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Impact of dietary supplements on reproductive function: Curcumin and Resveratrol

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Termino grata.

Abstract

Global infertility prevalence has been increasing in recent decades. Among various factors, advanced reproductive age of women is a major contributor to the widening of this condition. Concerned women look for dietary supplements with antioxidant properties advertised as a natural way to increase fertility. Curcumin (CUR) and resveratrol (RES) are two highly acclaimed polyphenols with antioxidant and anti-inflammatory properties widely described in different cell types. Curcumin, isolated from the roots of the *Curcuma Longa* plant, is a yellow pigment commonly used in Asian countries, especially in India. In addition to its antioxidant and anti-inflammatory properties, CUR elicits apoptotic cell death as evidenced in some tumor cells. Resveratrol, in turn, is produced by plants in response to UV rays, toxins, fungal attacks or pathogens. It is mainly present in red wine, cocoa, peanuts or blueberries. In recent years, it has been extensively studied and its importance increased due to its biological and pharmacological activities that have proved to be effective in cardiovascular and brain protection. In this work, the effect of both compounds on granulosa cells (GC) was studied. GC surround the oocyte and maintain a direct contact with it through the nutrient exchange and hormone production necessary for its development. Their normal function is crucial, since defects in these cells can contribute to women infertility. For this purpose, COV434 cell line and primary human granulosa cells (hGC) cultures from patients undergoing assisted reproductive technology (ART) were used. During the experiments, GC were treated with different concentrations of CUR and RES (0.001-50μM) at different times (24-72h). Low concentrations of both compounds showed an increase on cell viability and did not exert visible changes on cell morphology. Likewise, they lead to a decrease in ROS formation after stress induction, suggesting a protective role of CUR and RES. Changes in hormonal levels were not observed at these doses. In contrast, high concentrations of both compounds triggered a reduction on cell viability, accompanied by LDH release, suggesting cytotoxic effects. Allied to the above results, high doses of CUR and RES affected hormonal function of GC. CUR has a more evident dose-response effect; however, at 1-5 μM a reduction on cell viability without LDH release was observed, suggesting a programmed cell death mechanism. This work reinforces the importance of dietary supplements, namely CUR and RES, on the functions of GC and, consequently, on reproductive success.

Keywords: Dietary Supplements; Curcumin; Resveratrol; Fertility; Granulosa cells

Resumo

A prevalência global da infertilidade feminina tem vindo a aumentar nas últimas décadas. Entre diversos fatores, a idade reprodutiva avançada das mulheres é uma das principais razões que contribuem para o aumento desta condição. Com o objetivo de amenizar estes efeitos tem sido proposta a introdução na alimentação de suplementos com propriedades antioxidantes de forma a promover o aumento da fertilidade. Neste contexto é cada vez maior a procura de substâncias naturais em detrimento de compostos sintéticos. Atualmente, a curcumina (CUR) e o resveratrol (RES), na sua forma pura ou em conjugação com outras substâncias naturais, são dos suplementos alimentares mais procurados. A curcumina e o resveratrol são dois polifenóis com propriedades antioxidantes e anti-inflamatórias já demonstradas em diferentes tipos de células.

A curcumina (1,7-bis-(4-hydroxy3-methoxyphenyl) -1,6-hepadiene-3,5-dione), é o principal ingrediente ativo isolado das raízes da planta *Curcuma Longa* também conhecida por açafrão-da-terra, açafrão-da-Índia ou gengibre amarelo. A CUR, pigmento responsável pela tonalidade alaranjada característica da curcuma, além de apresentar propriedades antioxidantes e anti-inflamatórias, provoca a morte celular por apoptose em células tumorais. Adicionalmente, a CUR tem sido correlacionada com a prevenção de doenças como a doença de Alzheimer, depressão, doenças cardiovasculares, artrite e outras doenças crónicas relacionadas com o envelhecimento. Por outro lado, estudos *in vitro* utilizando CUR, sugerem uma diminuição na viabilidade das células cancerígenas, assim como uma diminuição da angiogénese. Para comprovar estes efeitos será necessário aumentar a investigação nesta área de modo a confirmar a relação entre a toma deste suplemento e um melhoramento na prevenção/combate do cancro.

O resveratrol (3,5,4'-trihydroxystilbene) é sintetizado por diversas plantas em resposta aos raios UV, toxinas, fungos ou agentes patogénicos. Este composto, encontrado principalmente em vinho tinto, cacau, amendoim ou mirtilos, apresenta atividades biológicas e farmacológicas que se revelaram eficazes na proteção cardiovascular e cerebral. Contudo, uma das maiores dificuldades no uso da CUR e do RES está relacionada com a biodisponibilidade que apresentam. Estudos indicam, que os valores detetáveis no plasma após o consumo de CUR ou RES são bastante reduzidos, sendo a sua eliminação rápida e a absorção residual.

Este trabalho surge com o objetivo de entender o efeito destes dois compostos no ciclo ovárico, sendo realizado com recurso a células da granulosa. As células da granulosa rodeiam o ovócito e mantém uma relação direta com o mesmo através de troca de nutrientes e produção de hormonas necessárias à sua manutenção e desenvolvimento. Entre outros fatores, defeitos nestas células podem contribuir para casos de infertilidade feminina sendo pertinente o estudo da CUR e do RES nas suas principais funções.

Os principais objetivos deste projeto prendem-se com i) o estudo do impacto direto de diferentes concentrações de CUR e RES na viabilidade das células da granulosa ii) a avaliação de ambos os compostos ao nível da proteção contra o stress oxidativo iii) com possíveis alterações ao nível da função hormonal destas células.

Deste modo, foi utilizada a linha celular COV434 com as quais se procedeu à análise do efeito de diferentes concentrações de CUR e RES. Apesar da vasta utilização de linhas celulares em diversas áreas de investigação, devido ao seu papel de relevo no complemento de estudos *in vivo* e no estudo de processos celulares, estas podem diferir fenotípica e geneticamente do tecido de origem. Em contrapartida, culturas de células primárias retêm diversas características e funções observadas *in vivo*, sendo um valioso modelo na transição para a área clínica. Após ensaios com ambos os compostos na

linha celular, procedeu-se a igual avaliação utilizando culturas primárias de células da granulosa de pacientes submetidas a tratamentos de reprodução medicamente assistida (TRA). Durante a colheita dos ovócitos, o líquido folicular foi recolhido e os ovócitos isolados para posterior fertilização *in vitro* ou injeção citoplasmática (ICSI). Após o consentimento informado das pacientes, o restante líquido folicular foi transportado para laboratório onde se procedeu ao isolamento e purificação das células da granulosa.

De acordo com os nossos resultados, os ensaios de viabilidade demonstram que a CUR exerce um efeito duplo. Através da realização de ensaios de MTT e LDH, verificamos que em baixas concentrações, a CUR provoca um ligeiro aumento na viabilidade das células da granulosa, enquanto que em concentrações elevadas provoca necrose, a viabilidade diminui e a libertação de LDH aumenta em ambos os modelos celulares. Contudo, nas concentrações de 1 e 5 μM de CUR, ocorre uma diminuição na viabilidade celular sem libertação de LDH, sugerindo apoptose. Este facto foi explorado através da análise da morfologia, utilizando microscopia de contraste de fase, colorações de Giemsa e Höechst e análise do potencial da membrana mitocondrial. Em seguida, foi avaliada a atividade da PARP-1 e das caspases -3/7 e caspase -9. Os resultados sugerem apoptose nas células primárias da granulosa, contudo mais estudos são necessários.

As espécies reativas de oxigénio (ROS) foram de igual modo analisadas, pois o aumento da sua produção está associado a um incremento no stress oxidativo das células, que se pode refletir na qualidade dos ovócitos. Após tratamento das células da granulosa com baixas concentrações de CUR e posterior indução de stress com recurso ao hidroperóxido de terc-butila (TBHP), a produção de ROS foi verificada. Identificamos uma diminuição nos níveis de ROS nas células previamente incubadas com CUR quando comparados com os níveis de ROS das células tratadas isoladamente com o indutor de stress. Estes resultados enaltecem as propriedades antioxidantes deste composto. Contudo, apesar de não se verificarem alterações na viabilidade celular após adição de baixas concentrações de CUR, procedemos ao estudo da função hormonal das GC de modo a garantir que esta não se encontra afetada. Foram identificadas diferenças na produção de estradiol e progesterona e na expressão dos genes associados, StAR, CYP11A1, 3β-HSD e aromatase (CYP19A1), que desempenham um papel chave na síntese das hormonas. Com baixas concentrações não são detetadas diferenças ao nível da expressão dos genes estudados, contudo a concentrações supra fisiológicas, a utilização de CUR sugere uma interferência na síntese de estradiol.

No entanto, os resultados da adição de diferentes concentrações de RES na linha celular não são concordantes com os obtidos nas células primárias da granulosa, sugerindo que estas últimas são menos sensíveis ao composto fenólico. Além disso, a análise da produção de ROS, o estudo do potencial da membrana mitocondrial e os estudos morfológicos realizados, não sugerem ocorrência de apoptose nas células da granulosa. O potencial antioxidante do RES foi igualmente avaliado e, tal como na CUR, não verificamos alterações dos níveis basais de ROS. No entanto, sugere algum tipo de proteção das células da granulosa após indução de stress. Por fim, no que diz respeito à função hormonal das células, os nossos resultados indicam que não existem alterações nos níveis de estradiol e progesterona a baixas concentrações, o que não se verifica a altas doses de RES, que revelam um aumento do estradiol. Por conseguinte, estes resultados sugerem que o RES altera a função hormonal das células da granulosa.

Em síntese, este estudo sugere que o consumo moderado de suplementos de RES e/ou CUR, promove melhoria na qualidade dos ovócitos. No entanto, quando em conjunto com coadjuvantes, como é o caso da piperina, a biodisponibilidade de ambos os compostos aumenta. O aumento da biodisponibilidade pode traduzir-se em consequências negativas para as células da granulosa, uma que vez que

concentrações elevadas de CUR e RES levam à diminuição da viabilidade celular e comprometem a função hormonal.

Palavras-chave: Suplementos alimentares; Curcumina; Resveratrol; Fertilidade; Células da granulosa

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List of abbreviations

ART- Assisted reproductive technology

3β-HSD- 3-beta-hydroxysteroid-dehydrogenase

AB-AM - Antibiotic-antimycotic

ADDS - Advanced Drug Delivered Systems

CCCP- carbonyl cyanide m-chlorophenylhydrazone

cDNA- Complementary DNA

CUR – Curcumin

DCFH-DA- 2’7’-dichlorodihydrofluorescein

DIOC6- 3,3’- dihexyloxacarbocyanine iodide

DMEM/F12- Dulbecco’s Modified Eagle Medium-F12

DMSO - Dimethyl sulfoxide

DNA- Deoxyribonucleic acid

EDTA- Ethylenediamine tetraacetic acid

ETOP- Etoposide

FF- Follicular Fluid

FSH - Follicle-Stimulating Hormone

GC - Granulosa Cells

H_2O_2 - Hydrogen Peroxide

IVF- *In Vitro* Fertilization

LH - Luteinizing Hormone

MTT- Methyltiazolyldiphenyl- Tetrazolium Bromide

PARP- Poly (ADP-ribose) polymerase

PBS- Phosphate Buffered Saline

RES - Resveratrol

RNS - Reactive Nitrogen Species

ROS - Reactive Oxygen Species

RT-qPCR- Quantitative Reverse Transcription PCR

StAR- Steroidogenic Acute Regulatory Protein

TBHP- tert-Butyl Hydroperoxide

ZEA -Zearalenone

Chapter 1: Introduction

1.1. Dietary supplements

Dietary supplements consume is widespread, despite the lack of scientific evidence suggesting benefits of a regular use. In most cases, reasons for consuming dietary supplements are varied and can range from social and psychological to economic factors [1-3]. Also, natural products are generally perceived by the public as being safer to humans rather than synthetic drugs [4].

