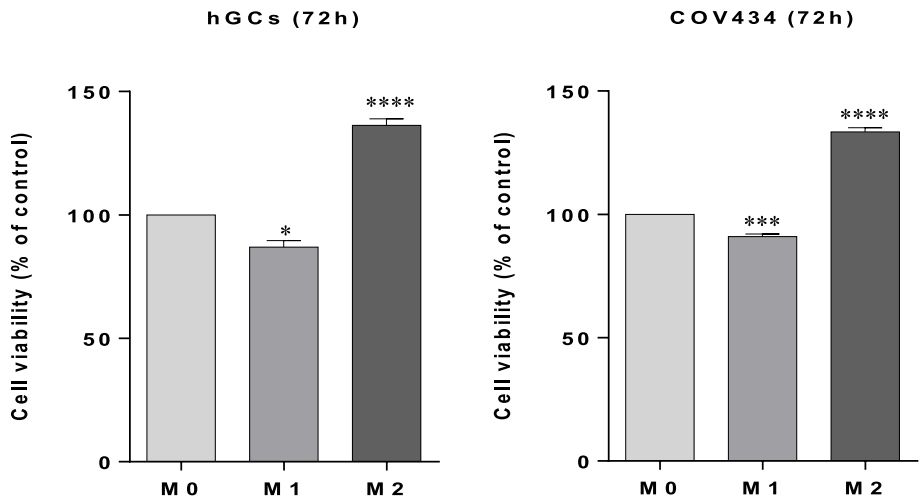

Fig. 2 Effects of conditioned media obtained from culture of M1 and M2 macrophages in hGCs and COV434 cells viability. MTT assay after interaction between CMs obtained from culture of M1 and M2 macrophages and hGCs and COV434 cells at 72 h. Results are compared to the control (M0) and expressed as means ± SEM (at least $n = 3$, in triplicate). Statistical analysis was based on one-way ANOVA, followed by a post hoc Bonferroni's multiple comparisons test. ns: not significant. * $p < 0.05$, *** $p < 0.001$ or $p <$ **** 0.0001: significantly different from the control (M0)

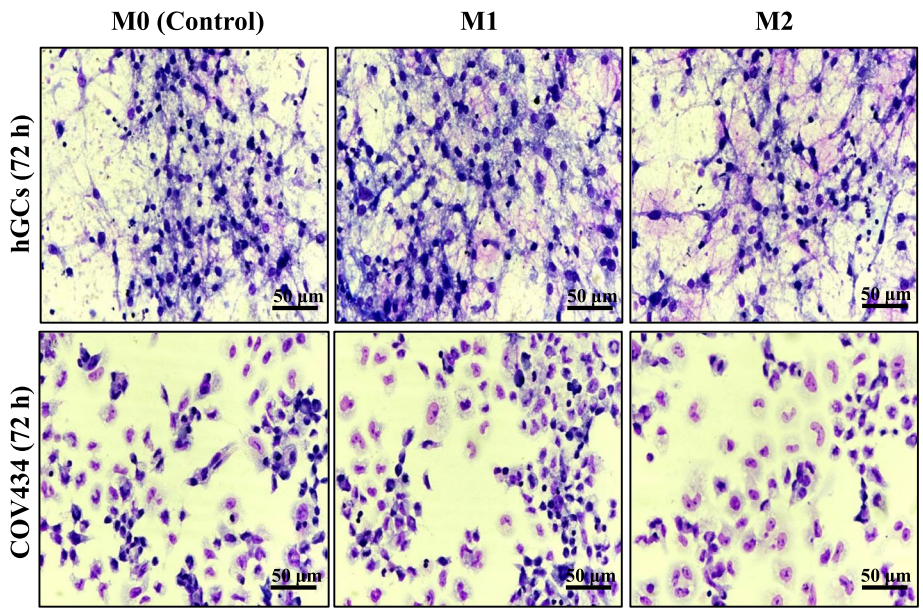

Fig. 3 Effects of conditioned media obtained from culture of M1 and M2 macrophages in hGCs and COV434 cells morphology. Cell morphology was analyzed after 72 h using Giemsa stain. Untreated cells containing only the media (RPMI 1640 10% FBS) were used as control (M0). Results are shown from single representative of three independent experiments