In the last years, much attention has been focused on the use of active dietary ingredients such as phytochemicals [5, 6]. These are a powerful group of compounds, belonging to plant secondary metabolites, which are present on a broad variety of foods including vegetables, fruits, nuts and cocoa as well as juice, tea, coffee and wine [7, 8]. Many of these plant metabolites have been tested on animal and human cells showing very intriguing biological activities. Usually grouped according to their chemical structure (e.g. polyphenols, terpenoids, alkaloids, nitrogen and sulfur compounds), the dietary supplements consumption is widespread, despite the lack of scientific evidence on the benefits of their regular use. [9, 10].

Among various phytochemicals, curcumin (CUR) and resveratrol (RES) are two naturally occurring polyphenols that are used as dietary supplements. However, both compounds exhibit low bioavailability due to their low absorption, rapid metabolism and rapid systemic elimination [11]. To increase intracellular intake, higher doses or combination with adjuvants, like piperine or Advanced Drug Delivered Systems (ADDS) are required [11].

1.1.1. Curcumin

Turmeric (*Curcuma longa)* is a golden spice, member of the ginger family (Zingiberaceae), commonly used in Asia [12]. Curcumin (1,7-bis-(4-hydroxy3-methoxyphenyl)-1,6-hepadiene-3,5-dione), has been identified as the active principle present in turmeric rhizome [13]. CUR is a low molecular weight polyphenol and presents a characteristic yellow color. Several clinical studies have focused on the safety of CUR for human consumption, leading to the approval by the U.S Food and Drug Administration (FDA) [14]. CUR has a broad spectrum of biological and pharmacological properties, including anti-inflammatory and antioxidant [4, 8]. In addition, studies suggest that CUR has stimulatory effects on the reproductive system [15, 16].

1.1.2. Resveratrol

RES (3,5,4'-trihydroxystilbene) is a phytoalexin present in plant-derived foods, including grape skin, cocoa and peanuts [17]. It is an important antioxidant compound that has been correlated with the cardiovascular protection effects of red wine ("French Paradox") [18]. Although there is no unequivocal evidence that RES intake can have benefits for human health, several studies have shown that RES can prevent a wide range of age-related diseases, including cancer, cardiovascular disease, diabetes and neurodegeneration [19-21] In several cancer cell lines and primary cell culture systems, resveratrol has been shown to exert anti-proliferative and pro-apoptotic properties [22]. However, little is known about the role of resveratrol in such vital biological functions such as reproduction and ovarian function.

1.2. Female reproductive system

Women are born with their cohort of primordial follicles. These are composed of the primary oocytes, surrounded by single layers of follicular epithelial cells, with a basal lamina delimiting this structure [23] . In subsequent years, some of them further develop into one or more of the subsequent follicular stages under the influence of sex hormones [24]. For instance, the most common fate of a follicle or female germ cell is atresia [25]. Ovulation represents an exceptional fate that occurs once a month, by the development of one (sometimes two) dominant follicle [24].

When primary follicles survive and the *zona pellucida*, a glycoprotein layer between the oocyte and the follicular epithelium, becomes visible, secondary follicles are formed [26]. At this stage, granulosa cells (GC) gain great importance in maintaining the oocyte, by producing important factors that reach the oocyte through the *zona pellucida* [27]. Meanwhile, outside the basal lamina, the ovarian stroma is organized to become theca follicular cells [28].

If the secondary follicles continue their development, tertiary follicles will be formed, and recognized by a fluid-filled cavity, the *antrum* [29]. The oocyte lies at the edge of the follicle, surrounded and connected by an extension of granulosa epithelial cells called the *cumulus oophorus* [30]. This last stage corresponds to an especially large tertiary follicle, formed once a month, which is expected to ovulate (figure 1.1) .

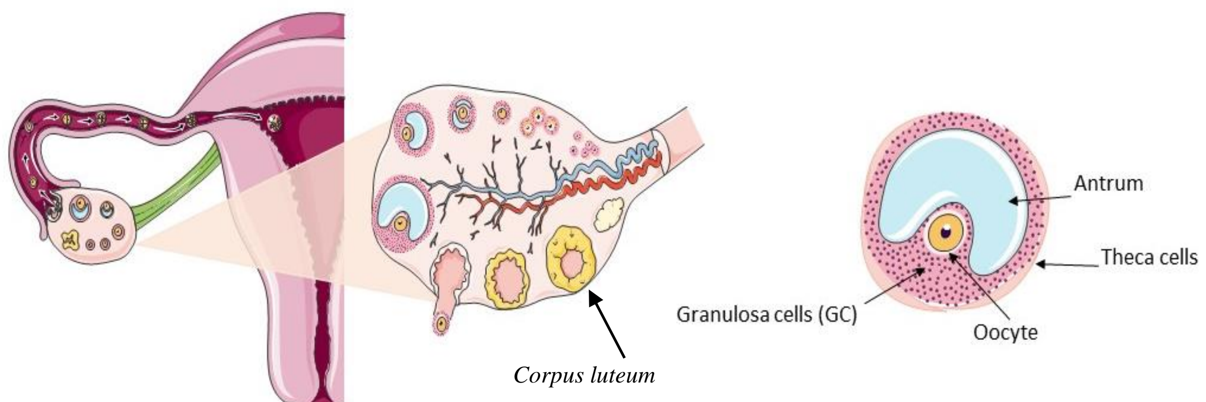

Figure 3.1 – **Representation of physiological changes during ovarian cycle and Graafian follicle morphology**.

1.2.1. Hormone synthesis

As previously mentioned, the development of follicles in subsequent follicular stages occurs under the influence of sex hormones. GC play a key endocrine role, by producing estrogen and growth factors throughout the development of the follicle [31]. In that process, in the antral follicles, FSH acts mainly on GC and LH on theca cells as GC express FSH receptors, but no LH receptors, and the opposite occurs in theca cells [31]. FSH will stimulate GC to proliferate, to produce estrogen and to encourage the enlargement of the antrum. LH will stimulate theca cells to proliferate and produce androgens that are precursors of estrogen which will diffuse to GC and then be converted by aromatase into estradiol (estrogen) [31]. As the antral follicle matures to a Graffian follicle, LH receptors begin to be expressed on GC [32].Thus, estrogens are the main regulators of follicle development, being crucial for ovulation to occur and aromatase is a key enzyme in these process (figure 1.2).

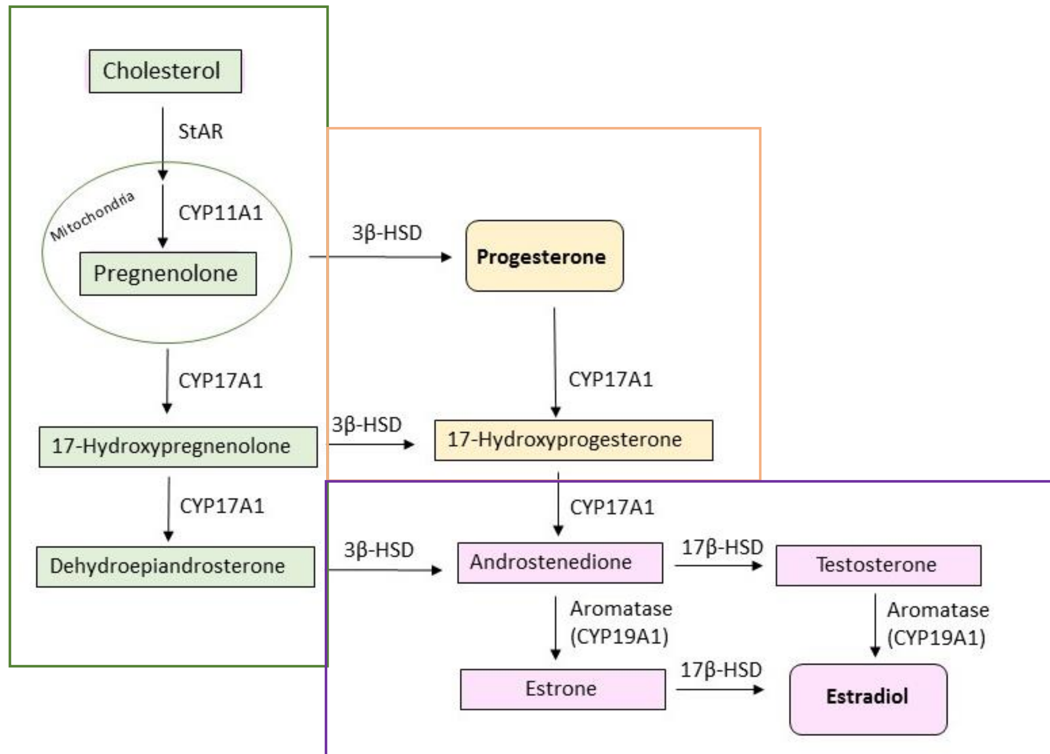

Figure 1.2 - **Estradiol and progesterone synthesis pathway**. Estradiol and progesterone are synthesized from the cholesterol precursor in response to LH and FSH. Steredoigenic acute regulatory protein (StAR) mediates the transport of cholesterol from outside to inside the mitochondrial membrane, where enzyme CYP11A1 catalyzes the conversion to pregnenolone. Pregnenolone is converted to progesterone by 3β-HSD. Aromatase (CYP19A1) is the final enzyme that results in the production of estrone and estradiol. Alterations in any of these steps are associated with impaired steroidogenesis. Different colors indicate the location where the process occur; Green – Theca cells; Yellow; Corpus luteum; Pink – GC. Adapted from Machtinger et al.

1.3. Aim

CUR and RES are becoming a part of the daily life for those who want to maintain a healthy lifestyle and seek for natural products instead of synthetic drugs. Due to their biological properties, both compounds are described as beneficial under certain conditions and are correlated with female fertility. However, there is still a lack of information on the possible effects of CUR and RES on GC and consequently, on follicular development and human oocyte quality. GC are essential for ovarian follicle development, playing a key role in estradiol production, among other factors that influence the reproductive cycle and, indirectly, interfere with female reproductive potential.

Therefore, the main objective of this project was to study the direct effects of increasing doses of CUR and RES on main GC functions *in vitro*. In addition, investigate whether both phenolic compounds exert a protective effect on GC against reactive oxygen species (ROS) and whether the expression of the key steroidogenesis-associated genes remain intact after CUR and RES incubation. Although there are granulosa cell lines that can be used to perform such studies, primary human granulosa cell (hGC) cultures immediately obtained from patient populations are especially valuable in translational clinical research. Thus, all the experiments were performed on granulosa cell line (COV434) and hGC.

This work may provide new insights into the mechanisms underlying the impact of CUR and RES consumption on reproductive success.

Chapter 2: Material and Methods

2.1. Material

Dulbecco's Modified Eagle Medium/F12 (DMEM/F12), methylthiazolyldiphenyltetrazolium bromide (MTT), hydrogen peroxide (H_2O_2), Trypan Blue Solution 0,4%, protease inhibitor cocktail (PI), Höechst 33342, carbonyl cyanide m-chlorophenylhydrazone (CCCP), Dimethyl Sulfoxide (DMSO), EDTA, Giemsa, sucrose, paraformaldehyde , fluoroshield, Curcumin, Resveratrol, tert-Butyl Hydroperoxide (TBHP) and dichlorodihydrofluorescein diacetate (DCDHF-DA), were from Sigma–Aldrich Co. St. Louis, MO, USA. Fetal bovine serum (FBS) was from Biochlome. Antibiotic-antimycotic (AB-AM) was from Grisp. Trypsin (2,5%) and 3,3′- dihexyloxacarbocyanine iodide (DiOC6) was from Gibco/Invitrogen Corporation, Carlsbad, CA, USA. Pierce LDH cytotoxicity assay kit (LDH) was from Thermo Fisher, Life Technologies. DPX was from VWR-Prolabo. Plates (6, 24 and 96-well) were from Falcon, culture flasks were from Sarstedt and all the other plastic material used in cell culture techniques were from Falcon TM, SD, USA or Nerbe plus. CytoTox 96 nonradioactive cytotoxicity assay kit, Caspase-Glo® (Promega, Madison, WI, USA); Percoll was from GE Healthcare, Buckinghamshire, UK).

2.2. Methods

2.2.1. COV434 cell culture

COV434 is a human ovarian granulosa cell line established from a solid primary tumour [33]. Cells were cultured in DMEM/F12 medium supplemented with 10% (v/v) FBS and an antibiotic-antimycotic solution (AB-AM), incubated at 37 °C and 95% air/5% CO_2 humidified atmosphere. For the experiments, cells were seeded in 96- , 24- or 6-well plates at densities 5×10^4, 30×10^4 and 80×10^4 cells/well, respectively, in DMEM/F12 medium supplemented with 5% (v/v) FBS, 1% AB-AM. After adherence (24 h), cells were treated with different concentrations of CUR and RES in cell culture medium with 2% (v/v) FBS, 1% AB-AM.

2.2.2. Isolation and primary cultures of human granulosa cells (hGC)

GC were obtained from human FF samples collected from patients undergoing *In Vitro* Fertilization (IVF), with their informed consent (attachment A), at Unidade de Medicina da Reprodução Dra. Ingeborg Chaves - Centro Hospitalar de Vila Nova de Gaia/Espinho. All the procedures were conducted in accordance with the Ethical Committee of Centro Hospitalar de Vila Nova de Gaia/Espinho and authorized by Comissão de Proteção de Dados (Proc. no.764/2017). Human granulosa cells (hGC) were isolated as described previously by Sluss et al. [34]. During oocyte aspiration, follicular fluid (FF) was collected and the oocytes isolated and removed for IVF. The remaining FF was transported to the laboratory for isolation and purification of hGC. Briefly, FF samples were centrifuged at 300g, for 10 min at 4ºC and the pellet was added to a Percoll density gradient (1:1 in PBS) hGC were collected at the interface of the FF and Percoll, washed and resuspended, and then seeded and incubated in DMEM/F12 medium supplemented with 10% (v/v) of FBS and 1% AB-AM at 37 °C in 95% air/5% CO_2 humidified atmosphere. For the experiments, cells were seeded in 96- or 24- well plates at densities 7.5×10^4 and 70×10^4 cells/well, respectively. After adherence (24 h), cells were treated with different concentrations of CUR or RES in cell culture medium with 2% (v/v) FBS.

2.2.3. Cell viability and cytotoxicity assays

COV434 cells and hGCs were plated in 96-well plates and incubated in DMEM/F12 medium with 2% FBS and 1% AB-AM, in the absence or presence of CUR (0.001–50 μM) or RES (0.001-200 μM) for 24h, 48h and 72h. Serial dilutions of CUR and RES were dissolved in DMEM/F12. CUR and RES vehicle (DMSO), did not affected cell viability. The yellow tetrazole MTT (0.5 mg/ml final concentration) was added and cells were incubated at 37 °C for 3 h. The formed purple formazan was dissolved in a solution of DMSO: isopropanol (3:1) and spectrophotometrically quantified at 540 nm.

The activity of the cytoplasmic enzyme lactate dehydrogenase (LDH) released into the culture medium was evaluated using the CytoTox 96 nonradioactive cytotoxicity assay kit, according to the manufacturer's instructions.

2.2.4. Morphological studies

Morphological alterations were evaluated by phase-contrast microscopy, Giemsa and Höechst staining. COV434 and hGC were cultured in 24-well culture plates with coverslips and treated with CUR or RES for 24h, 48h and 72h. Cells were observed under a phase contrast microscope (Eclipse 400, Nikon, Japan) equipped with an image analysis software Nikon NIS Elements. For Giemsa staining, cells were fixed with 4% paraformaldehyde solution, stained with Giemsa stain solution for 30 min and observed under a bright field microscope (Eclipse E400, Nikon, Japan) equipped with image analysis software LeicaQWin. For Höechst staining, cells were exposed to 0.5 μg/ml Höechst 33342 for 20 min and examined under a fluorescence microscope (Eclipse CI, Nikon, Japan) equipped with an excitation filter with maximum transmission at 360/400 nm. Images were processed by Nikon NIS Elements Image Software.

2.2.5. Mitochondrial membrane potential (ΔΨm) and intracellular reactive oxygen and nitrogen species (ROS/RNS)

For the assessment of the mitochondrial membrane potential ($\Delta\Psi_m$) and intracellular reactive oxygen and nitrogen species (ROS/RNS) production, COV434 and hGCs cells were seeded in 96-well black plates and treated with CUR (0-10 μM) and RES (0-50 μM). For $\Delta\Psi_m$ studies, cells were incubated with a 100 nM dihexyloxacarbocyanine iodide (DiOC6) solution for 30 min, at 37 °C, in the dark. For positive control, cells were incubated with the mitochondrial membrane-depolarizing agent carbonyl cyanide m-chlorophenylhydrazone CCCP (10 μM) for 15 min before addition of $DiOC_6$. Fluorescence was measured by the use of a Microplate Fluorimeter (BioTek Instruments, Winooski, VT, USA) (excitation: 488 nm; emission: 525 nm). The results are expressed in relative fluorescence units (RFU).

For the quantification of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) generated by CUR and RES, cells were incubated with the probe 2′-7′-dichlorodihydrofluorescein (DCDHF-DA) for 1 h at room temperature. Fluorescence, proportional to the cellular levels of ROS/RNS, was measured using the Microplate Fluorimeter (BioTek Instruments, Winooski, VT, USA) (excitation: 485 ± 10 nm; emission: 530 ± 12.5 nm). The stress inducer H_2O_2 (200 μM) was used as a positive control. The results are expressed in relative fluorescence units (RFU).

2.2.6. Evaluation of caspase-3/-7 and -9 activities

To detect caspase-3/-7 and -9 activities, COV434 and hGCs cells were seeded in a 96-well white plates and incubated for 24h, 48 h and 72h in the absence or presence of Curcumin (1-5 μM). At the end of

the incubation time, Caspase-Glo -3/-7 or -9 reagent was added, according to the manufacturer's instructions. The plates were incubated for 1 h and the resultant luminescence was measured in the Microplate Luminometer (BioTek Instruments, Winooski, VT, USA). A positive control assay was conducted using Etoposide (Etop. 0.1 μM), which was added 12 h before the end of the experiment. The results are presented as relative light units (RLU).

2.2.7. Protein extraction and Western Blot analysis

COV434 cells were seeded in 6-well plates and treated with Curcumin for 48 h. Cell extracts were prepared in Lysis buffer (50 mM Tris–HCl pH 7.4, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris, 150 mM NaCl, 2 mM EDTA, 50 mM NaF) containing a cocktail of protease inhibitors (PI). Total protein concentrations were determined by Bradford assay. Protein samples (30 μg) were subjected to 10% SDS–polyacrylamide and transferred onto nitrocellulose membranes. Membranes were incubated with antibodies against rabbit-PARP (1:150; -9542S Cell Signaling Technologies, Leiden, Netherlands) at 4 °C overnight. Membranes were then washed and incubated with secondary antibody anti-rabbit (1:1000; Santa Cruz Biotechnology, CA, USA) and detected by enhanced chemiluminescence. The membranes were then stripped and reincubated with anti-β-actin (1:500; Santa Cruz Biotechnology, CA, USA) for loading control. Etoposide (ETOP) was used as positive control.

2.2.8. DNA isolation and hormonal quantification by ELFA

After isolation, hGCs were plated in 24-well plates for 24h and then treated with CUR or RES, with addiction of 1 unit of follicle-stimulating hormone (FSH) and androstenedione (Sigma-Aldrich,USA) as an androgenic substrate for the production of estrogen, for 72h. Cell culture media were collected, centrifuged and stored at − 80 °C. Estradiol and progesterone secretion was evaluated by ELFA (Enzyme Linked Fluorescent Assay), using VIDAS® Progesterone and VIDAS® Estradiol II kits (bioMérieux SA, Marcy l'Etoile, France), according to the manufacturer's instructions. DNA isolation was performed using TripleXtractor reagent, (GRiSP Research Solutions, Porto, Portugal), according to the manufacturer's instructions and quantified in the NanoDrop ND-1000 Spctrophotometer (NanoDrop Technologies,Inc., Wilmington, DE, USA Hormone levels were standardized to cell DNA.

2.2.9. RNA isolation and gene expression analysis by RT-PCR

To investigate the influence of CUR and RES on Steroidogenic Acute Regulatory Protein (StAR), CYP11A1, 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4-isomerase (3β-HSD) and aromatase (CYP19A1) gene transcription, cells were seeded in 24-well plates and treated with different concentrations of CUR (0.001-5 μM) and RES (0.001-5 μM). Also, FSH ($1x10^{-4}$IU/mL) and androstenedione (50nM) were added to the cells. Then, cells were harvested using TripleXtractor reagent, (GRiSP Research Solutions, Porto, Portugal) and total RNA was extracted according to the manufacturer's instructions. RNA was quantified in the NanoDrop ND-1000 Spctrophotometer (NanoDrop Technologies,Inc., Wilmington, DE, USA). RNA was reverse transcribed using the iScript™ select cDNA Synthesis Kit (Bio-Rad Laboratories, USA), and the resultant cDNA was amplified with specific primers, using KAPA SYBR® FAST qPCR Master Mix 2 × Kit (Kapa Biosystems, Woburn, MA, USA) in MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratories, USA), according to the kit protocol. Primer sequences and RT-PCR conditions are summarized in Table 1. The specificity of the amplified RT-PCR product was assessed by the melting curve analysis. Relative quantification of gene expression was calculated by the formula 2-$\Delta\Delta C_t$ method, using the expression of GAPDH as housekeeping gene. As similar results were obtained with both housekeeping genes, the RT-PCR data presented were normalized to GAPDH.

Table 2.1 -Primer sequences and qPCR conditions used to assess the expression of genes encoding StAR, 3β-HSD, CYP11A1 and aromatase (CYP19A1). AT- annealing temperature; MT- melting temperature; AL- amplicon length.

Gene	GenBank	Primer sequence (5'-3')	AT (ºC)	MT (ºC)
StAR	NM_000349	F: ATCAAGCTGTGCTGGGAGC R: TGGCCATCACGCCTGTTGCC	60.0	84.0
3β-HSD	NM_000198	F: GTCATCCACACCGCCTGTAT R: CACAGGCCTCCAACAGTAGC	60.0	82.0
CYP11A1	NM_000781	F: TGGGTCGCCTATCACCAG R: CCACCCGGTCTTTCTTCC	60.0	78.0
CYP19A1	NM_000103	F: TGCAAAGCACCCTAATGTTG R: TGGTACCGCATGCTCTCATA	60.0	84.5
GAPDH	NM_001289745.1	F: CGCGAAGCTTGTGATCAATGG R: GGCAGTGATGGCATGGACTG	55.0	83.0

2.2.10. Statistical analysis

Statistical analysis was carried out using ANOVA. The dependent variables, whose means were under comparison, were quantifications of cell viability, LDH release, mitochondrial membrane potential, reactive oxygen species and caspases -3/7 and -9 activity measurement. One-way ANOVA was used when one independent variable was involved, namely CUR or RES dose levels, and two-way ANOVA was used for two independent variables simultaneously, like doses and timings. When the null hypothesis was rejected, pairwise comparisons were conducted by the post-hoc Tuckey's and Bonferroni test, respectively for one- or two-way ANOVA. The means under comparison were drawn from at least three independent experiments carried out in triplicate. The results shown graphically, with means and their SEM (standard error mean), and differences were considered statistically significant when $p < 0.05$, although other p values are also reported. All statistical analysis were performed using GraphPad Prism software 7.0 (GraphPad PRISM v. 7.0, GraphPad Software, Inc., San Diego, CA, USA).