However, although the THP-1 cell line is widely recognized as a suitable model for assessing macrophage functions and responses to foreign stimuli in vitro, there is currently no standardized protocol for the differentiation of THP-1 monocyte into macrophages using PMA. The lack of a standard protocol has a significant impact on the interpretation of results and comparison of studies. The differentiation protocol used in this study was based on the one previously published by our research group. We initiated THP-1 differentiation by exposing the cells to PMA (25 ng/mL) for 48 h, followed by a 24 h resting period [18]. Differentiation of monocytes into resting M0 was confirmed by observation that macrophage-like cells adhered to tissue culture flask, adopting a stellate

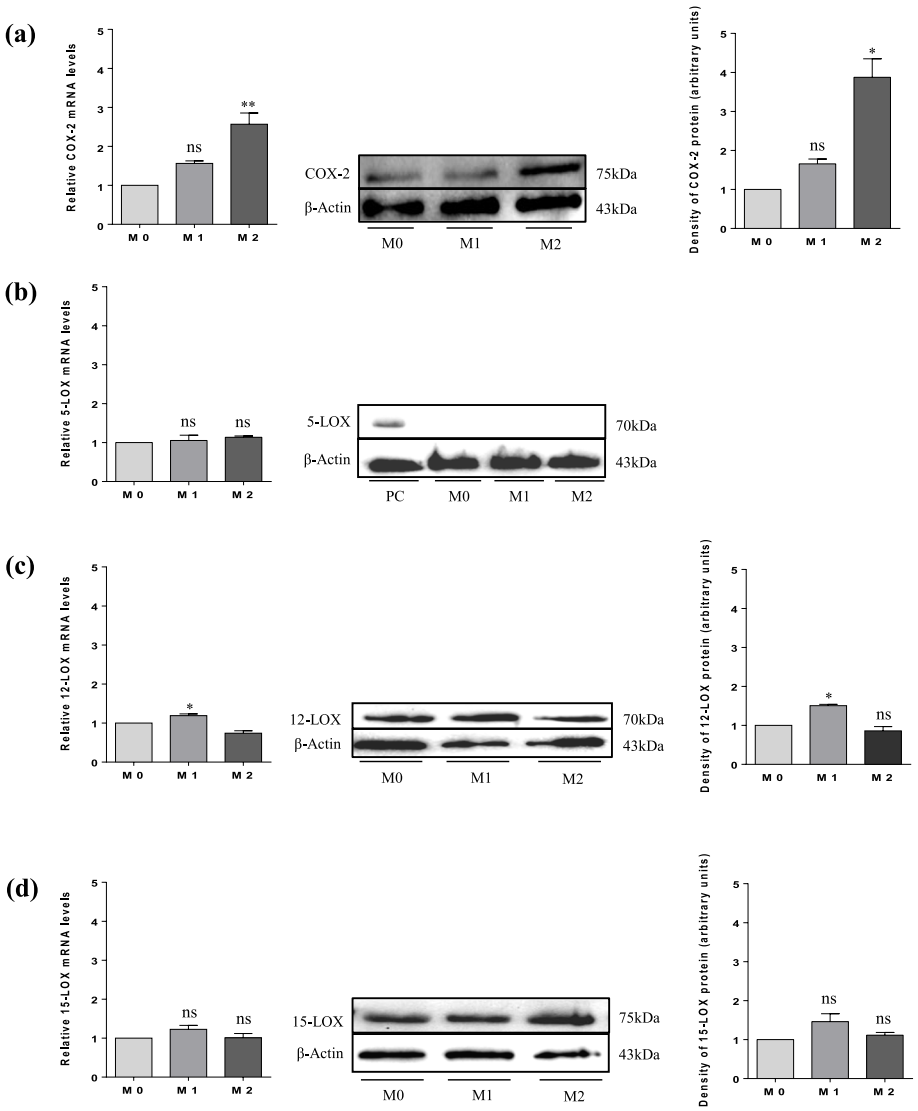

Fig. 4 Expression of COX-2 and 5-, 12-, and 15-LOX in hGCs by mRNA and protein levels. (**a**) COX-2 expression; (**b**) 5-LOX expression; (**c**) 12-LOX expression; and (**d**) 15-LOX expression. Results are compared to the control (M0) and expressed as means ± SEM. For PCR results ($n = 3$, in duplicate) and for western blotting (a single representative of three independent experiments). Statistical analysis was based on one-way ANOVA, followed by a post hoc Bonferroni's multiple comparisons test. ns: not significant. * $p < 0.05$, ** $p < 0.01$