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Chapter 3: Results

A. The effect of CUR and RES on COV434 cells

3.1. CUR and RES on cell viability

In order to analyze the effect of CUR and RES on cell viability, respectively, it was performed MTT assay and measured LDH released in the culture medium. Both compounds induce a decrease in cell viability, dependent on compound concentration and exposure time

Cell treatment using CUR at different concentrations (0.001-50 µM) and times (24h, 48h and 72h) induce a significant decrease in COV434 cell viability over 10 µM at 24h and 5 µM at 48h and 72h (figure 3.1A). Additionally, CUR induces cell cytotoxicity at 20 µM at 24h and 48h and 10 µM by72h (figure 3.1B).

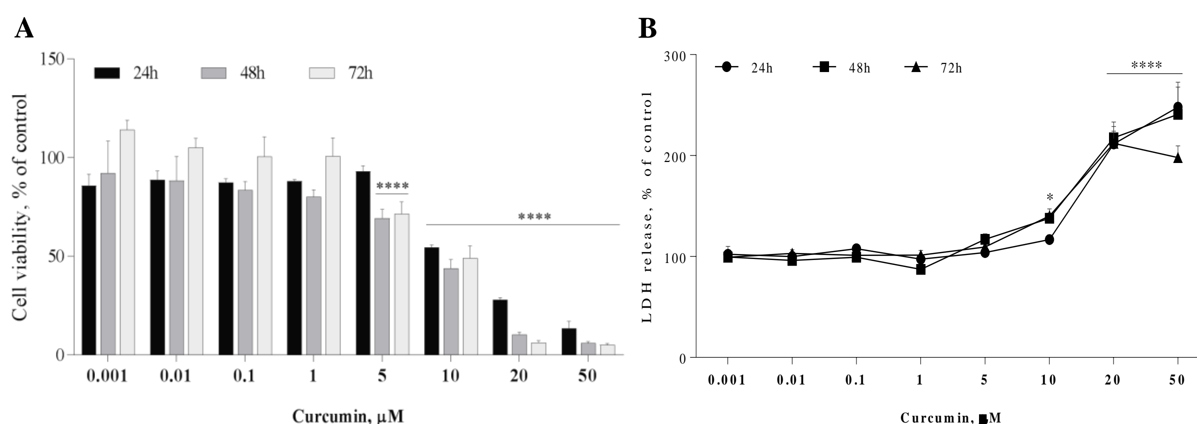

Figure 3.1- **Effects of CUR on COV434 cell viability.** Cell viability of CUR-treated cells with different concentrations (0.001-50 µM) at 24h, 48h and 72h of treatment, assessed by (**A**) MTT assay and (**B**) LDH release. Results are expressed as mean ± SEM of at least five independent experiments performed in triplicate. Significant differences between control and treated cells are denoted as * (p<0.05), ** (p<0.01) and **** (p<0.0001).

On the other hand, cell treatment using RES at different concentrations (0.001-200 µM) and at different times (24,48 and 72h), induces a significant decrease in COV434 cell viability over 100 µM at 24h and

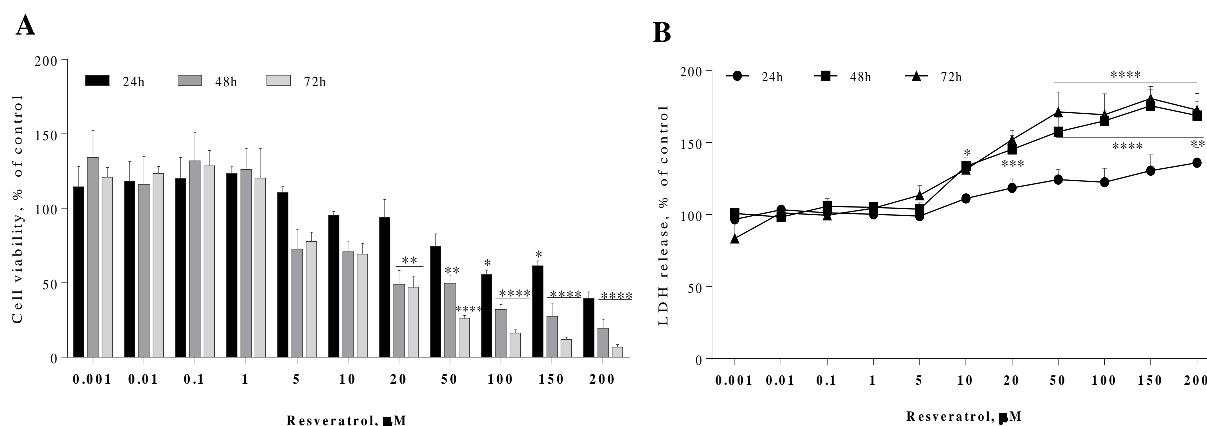

Figure 3.2- **Effects of RES on COV434 cell viability.** Cell viability of RES-treated cells with different concentrations (0.001-200 µM) at 24h, 48h and 72h of treatment, assessed by (**A**) MTT assay and (**B**) LDH release. Results are expressed as mean ± SEM of at least five independent experiments performed in triplicate. Significant differences between control and treated cells are denoted as * (p<0.05), ** (p<0.01), *** (p<0.001) and **** (p<0.0001).

over 20 μM at 48h and 72h (figure 3.2A). Cytotoxicity is demonstrated at a concentration above 150 μM at 24h and 10 μM after 48h and 72h of treatment (figure 3.2B).

To examine the morphological changes that might be induced by CUR on COV434, cells were treated with CUR at different concentrations for 48 hours and then observed under a phase contrast microscope (figure 3.3 A, B, C) or stained with either Giemsa (figure 3.3 D, E, F) or Höechst (figure 3.3, G, H, I). According to previous cell viability studies, concentrations of 1 μM and 5 μM were chosen. Treatments with CUR 1 μM do not cause significant change in cell morphology. However, when we incubate with CUR at a higher dose (5 μM), COV434 cells density significantly decreases. The results shown in figure 3.3 are representative of all assays. In figure 5A, the typical appearance of control (untreated) COV434 cells is presented.

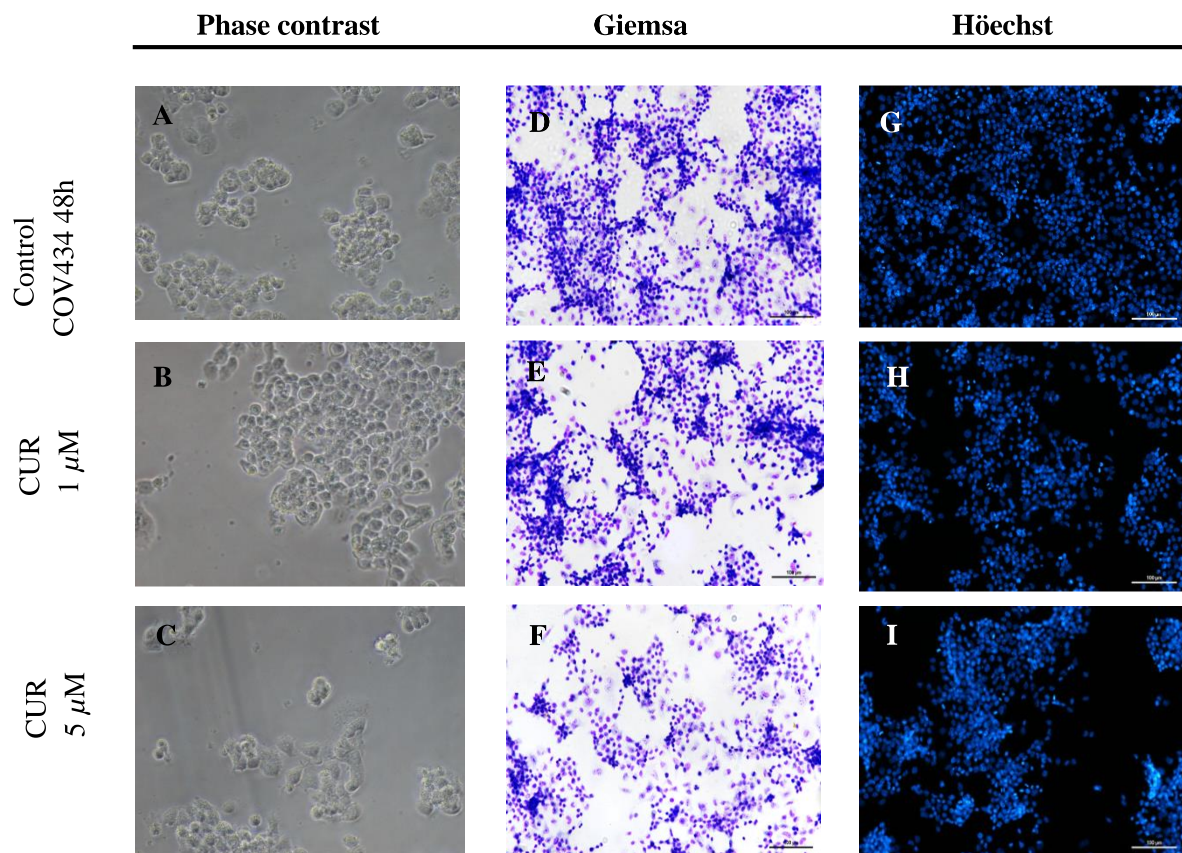

Figure 3.3- **Effects of CUR on COV434 cell morphology after 48h**.
(**A, B, C**) Phase contrast microscopy; (**D, E, F**) Giemsa staining; (**G, H, I**) Höechst staining. COV434 cells morphology was analyzed in the absence (control) or presence of CUR (1 μM and 5 μM) after 48 hours. Results are shown from single representative of three independent experiments. Total magnification 200x.

On the other hand, COV434 cells treated with RES after 48 hours were subjected to the same morphological studies (figure 3.4). According to MTT and LDH assays, concentrations of 1 μM and 5 μM were chosen. Treatment with RES 1 μM shows a slight increase in cell density when compared to the control. In addition, no nuclear condensation or morphological changes are identified. At RES 5 μM there is a decrease on cell density, however, Höechst staining shows no signs of apoptosis (nuclear condensation or fragmentation).

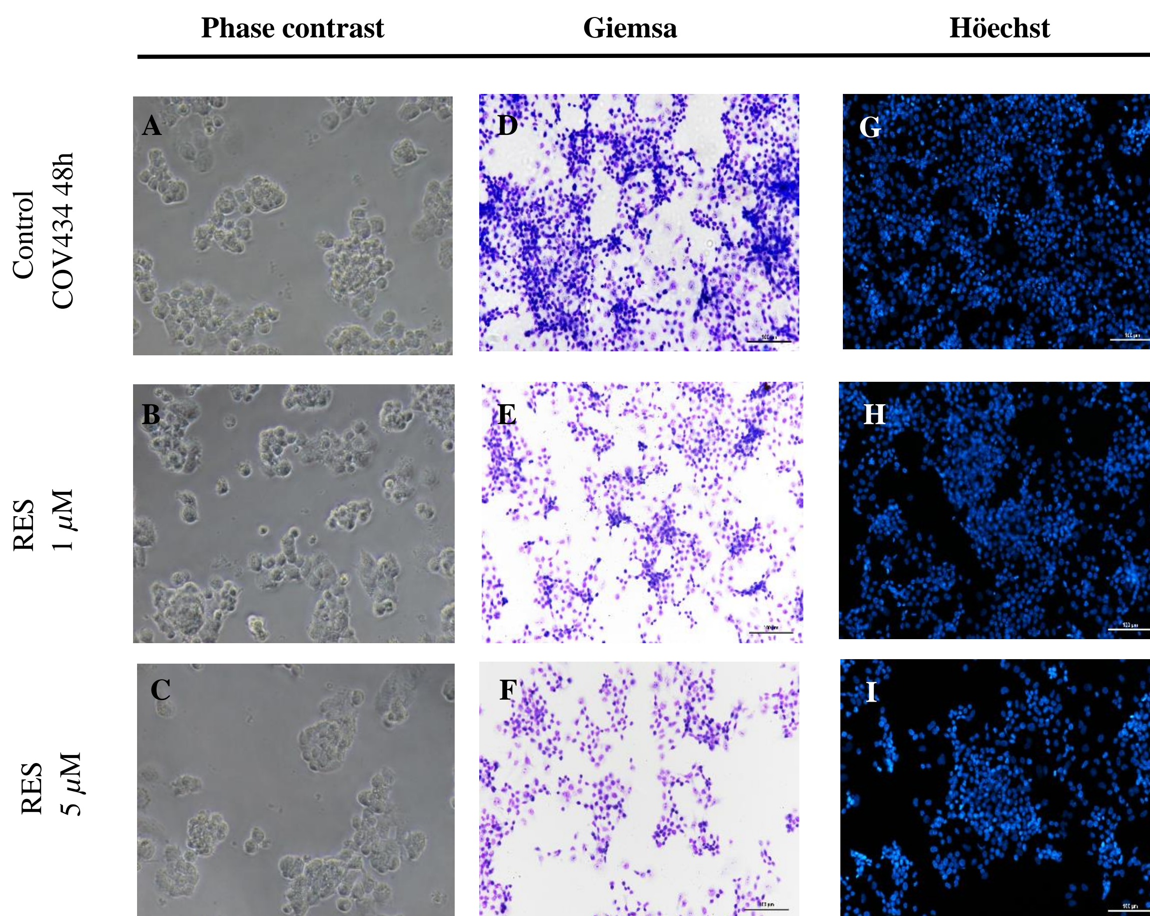

Figure 3.4 - **Effect of RES on COV434 cell morphology after 48 hours.**
(**A, B, C**) Phase contrast microscopy; (**D, E, F**) Giemsa staining; (**G, H, I**) Höechst staining. COV434 cells morphology was analyzed in the absence (control) or presence RES (1 μM and 5 μM) after 48 hours. Results are shown from single representative of three independent experiments. Total magnification 200x.

To study the reduction in cell viability observed for higher concentration of either CUR or RES, mitochondrial membrane potential (Δψm) on COV434 was measured using a $DiOC_6$ probe.

COV434 cells were treated with CUR at different concentrations (5-10 μM) and at different times (24h, 36h and 48h). When compared with the control, results demonstrate a decrease around 30% in Δψm over 5 μM of CUR and 34% at a concentration of 10 μM, after 48h (figure 3.5A). These results are in agreement with the cell viability results previously discussed, suggesting an association of decreased cell viability with mitochondrial dysfunction.

In contrast, treatment with RES (5-20 μM) at 24h, 36h and 48h, do not induce significant differences in Δψm (figure 3.5B).

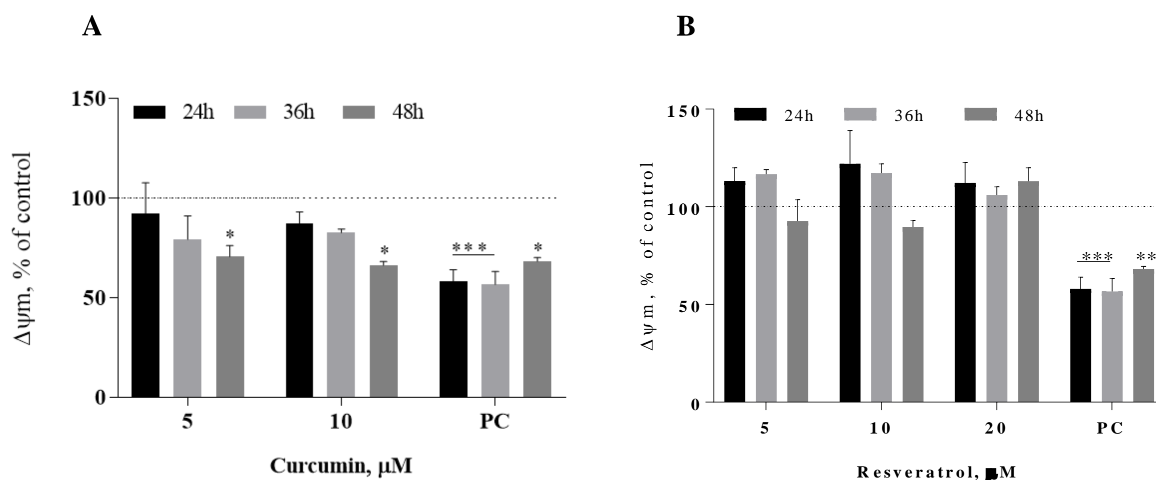

Figure 3.5- **Effect of CUR and RES on Δψm of COV434 at 24h, 36h and 48h after treatment**. Δψm in cells treated with CUR (5-10 μM) at 24h, 36h and 48h in comparison with untreated cells, assessed by fluorescence assay with DiOC6 probe. CCCP (10 μM) was used as positive control (PC). Significant differences between control and treated cells are denoted as * (p<0.05), ** (p<0.01), *** (p<0.001) and **** (p<0.0001).

Unlike the RES, COV434 cell line in the presence of CUR shows a decrease in cell viability at 1 and 5 μM without associated LDH release and a decrease in Δψm at 48 hours, suggesting the involvement of programmed cell death. In order to investigate the latter, caspase -3/7 and caspase 9 activities were measured. Caspases are specific proteases that play a determinant role in apoptosis. The results show no statistical differences on caspase -3/7 (figure 3.6A) and caspase 9 (figure 3.6B) activities after 48 hours of treatment between using CUR at a concentration of 1 and 5 μM.

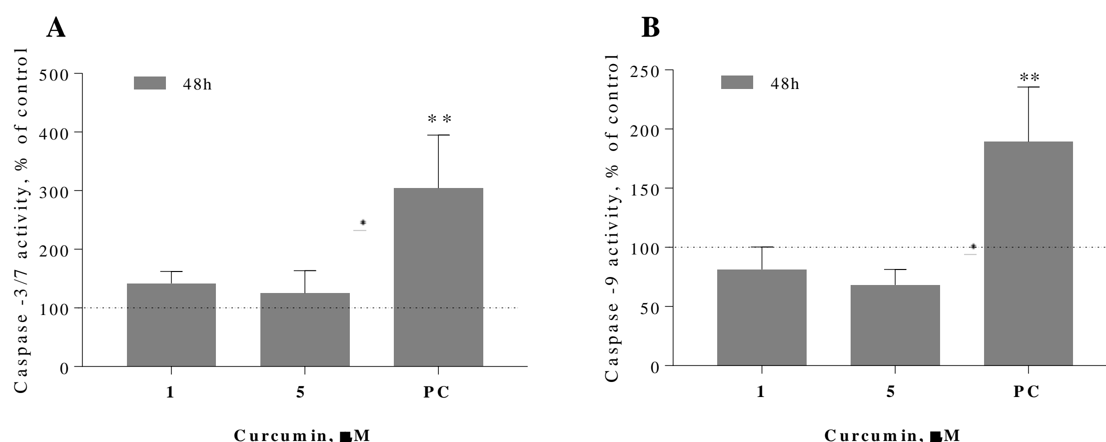

Figure 3.6 - **Effect of CUR on COV434 cells caspase 3/7 (A) and -9 (B) activity at 48h.** Etoposide (ETOP) was used as positive control. Significant differences between control and treated cells are denoted as * (p<0.05), ** (p<0.01), *** (p<0.001) and **** (p<0.0001).

Furthermore, Poly (ADP-ribose) polymerase (PARP) expression was evaluated by western blot (figure 9). PARP-1 is the first well characterized member of PARPs family. PARPs are a family of associated enzymes that are responsible for various cellular processes, including DNA repair. Increased of PARP may indicate accumulation of DNA damage [35].

Results show an increase in PARP-1 expression after 1µM and 5 µM CUR treatment when compared to the control. In line with caspase -3/7 results, no cleaved PARP-1 is observed (figure 3.7). These results suggest a PARP-1 overactivation resulting in a caspase-independent cell death pathway.

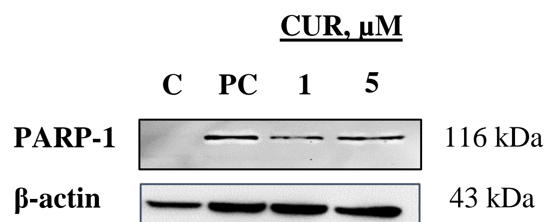

Figure 3.7 – **PARP-1 expression on COV434 cells**. Western blot analysis in the absence (control) or presence of CUR (1-5µM) after 48 hours. ETOP was used as positive control. β-actin was used as loading control.

To further explore the mechanisms underlying cell viability loss after treatment with CUR or RES, reactive species production was evaluated, which is often associated with mitochondrial dysfunction and oxidative stress. Two fluorescence assays with DCFH-DA were performed. Short and long-term effects on ROS as result of CUR and RES exposure was assayed. Firstly, cells were treated with CUR and RES at higher concentrations and ROS immediately measured for 4 hours. On the contrary, in the second assay, cells were treated with CUR and RES in concentrations between 1 and10 µM, both at 24, 48 and 72 hours and then the probe DCFH-DA was added.

The results show an immediate increase in ROS production at 10 µM of CUR and a decrease after 72h with the same concentration. On the other hand, significant changes are observed after 2 hours of treatment over 5 µM RES, but the effect is not confirmed after 48 or 72h. These results suggest an immediate effect on ROS production, but at long term that is not observed (figure 3.8).

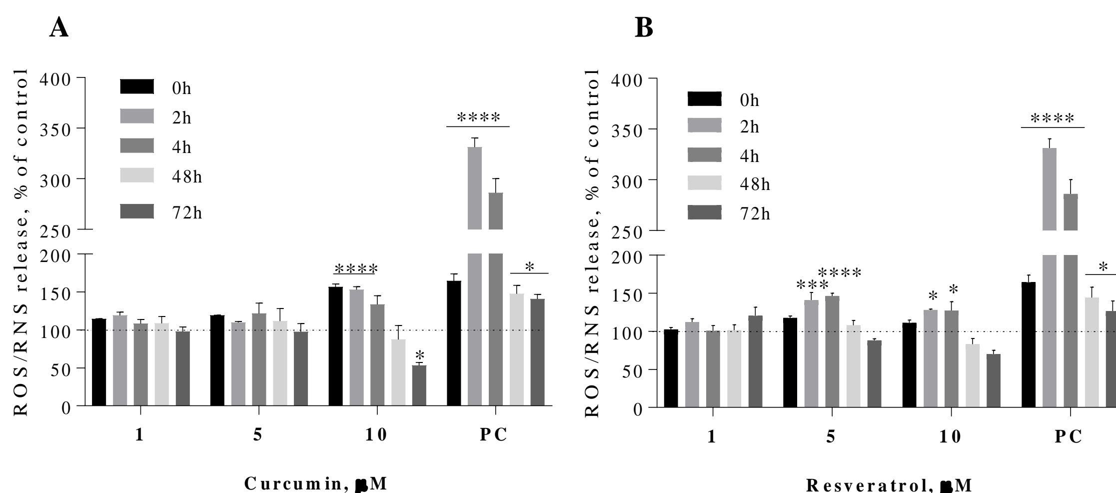

Figure 3.8 - **Effect of CUR and RES on COV434 ROS formation.** ROS production in cells treated with CUR (**A**) and RES (**B**) at different concentrations (1 -10 µM) from 0h to 72h in comparison with untreated cells (control), assessed by fluorescence assay with DCFH-DA probe. H_2O_2 (200 µM) was used as positive control (PC). Significant differences between control and treated cells are denoted as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and **** ($p<0.0001$).