morphology. After that, resting M0 were primed for 24 h with fresh medium supplemented with IFN-γ (20 ng/mL) + LPS (10 pg/mL) to differentiate into M1 and by IL-13 (20 ng/mL) for M2. Transcriptional markers previously used to characterize distinct subsets of macrophages were identified in the literature and used in the present study [24, 25]. As stated earlier, a distinctive hallmark of M1 polarization is the high production of pro-inflammatory cytokines, while M2 polarization regulates the production of pro-resolving mediators. Thus, M1 and M2 macrophages have been shown to be distinguished by distinct chemokine arrays. Studies conducted in ex vivo human systems reported that IL-1β and TNF-α genes were expressed in the M1 macrophages [26]. Our results showed a very similar array of up-regulated genes in M1 macrophages, suggesting a successful differentiation. In turn, the chemokines CCL18 and CCL22 are categorized according to the M2 state [25, 26]. Our findings show that M2 macrophages expressed all the chemokines mentioned above (Fig. 1).

Inflammatory conditions can severely disrupt normal ovarian function and oocyte quality. In vitro models that adequately simulate the ovarian microenvironment may provide a helpful tool to study the mechanisms by

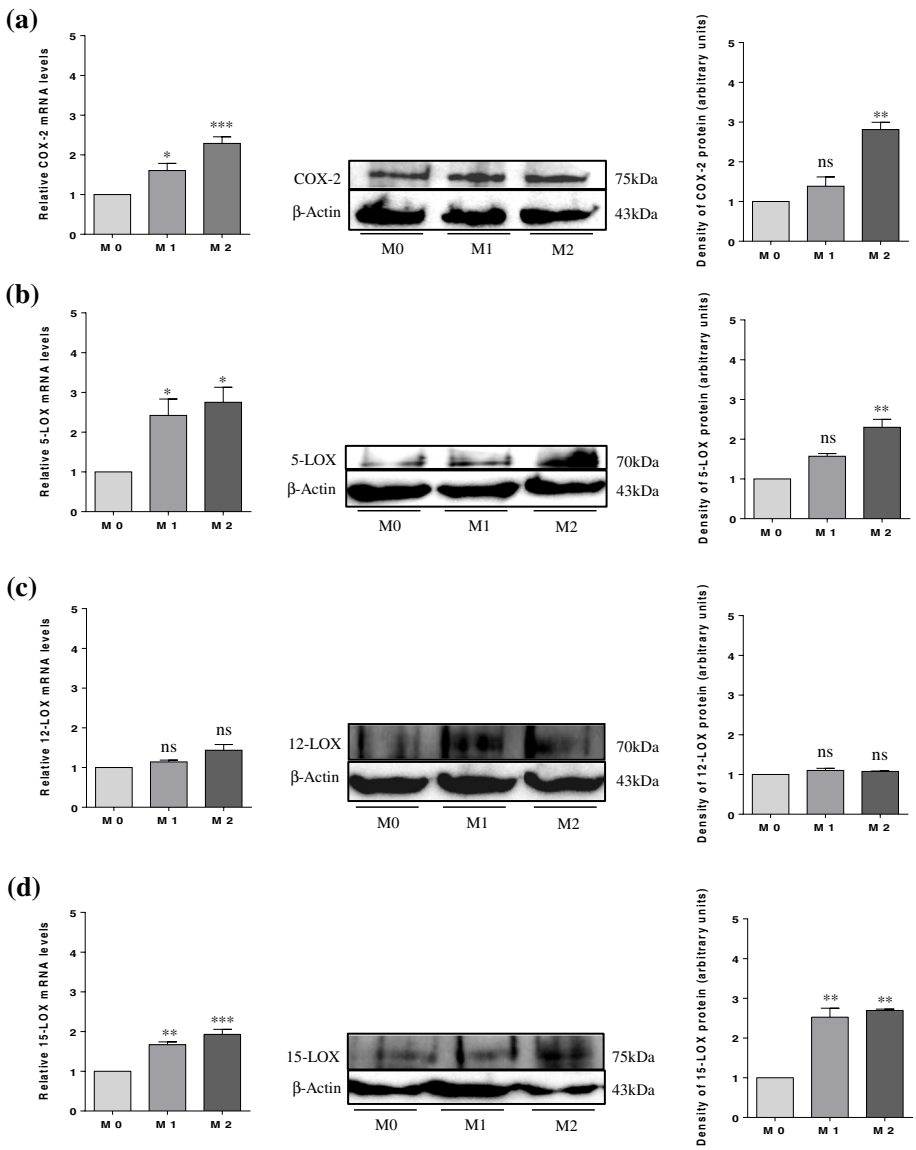

Fig. 5 Expression of COX-2 and 5-, 12-, and 15-LOX in COV434 by mRNA and protein levels. (**a**) COX-2 expression; (**b**) 5-LOX expression; (**c**) 12-LOX expression; and (**d**) 15-LOX expression. Results are compared to the control (M0) and expressed as means ± SEM. For PCR results ($n = 3$, in duplicate) and for western blotting (a single representative of three independent experiments). Statistical analysis was based on one-way ANOVA, followed by a post hoc Bonferroni's multiple comparisons test. ns: not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

which pro-inflammatory M1 and pro-resolving M2 macrophages may affect the hGCs functions. At this point, we established an interaction model between CMs obtained from culture of M1 or M2 macrophages and GCs to mimic the ovary in inflamed states. This model of interaction has long been widely used to study fundamental cellular interactions of any kind. These systems are highly relevant for drug discovery as they provide a more representative in vivo model of human tissue than animal models [27]. Culture models using CMs obtained from another cell type incorporate a physical barrier between cell types, allowing only signalling through the cell secretome (CS). The CM is commonly employed, where the medium is used first to culture one cell type and then transferred to the second cell type. The CM is constituted by the CS of the first cell type that contains soluble factors and can affect the behaviour of the second cell type in a positive and/or negative way [28].

In the culture model adopted in this study, we decided to use only primary human GCs isolated from women

who had male factor as a cause of infertility. Exclusion criteria for women with endometriosis or PCOS, for example, were based on eliminating cells obtained from inflammatory-associated pathologies and, therefore, investigating only the contribution of macrophages to physiological ovarian inflammation. An immortalized hGCs line could be helpful in studying many of the processes that are implicated in human follicle development [29]. We chose to employ the COV434 cell line due to its ability to synthesize estradiol from FSH stimulation, presence of specific markers of apoptosis enabling the induction of follicular atresia, and capacity to form intercellular connections with cells surrounding an oocyte [29, 30].

Regarding cell viability, we observed that CMs obtained from culture of M1 and M2 macrophages affected the viability of both hGCs and COV434 cells, when compared to the resting macrophage (M0), which was used as a control. However, no morphological changes were observed in both cells. As seen in Fig. 2, when hGCs and COV434 cells are exposed to CM of M1 macrophages, a significant decrease in cell viability occurs, suggesting that the pro-inflammatory environment may be influencing cells' viability. However, it is possible to notice a greater significant difference in the decrease of hGCs viability ($p < 0.05$) in comparison to COV434 ($p < 0.001$), which in part can be explained by the fact that hGCs are originated from a previous inflammatory environment, such as ovaries. Furthermore, it is known that pro-inflammatory M1 macrophages can produce ROS/RNS, which can induce apoptosis in hGCs [31]. However, this observation requires further investigation. Thus, the pro-inflammatory environment can affect the ovarian oxidative balance. Regarding cell viability when hGCs and COV434 cells are exposed to the CM of M2 macrophages, it can be observed that it is affected in a similar statistical proportion for both cells ($p < 0.0001$) when compared to the M0 control, suggesting that the anti-inflammatory environment provided by M2 macrophages can, to some extent, contribute to cell proliferation.

In the present study, the interaction between CM obtained from culture of M2 macrophages and hGCs and COV434 cells for 72 h induced a significant increase in mRNA and protein levels of COX-2. However, no significant increase was observed when cells were exposed to CM obtained from culture of M1 macrophages (Figs. 4a and 5a). Narki et al. [32] previously reported that the expression of COX-2 in GCs isolated from women undergoing ART was induced by the pro-inflammatory cytokine IL-1β, contrasting our results. From these findings, it is possible to suggest that the induction of COX-2 expression by the CM of M2 macrophages may increase the production of pro-resolving mediators by hGCs and COV434 cells, leading to the resolution of inflammation.