3.2. Antioxidant potential of CUR and RES on COV434 cells

In order to explore the long-term antioxidant potential of CUR and RES on COV434 cells, ROS production was measure using the lowest concentrations (0.001 -0.1 µM) of both compounds. Both compounds show no significant results in ROS production (figure 3.9).

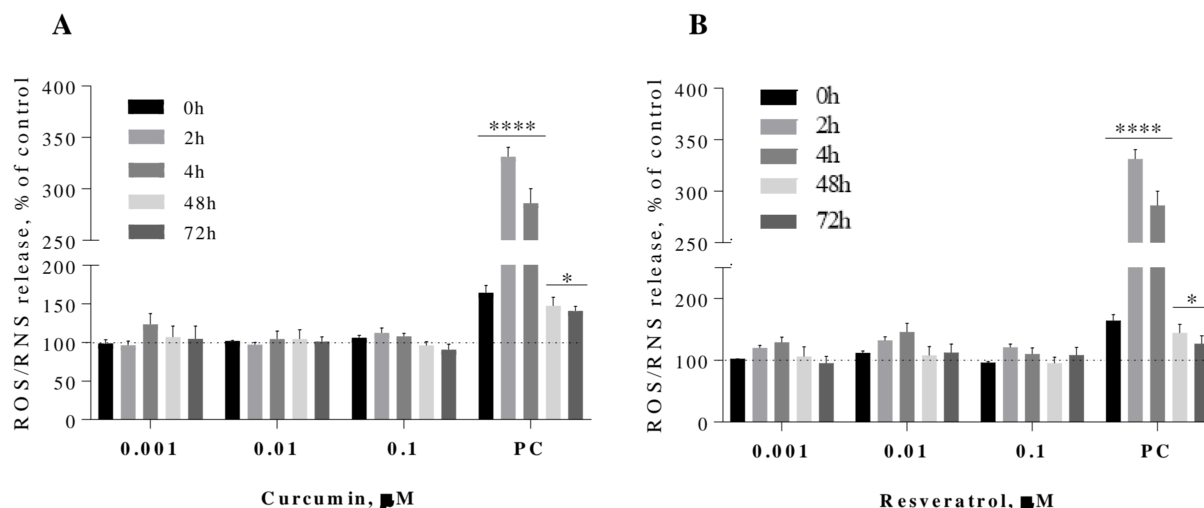

Figure 3.9 -**Effect of CUR and RES on COV434 ROS formation**. ROS production in cells treated with CUR (**A**) and RES (**B**) at different concentrations (0.001 -0.1 µM) from 0h to 72h in comparison with untreated cells (control), assessed by fluorescence assay with DCFH-DA probe. H_2O_2 (200 µM) was used as positive control. Results are expressed as mean of three independent experiments performed in triplicate. Significant differences between control and treated cells are denoted as *** (p<0.001) and **** (p<0.0001).

Then again, when COV434 cells are treated with CUR and RES for 72 hours and then co-treated with TBHP 5 µM (stress inducer), there is a reduction in ROS production when compared to the TBHP alone. These results suggest that CUR and RES play a protective effect against ROS stimulation (figure 3.10).

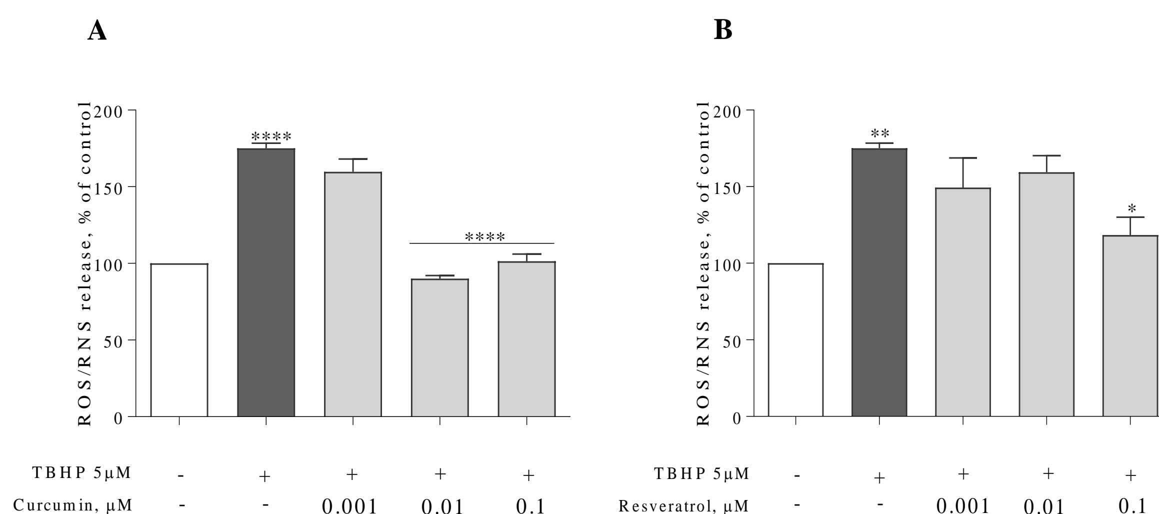

Figure 3.10 - **ROS formation after stress induction**. ROS production in cells treated with CUR (**A**) and RES (**B**) at different concentrations (0.001 -0.1 µM) for 72 hours and then co-treated with TBHP 5µM in comparison with TBHP alone, assessed by fluorescence assay with DCFH-DA probe. Results are expressed as mean of three independent experiments performed in triplicate. Significant differences between control and treated cells are denoted as *** (p<0.001) and **** (p<0.0001).

3.3. The role of CUR and RES on endocrine function

Finally, it was considered the impact of CUR and RES on COV434 cells endocrine function concerning the hormones that have a major influence on the female reproductive system, progesterone and estradiol. However, the granulosa cell line COV434 have a functional FSH but no LH receptors which leads to undetectable progesterone levels.

In order to accomplish the third aim of this study, in every treatment, FSH and androstenedione were added to the medium. FSH acts as an GC activator and androstenedione as a substratum for estradiol synthesis. To understand the possible impact of CUR and RES intake for long periods, 72 hours' treatment and two different concentrations, one lower and one higher (0.01 and 5 µM), were chosen for both compounds.

Moreover, the secretion of estradiol by COV434 cells into cell culture medium was also evaluated through the ELFA technique. Cells treated with 5 µM CUR show an extreme increase on estradiol levels (figure 3.11A). On the other hand, cells treated with RES also present a 3-fold increase at 5 µM suggesting that, although lower doses do not have an impact on hormonal levels, higher doses may influence estradiol production (figure 3.11B).

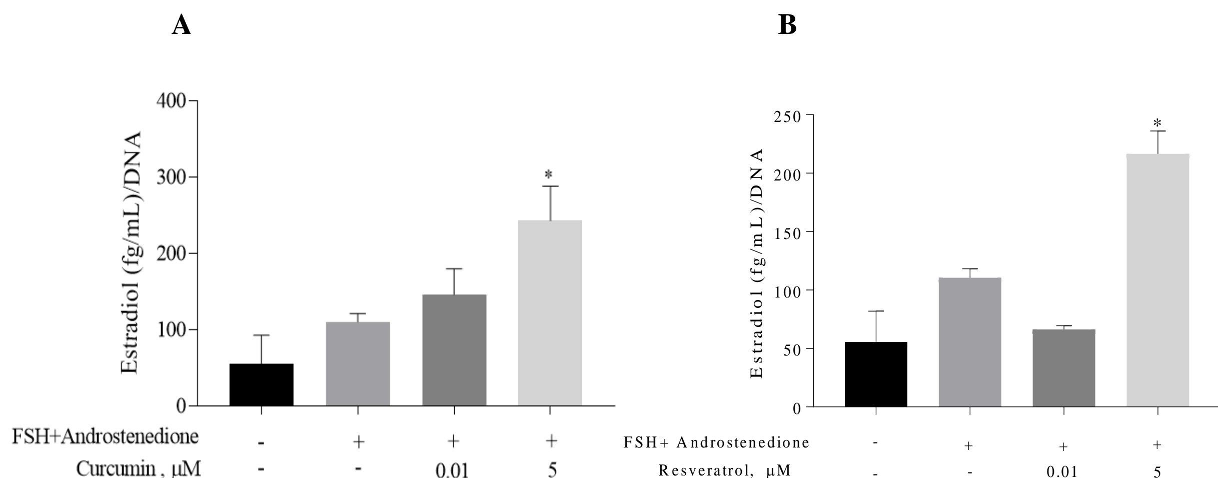

Figure 3.11 - **Effects of CUR and RES in the secretion of estradiol by COV434 cells after 72 hours of treatment**. Significant differences between control and treated cells are denoted as * ($p<0.05$).

In order to explore if CUR and RES induce alterations in the transcription levels of genes that encode for key enzymes responsible for progesterone and estradiol biosynthesis, qPCR was also performed using cDNA samples from COV434 cells treated with CUR for 72 hours.

The evaluation of StAR, CYP11A1, 3β-HSD and CYP19A11 mRNA levels indicate that CUR at 0.01 µM has no statistically significant influence on the transcripts when compared to COV434 cells treated with FSH and androstenedione alone (figure 3.12). However, at 5 µM there is severe decrease on StAR, CYP11A1 and 3β-HSD transcriptional levels. The decrease is not verified on aromatase (CYP19A1), which is the final enzyme to estradiol synthesis.

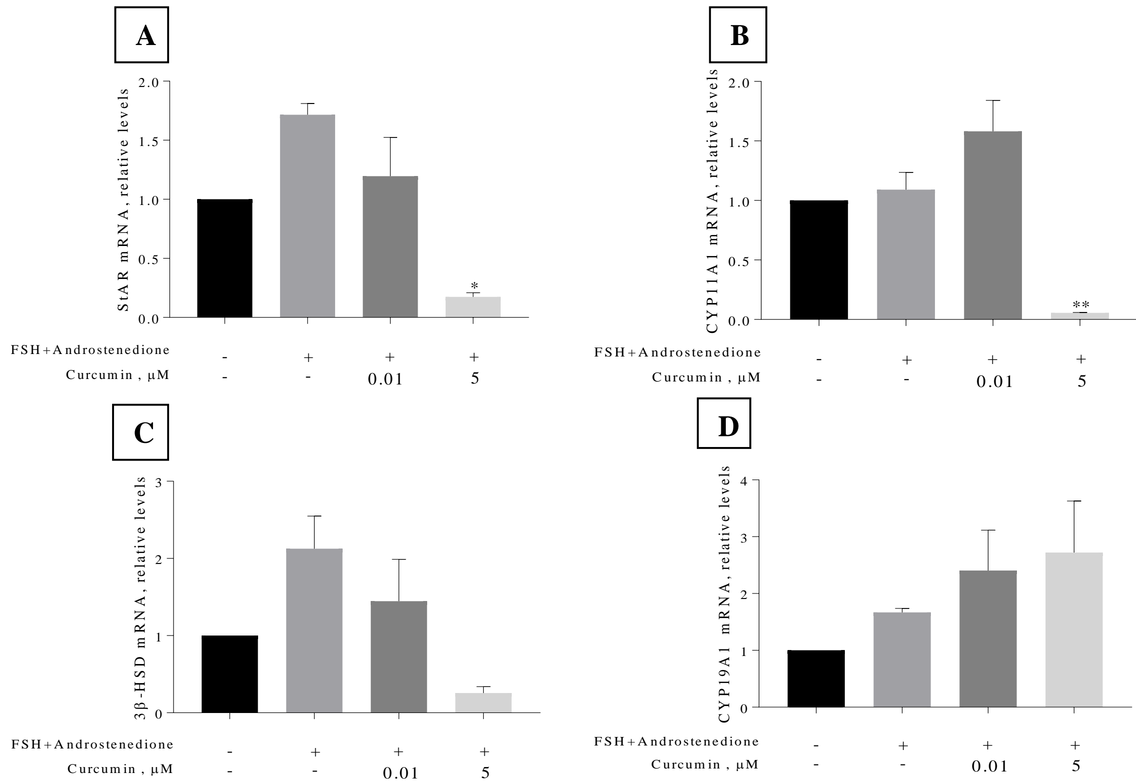

Figure 3.12 **- Effects of CUR on the transcriptional levels of StAR (A), CYP11A1 (B) 3β-HSD (C) and CYP19A1 (D) after 72h of treatment, on COV434 cells**. Significant differences between cells co-treated with FSH and androstenedione and cells treated with FSH, androstenedione and CUR are denoted as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and **** ($p<0.0001$).