Feldam et al. [33] demonstrated for the first time the expression of 5-, 12-, and 15-LOX in GCs isolated from women undergoing ART through the characterization of specific products derived from the metabolism of arachidonic acid (AA), such as 5-, 12-, and 15-hydroxyeicosatetraenoic acids (HETEs). In a recent study, Zhang et al. [8] reported that the pro-resolving mediator resolvin E1 (RvE1) improved oocyte quality by decreasing apoptosis rate of CCs and increasing cell viability and proliferation. Considering that COX-2 and LOXs, both involved in RvE1 production, when stimulated can produce pro-inflammatory and pro-resolving mediators, we also investigated their expression in hGCs and COV434 cells by CMs of M1 and M2 macrophages. From our data, it is possible to observe that neither CM of M1 nor that of M2 macrophages were effective in inducing 5-LOX expression in hGCs, both in terms of RNA and protein levels, when compared to the positive control (PC, placenta homogenate) (Fig. 4b). Regarding COV434 cells, a significant increase in 5-LOX mRNA levels is observed when exposed to CMs of M1 and M2 macrophages. However, the same was not observed according to the results of western blotting, where a significant increase in 5-LOX protein expression was observed only when the cells were exposed to the CM of M2 macrophages (Fig. 5b). Concerning 12-LOX expression, a significant increase in its expression is only observed when hGCs are exposed to the CM of M1 macrophages (Fig. 4c). However, 12-LOX expression is not observed when COV434 cells are exposed to CMs of M1 and M2 macrophages (Fig. 5c). These results can be explained, at least to some extent, by the fact that the hGCs are originated from an inflammatory environment per se. Thus, increased expression of 12-LOX may be a compensatory way in which cells produce pro-resolving mediators to overcome inflammation. Finally, CMs of M1 and M2 macrophages, despite 72 h of interaction with hGCs, were not able to significantly affect the 15-LOX expression when compared to control (M0) (Fig. 4d). This result contrasts with the one published by Liao et al. [34], in which the authors demonstrated that 15-LOX expression was up-regulated in GCs isolated from women with PCOS. In regard to COV434 cells, a significant increase in 15-LOX expression is observed when these cells are also exposed to CMs of M1 and M2 macrophages (Fig. 5d).

Conclusions

In conclusion, it can be inferred that M2 macrophages with anti-inflammatory characteristics were able to significantly influence the expression of COX-2 and 5-, 12-, and 15-LOX in hGCs. Therefore, it is possible to suggest

that the production of pro-resolving mediators by hGCs can, at least in part, reverse the physiological inflammation present in the ovaries. As far as we know, this model of interaction between CMs obtained from culture of M0, M1 and M2 macrophages and hGCs and COV434 cells that aims to investigate the expression of COX-2 and 5-, 12-, and 15-LOX had not been investigated earlier, which enabled us to contribute with new findings related to the role of differentiated macrophages found in the ovaries. However, the extent to which pro-resolving concentrations affect oocyte quality, maturation, fertilization potential, and embryonic development is unclear and requires further investigation.

Abbreviations

AA: Arachidonic Acid; ART: Assisted Reproductive Technology; BMI: Body Mass Index; CC: Cumulus Cell; CM: Conditioned Media; COX: Cyclooxygenase; CS: Cell Secretome; DMEM: Dulbecco's Modified Eagle Medium; DMSO: Dimethyl Sulfoxide; DPX: Dibutylphthalate Polystyrene Xylene; ECACC: European Collection of Authenticated Cell Cultures; EDTA: Ethylenediaminetetraacetic Acid; FBS: Fetal Bovine Serum; FF: Follicular Fluid; FSH: Follicle Stimulating Hormone; GAPDH: Glyceraldehydes 3-Phosphate Dehydrogenase; GnRH: Gonadotropin-Releasing Hormone; hCG: Human Chorionic Gonadotropin; HETE: Hydroxyeicosatetraenoic Acid; hGC: Human Granulosa Cell; ICSI: Intracytoplasmic Sperm Injection; IFN-γ: Interferon-γ; IL: Interleukin; IVF: In Vitro Fertilization; LH: Luteinizing Hormone; LOX: Lipoxygenase; LPS: Lipopolysaccharide; MGC: Mural Granulosa Cell; MTT: Methylthiazolyldiphenyl-tetrazolium bromide; PCOS: Polycystic Ovary Syndrome; PGE_2: Prostaglandin E_2; PMA: Phorbol 12-Myristate 13-Acetate; ROS/RNS: Reactive Oxygen/Nitrogen Species; RPMI: Roswell Park Memorial Institute; RT: Room Temperature; RT-PCR: Real-Time Polymerase Chain Reaction; RvE1: Resolvin E1; SPM: Specialized Pro-resolving Mediator; TC: Theca Cell; TNF-α: Tumor Necrosis Factor-alpha; WHO: World Health Organization.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12958-022-00983-6.

Additional file 1: Supplementary figures4 and 5. Uncropped gel from western blots shown in themain figures of the manuscript. Dotted line represents indirect co-culture ofconditioned media of M0, M1 and M2 with hGCs (figure 4) and COV434 (figure5).

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Author's contributions

T.M., B.F. and I.R. designed research and analyzed all data; T.M. wrote the paper and accomplished all experiments; Lia Costa helped with the collection of follicular fluid. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated through this study are included in this article.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Centro Hospitalar de Vila Nova de Gaia/Espinho) and by the National Data Protection Commission (authorization number 526/2017). Informed consents were signed by the patients.

Consent for publication

Not applicable.

Competing interests

The authors state that they have no financial or commercial conflicts of interest.

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Publisher's Note

Chapter III - Discussion and Conclusions

Infertility is a condition that affects a large number of couples atreproductive age. According to data published by WHO, it is believed that approximately 48 million couples, representing 13-15% of the world population, live with this condition [83]. Concerning the female reproductive system, infertility can be caused by a variety of abnormalities of the uterus, fallopian tubes, ovaries, endocrine system, as well as advanced age. Endometriosis and PCOS are the two greatest causes of infertility in women. These diseases are multifactorial, with endometriosis being mainly characterized by inflammatory aspects and PCOS resulting primarily from endocrine disorders, lifestyle/environment, and obesity [84, 85].

Endometriosis is not fully understood and can disrupt all stages of the reproductive processes. Inflammatory processes with increased release of cytokines, growth factors, and vasoactive agents into the peritoneal fluid by activated macrophages have been associated with impaired folliculogenesis and ovulation. According to the guidance from the National Institute for Health and Care Excellence, first-line endometriosis treatment includes NSAIDs, usually combined with progesterone therapy. While NSAIDs suppress the COX-2 function, reducing PG levels and pain; hormone therapy aims to inhibit ovarian activity and estradiol and LH production. However, both therapies have associated side effects [86].

The etiology of PCOS also remains unknown, but several reports have identified high OS markers levels in patients with this disease when compared to normal women. PCOS is typically marked by three main outcomes, such as hyperinsulinemia, hyperandrogenemia, and/or ovulation changes. There is a wide variety of therapeutic options for PCOS, including the oral contraceptive pill, metformin, and antiandrogen drugs. However, an increased risk of thrombosis, exacerbated inflammatory response, and reduced serum 25-hydroxyvitamin D levels are some side effects associated with long-term use of these medications [85].

Comprehensive clinical trials have suggested that nutrition and lifestyle practices can be involved in follicular growth and ovulation rates in women undergoing ART [87]. Current findings indicate that OS/NS, i.e. the imbalance between ROS/RNS production and antioxidants that cause cellular, protein, and lipid damage, plays a major role in endometriosis and PCOS progression to infertility [88]. Thus, antioxidant supplementation may contribute to overcome complications, such as immature oocyte and OS/NS in the human ART setting. Several studies evaluating the effects of polyphenols in the treatment of PCOS and endometriosis have been carried out [89, 90].

Polyphenols are compounds of natural origin with two or more phenolic unit(s), which are produced by plants as a defence mechanism against stress. Regarding their chemical structure, polyphenols can be divided into four groups: flavonoids, phenolic acids, stilbenes, and lignans. In recent years, the importance of polyhydroxyphenols has risen significantly due to its antioxidant and anti-inflammatory properties [89]. Our research group has been investigating the antioxidant effects of some polyphenols in hGCs functions. Moreira-Pinto *et al.* demonstrated that low doses of curcumin [91] and resveratrol [92] were able to increase cell viability, in addition to OS protection. These studies provide evidence that these compounds have a protective effect against OS on hGCs and, thus may enhance oocyte maturation and quality. Other recent papers also demonstrate the antioxidant and anti-inflammatory properties of polyphenolic compounds, such as rutin, quercetin, and catechins, in the treatment of endometriosis and PCOS [93-95].

Considering these aspects, the major goal of this thesis was to investigate the antioxidant and anti-inflammatory properties of a panel of 34 phenolic chalcones and its ability to balance OS/NS and inflammation present in the ovaries. Thus, these compounds may then be used as a supplement in women undergoing ART to improve oocyte quality and increase fertilization success rates. I categorized chalcones into 6 groups according to their structural similarities to allow us to infer a structure-activity relationship (SAR) based on the results obtained.