On the other hand, RES induces a decrease in mRNA levels of all studied genes (StAR, CYP11A1. 3β-HSD and CYP19A1) at 0.01 and 5 µM (figure 3.13).

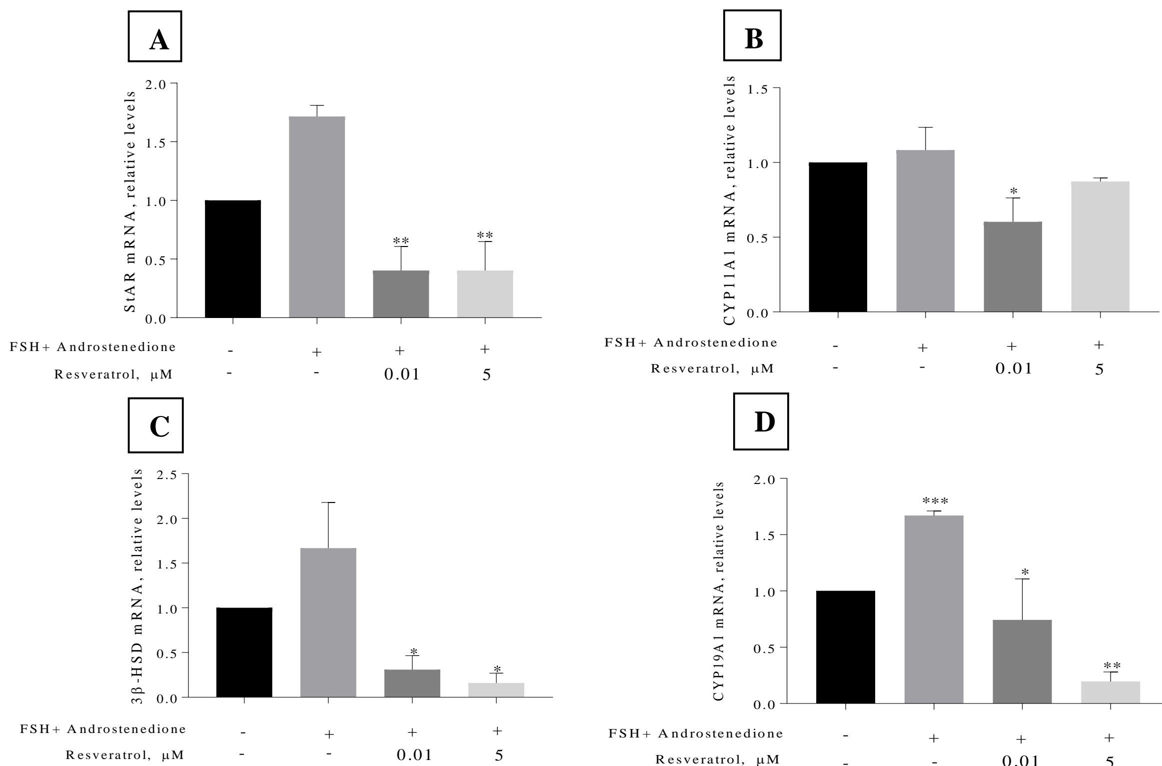

Figure 3.13 **- Effects of RES on the transcriptional levels of StAR, CYP11A1, 3β-HSD and CYP19A1 after 72h of treatment, on COV434 cells**. Significant differences between cells co-treated with FSH and androstenedione and cells treated with FSH, androstenedione and CUR are denoted as * ($p<0.05$), ** ($p<0.005$), *** ($p<0.0005$) and **** ($p<0.00005$).

B. The effect of CUR and RES on primary cultures of human granulosa cells (hGC)

After concluding that CUR and RES influence COV434 cell viability and play a role in oxidative stress protection and hormone synthesis, studies were performed in primary cultures of human granulosa cells from patients undergoing IVF treatments, in order to confirm the results previously obtained.

3.4. CUR and RES effect on hGC viability

Cell treatment using CUR at different times (24h, 48h and 72h) and concentrations (0.001-50 µM) induces a decrease in hGC viability over 50 µM at 24h and 20 µM at 72h (figure 3.14A). In addition, CUR induces cell cytotoxicity at 50 µM at 24h and 48h and 10 µM at 72h (figure 3.14B).

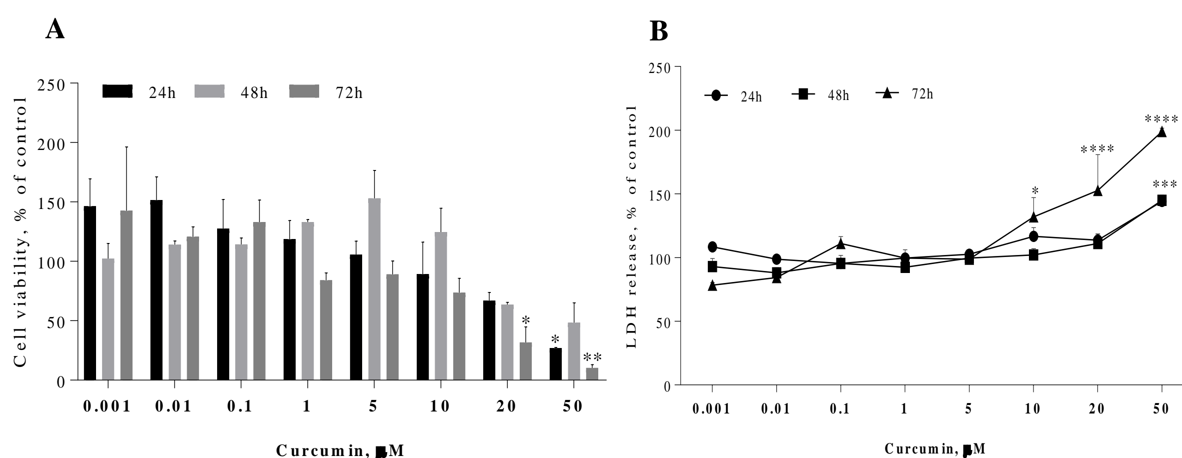

Figure 3.14 - **Effects of CUR on hGC viability.** Cell viability of CUR-treated cells with different concentrations (0.001-100 µM) at 24h, 48h and 72h of treatment, assessed by (**A**) MTT assay and (**B**) LDH release. Results are expressed as mean ± SEM of at least five independent experiments performed in triplicate. Significant differences between control and treated cells are denoted as * ($p<0.05$), ** ($p<0.01$) and **** ($p<0.0001$).

On the other hand, cell treatment using RES (0.001-50 µM) does not lead to any tendency on cell viability (figure 3.15A). However, cytotoxicity is demonstrated at a concentration of 50 µM at 72h (figure 3.15B). These results suggest that primary hGC are less sensitive to the compound compared to COV434 cells.

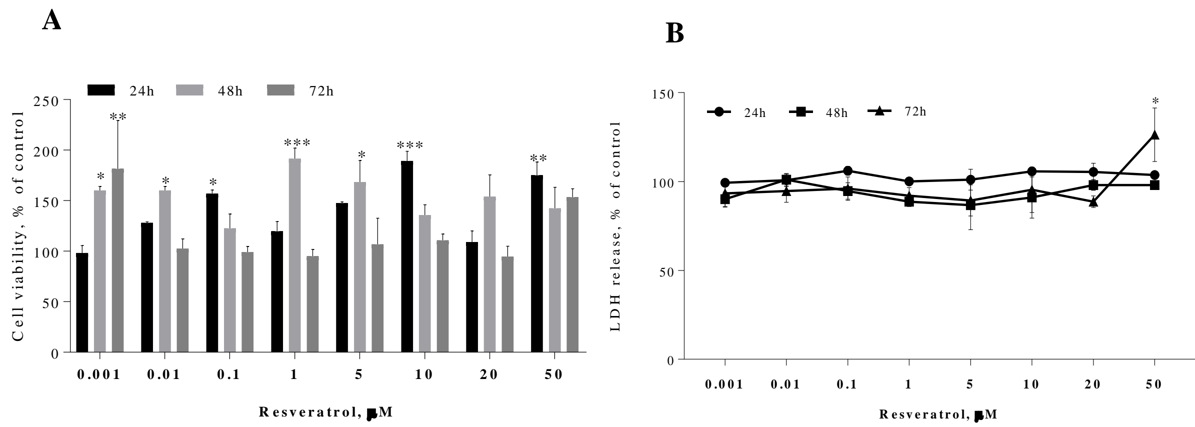

Figure 3.15 - **Effects of RES on hGC viability**. Cell viability of RES-treated cells with different concentrations (0.01-50 µM) at 24h, 48h and 72h of treatment, assessed by (**A**) MTT assay and (**B**) LDH release. Results are expressed as mean ± SEM of at least five independent experiments performed in triplicate. Significant differences between control and treated cells are denoted as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and **** ($p<0.0001$).

Moreover, to examine the morphological changes that can be induced by CUR and RES on hGC, cells were treated with concentrations between 1 and 5 µM for 48 hours and then observed under a phase contrast microscope. However, due to the difficulty in staining hGC, because of its week adherence and availability, only preliminary studies were performed. These results do not confirm any morphological changes (data not shown).

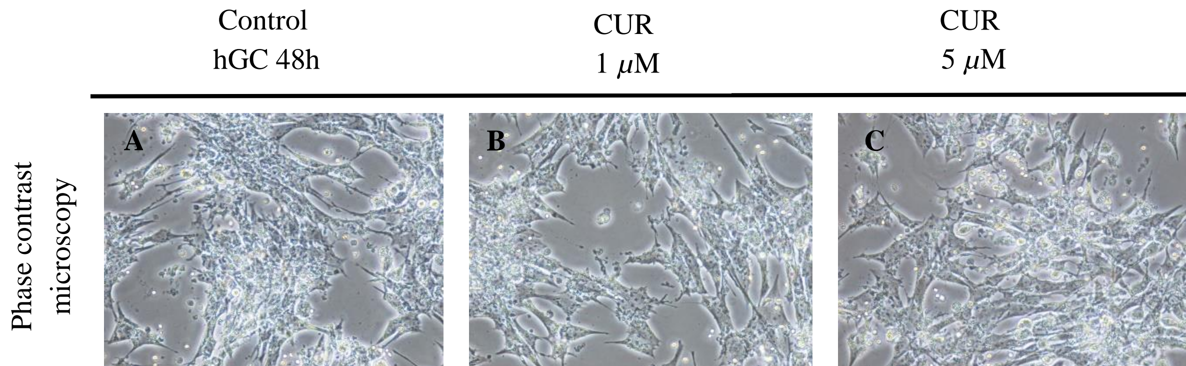

Figure 3.16 - **Effects of CUR on hGC morphology.** (**A, B, C**) Phase contrast microscopy. hGC morphology was analyzed in the absence (control) or presence of CUR (1 µM and 5 µM) after 48 hours. Results are shown from single representative of three independent experiments. Total magnification 200x.