I first evaluated the ability of chalcones to specifically scavenge HOCl, an RS with greater oxidizing power in a chemical system. The non-fluorescent probe DHR was used as an indicator of the antioxidant property of chalcones. This probe can be oxidized by HOCl present in the medium, culminating in the formation of the cationic compound rhodamine 123, which exhibit green fluorescence. If the chalcones show the ability to scavenge HOCl, the oxidation of DHR does not occur and it is possible to observe a decrease in the fluorescence. As expected, the pentahydroxylated chalcone showed the highest HOCl scavenging activity, supporting the theory that polyphenolic compounds present antioxidant activity [96].

Considering that OS/NS are conditions present in women with endometriosis and PCOS and that ROS/RNS formed can directly impact the quality of oocytes, I decided in a second moment to investigate the ability of these chalcones to inhibit neutrophils' oxidative burst. As mentioned, these cells are the first to be recruited at the onset phase of the inflammatory response, targeting the production of ROS/RNS. Since ovulation

presents characteristics of an inflammatory process, neutrophils are recruited to the ovaries where they begin to produce these RS. To evaluate the inhibition of the oxidative burst, neutrophils were isolated from human whole blood of patients who did not present an ongoing inflammatory process or were not taking any medication with anti-inflammatory action. After patient selection and neutrophil isolation, the oxidative burst was stimulated by PMA. The oxidative burst is characterized by the production of ROS generated by mechanisms dependent on the release of MPO from azurophilic granules. This oxidative metabolism of neutrophils is mediated by an enzymatic complex associated with cytoplasm and granule-specific membranes, the NADPH oxidase [97]. This enzyme is stimulated by PMA from the activation of protein kinase C (PKC). PKC hyperactivity promotes an increase in NADPH oxidase activity, through the phosphorylation of one of its subunits, leading to excessive production of $O_2^{\bullet -}$, deteriorating the OS/NS status. PKC activation can also occur in mitochondria, resulting in ROS generation that culminates in organelle dysfunction and cell death by apoptosis due to oxidative conditions [98]. Finally, PKC activation also induces increased activity of the NF-κB pathway, which is associated with transcription and production of several pro-inflammatory mediators and cytokines, as well as RS [99]. The PKC pathway works as a feedback loop, since this pathway results in the production of high levels of RS. The overproduction of RS can act on GCs, inducing its apoptosis and a reduction in oocyte quality.

When neutrophils are activated, the ROS generated can via oxidation mechanisms, convert molecules, such as luminol, to unstable and excited intermediate derivatives, such as aminophtalate. The latter when return to their fundamental state emit light in the form of photons. This production of light, during the chemical reaction is one of the oxidative burst characteristics, and may be an indicator of the activation of oxidative metabolism. Thus, luminol has been used as a chemiluminescent probe in the study of oxidative metabolism of neutrophils. Luminol is exclusively oxidised by H_2O_2 and its derivatives, such as HOCl, and the MPO enzyme, present in neutrophils, is an important catalyst in the chemical reaction. Thus, luminol can be used to assess ROS production during neutrophil stimulation [100]. If the chalcones show the ability to inhibit the neutrophils' oxidative burst, luminol oxidation does not occur and it is possible to observe a decrease in light output. ROS/RNS are produced within neutrophils, specifically, inside the phagosome. In order for chalcones to be able to cross the plasma membranes of cells, it is necessary that in their chemical structure there is a balance between hydrophilic and hydrophobic characteristics, since the membranes are formed by phospholipids. Supporting this theory, chalcones more active in inhibiting the neutrophils' oxidative burst were those presenting

hydrophilic groups, such as hydroxyl, and hydrophobic group, such as methoxyl and nitro and/or chloride atom[96].

Once the results on the antioxidant activity of chalcones were obtained, my second aim was to investigate this activity, in addition to the anti-inflammatory activity, in a specific clinical condition. Since the ovaries are an environment with inflammatory features and that all mediators found therein can impact on follicle growth and oocyte quality, I decided to investigate the influence of chalcones on female infertility. It is known that neutrophils and macrophages are the first immune cells to be recruited during inflammatory response. These cells migrate to the ovaries where they produce and/or activate biochemical pathways that contribute to proper inflammatory balance. Neutrophils, in turn, will be responsible for the ROS/RNS production in order to eliminate the initial inflammatory stimuli, whereas macrophages undergo a phenotype reprogramming. It involves macrophage differentiating into pro- (M1) and anti-inflammatory (M2), which will produce mediators that can exacerbate or control inflammation. In the ovaries, RS produced by neutrophils and mediators by macrophages can reach the follicle, and thus impact the functions of GCs. Because they are directly in contact with oocytes, GCs play a key role with regard to oocyte growth and maturation. It is from them that the oocytes obtain nutritional support to enhance their development. Therefore, the entire environment around the GCs, but also what these cells produce, are extremely important to ensure oocyte quality and fertilization.

On this basis, my aim was to evaluate whether chalcones were able to inhibit or promote the activation of inflammatory pathways in GCs exposed to pro- and anti-inflammatory environments. On a first step, I evaluated which pathways were activated by either M1 or M2 polarized macrophages, namely, COX-2 and 5-, 12-, and 15-LOX. For this, I simulated the ovary inflammatory environment by establishing an indirect co-culture system between GCs and conditioned media (CMs) from M1 and M2 macrophages.

For this second work, I used PMA differentiated THP-1 cells as a model for human monocyte derived macrophages followed by specific stimulus to obtain M1 and M2 macrophage phenotypes. After, PCR results confirmed the macrophage polarization: M1 expressed specific markers, such as TNF-α and IL-1β, and M2 expressed CCL18 and CCL22. In co-culture system, we chose to use only primary hGCs isolated from women who had male factor as a cause of infertility. An immortalised hGCs line may be helpful in the study of many of the processes that are involved in human follicle development. I chose to use the COV434 cell line due to its capacity to synthesise oestradiol. In our study,

co-culture between CM of polarised M2 macrophages and hGCs or COV434 cells induced an increase in COX-2 mRNA and protein levels. However, no increase was observed when cells were exposed to CM from polarized M1 macrophages. So, it is possible to propose that induction of COX-2 expression by M2 CM may increase the production of pro-resolving mediators by hGCs and COV434 cells, driving the resolution of inflammation. Given that COX-2 and LOX mediators, both implicated in RvE1 production, when stimulated can yield pro-inflammatory and pro-resolving mediators, I also assessed their expression in hGCs and COV434 cells. Our results revealed that neither CMs from M1 nor M2 were effective in the induction of 5-LOX expression in hGCs, both in terms of RNA and protein levels. With respect to COV434 cells, an increase in 5-LOX mRNA levels was noted when exposed to CMs from both M1 and M2 CMs. However, the same was not found at protein level. In western blotting, there was an increase in 5-LOX protein expression only in CM from M2. Concerning the expression of 12-LOX, an increase in its expression is only observed when hGCs are exposed to M1 CM. However, the expression of 12-LOX is not observed when COV434 cells are exposed to the CM of M1 and M2. These results can be explained, at least to some degree, by the fact that hGCs arise from an inflammatory environment per se. Thus, the increased expression of 12-LOX may be a compensation mechanism in which cells produce pro-resolving mediators to overcome inflammation. Lastly, CMs from M1 and M2 were not able to affect 15-LOX expression in hGCs. Regarding COV434 cells, an increase in 15-LOX expression is observed when these cells are also exposed to CMs from M1 and M2.

Conclusions and Perspectives

In summary, this thesis contributed to the search for an alternative to nutritional supplementation for women undergoing ART. These molecules showed promising antioxidant properties, as they act as HOCl scavengers and inhibitors of neutrophils' oxidative burst. The SAR discussed throughout this thesis provides a framework for the structural requirements of chalcones to act as antioxidant agents and that may allow the design and synthesis of new and promising anti-inflammatory chalcones.

Regarding the second objective of this thesis, it can be inferred that polarized M2 macrophages with anti-inflammatory features were able to influence the expression of COX-2 and 5-, 12-, and 15-LOX in hGCs. Therefore, it is reasonable to suggest that the production of pro-resolving mediators by hGCs can, at least in part, reverse the physiological inflammation present in the ovaries. To our knowledge, this model of indirect co-culture between hGCs and COV434 cells with the CMs of M0, M1 and M2 aimed to investigate the expression of COX-2 and 5-, 12-, and 15-LOX have not been investigated, allowing us to contribute with new findings related to the role of polarized macrophages found in the ovaries. However, to what extent do pro-resolving lipid concentrations affect oocyte quality, maturation, fertilization potential, and embryo development is unclear and requires further investigation.

As perspectives of this thesis, the evaluation of the possible antioxidant and anti-inflammatory activities of chalcones with regard to the modulation of inflammatory pathways activated in GCs, namely COX-2 and 5-, 12-, and 15-LOX, when exposed to pro- and anti-inflammatory environments. In addition, evaluate the activation of these inflammatory pathways in women with inflammatory diseases, such as endometriosis and PCOS.

Chapter IV - References

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