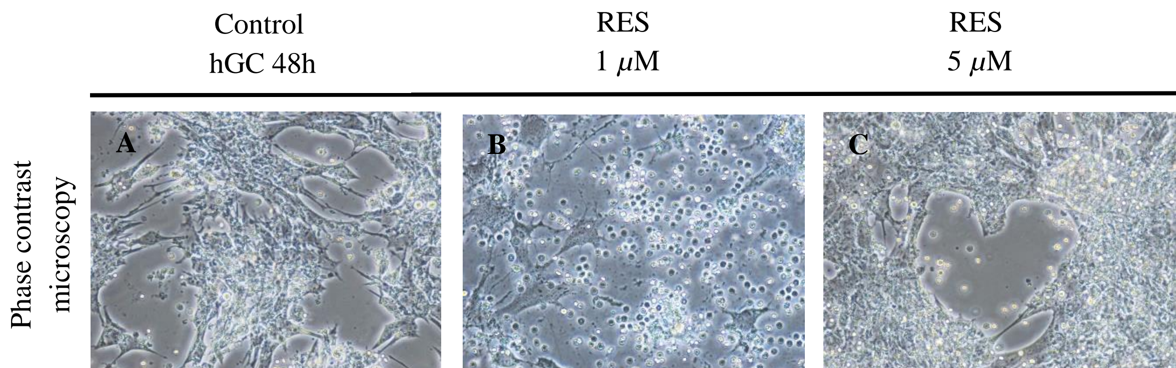

Figure 3.17- **Effects of RES on hGC morphology.** (**A, B, C**) Phase contrast microscopy. hGC morphology was analyzed in the absence (control) or presence of RES (1 µM and 5 µM) after 48 hours. Results are shown from single representative of three independent experiments. Total magnification 200x.

After suggestion of apoptotic death in COV434 cells resulting from CUR addition, caspase -3/7 and -9 activities were also measured 48 hours after treatment, in order to clarify if the same occur in primary cells. Results show no differences in caspase 3/7 activation at any tested concentration (figure 3.18A). There is however an increase in caspase 9 activity dependent on its concentration, being statistically significant at 5 µM (figure 3.18B).

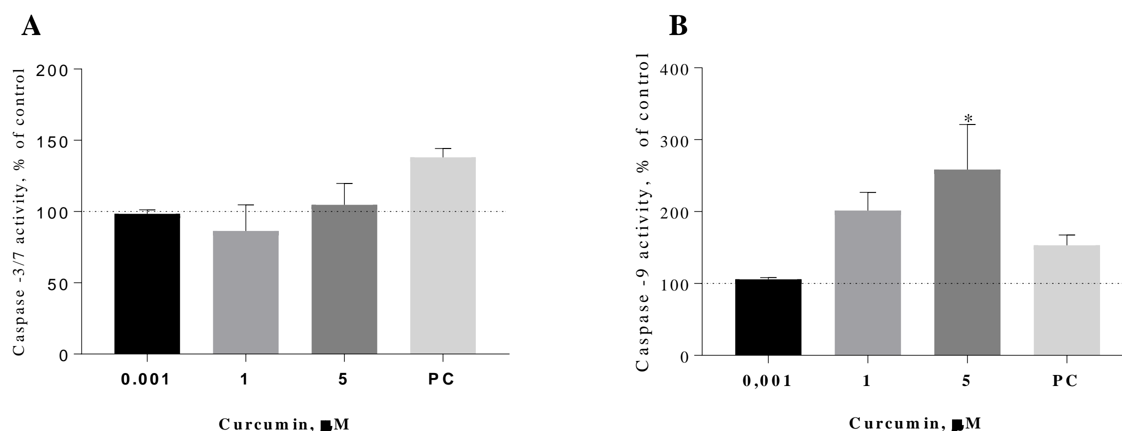

Figure 3.18 - **Effect of CUR on primary hGC caspase 3/7 and -9 activity after 48h**. Etoposide (ETOP) was used as positive control. Significant differences between control and treated cells are denoted as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and **** ($p<0.0001$).

Then, ROS/RNS release was measured. Due to the low availability of primary hGC the experiments were only performed after 48 hours of treatment. The results show no significant differences. Still, the concentration of CUR 0.001 µM appears to induce a decrease in ROS production (figure 3.19). These results suggest an antioxidant effect only at lower doses of this compound.

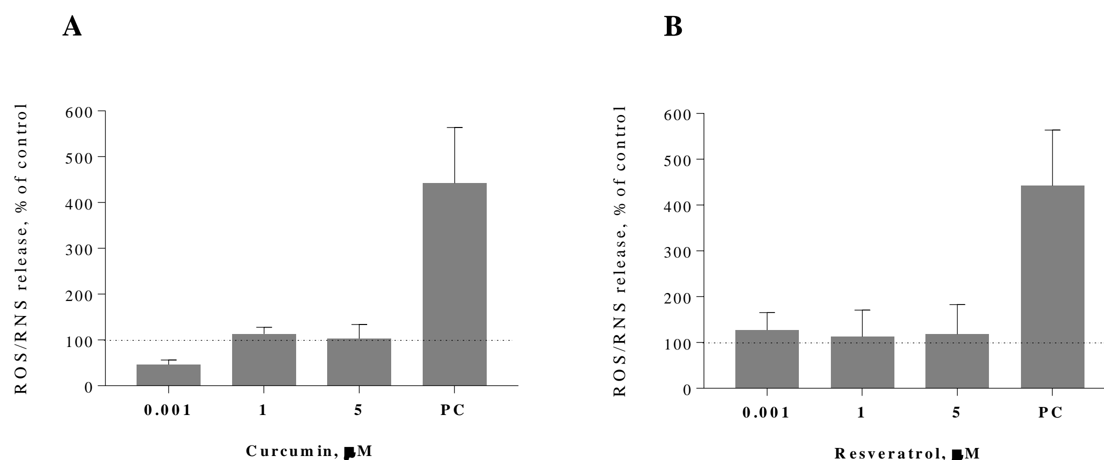

Figure 3.19- **Effect of CUR and RES on hGC ROS formation.** ROS production in cells treated with CUR (**A**) and RES (**B**) at different concentrations (0.001 -5 µM) at 48h in comparison with untreated cells (control), assessed by fluorescence assay with DCFH-DA probe. H_2O_2 (200 µM) was used as positive control. Results are expressed as mean of three independent experiments performed in triplicate. Significant differences between control and treated cells are denoted as *** ($p<0.001$) and **** ($p<0.0001$).

3.5. Antioxidant potential of CUR and RES on primary hGC

In order to investigate the antioxidant potential of CUR and RES in primary hGC, cells were treated for 72 hours and co-treated with TBHP 5 µM. CUR exerts a reduction in ROS production when compared to TBHP alone. CUR at a concentration of 0.1 µM presents statistically significant results. In contrast, there are no significant differences regarding RES but there is a slight decrease in ROS production at 0.001 and 0.01 µM of RES. These outcomes suggest that CUR and RES play a protective effect against ROS stimulation, not only in COV434, but also in primary cells (figure 3.20).

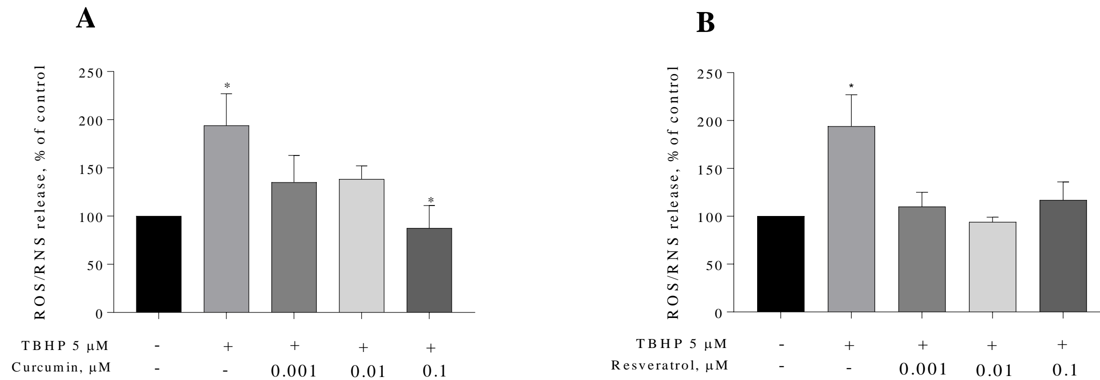

Figure 3.20 - **ROS formation after stress induction.** ROS production in cells treated with CUR (**A**) and RES (**B**) at different concentrations (0.001 -0.1 µM) for 72 hours and then co-treated with TBHP 5µM in comparison TBHP alone, assessed by fluorescence assay with DCFH-DA probe. Results are expressed as mean of three independent experiments performed in triplicate. Significant differences between control and treated cells are denoted as *** ($p<0.001$) and **** ($p<0.0001$).

3.6. The role of CUR and RES on endocrine function

The secretion of estradiol by hGC was also evaluated by the ELFA technique. CUR-treated cells show no change in estradiol levels (figure 3.21A). In contrast, cells treated with RES present a dose-dependent increase, being significant at 5 µM (figure 3.21B).

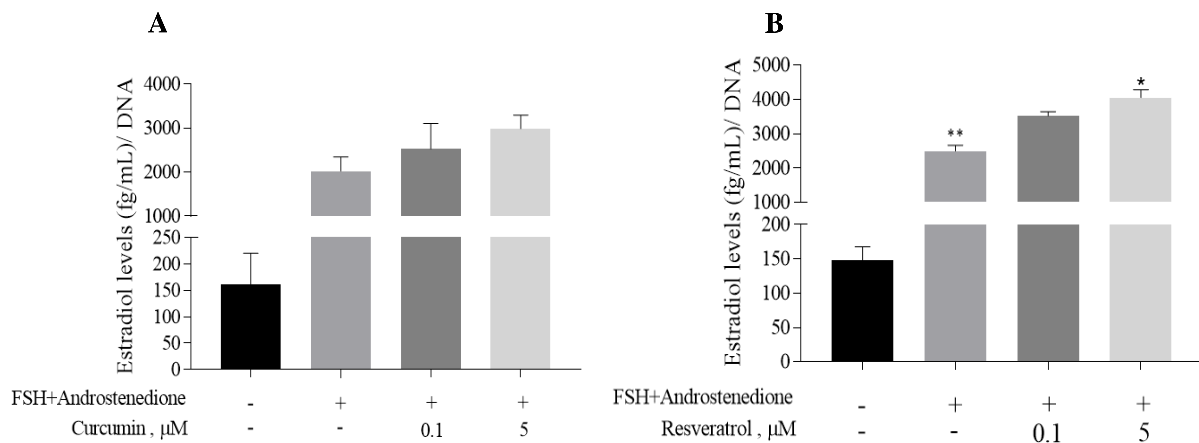

Figure 3.21- **Effects of CUR and RES in the secretion of estradiol by hGC after 72 hours of treatment**. Significant differences between control and treated cells are denoted as * ($p<0.05$), ** ($p<0.01$).

Therefore, typical IVF-derived GC are luteinized and produce progesterone like their *in-situ* counterparts [36]. Taking this into account, progesterone levels were measured. No significant changes were observed after CUR or RES addition though (figure 3.22).

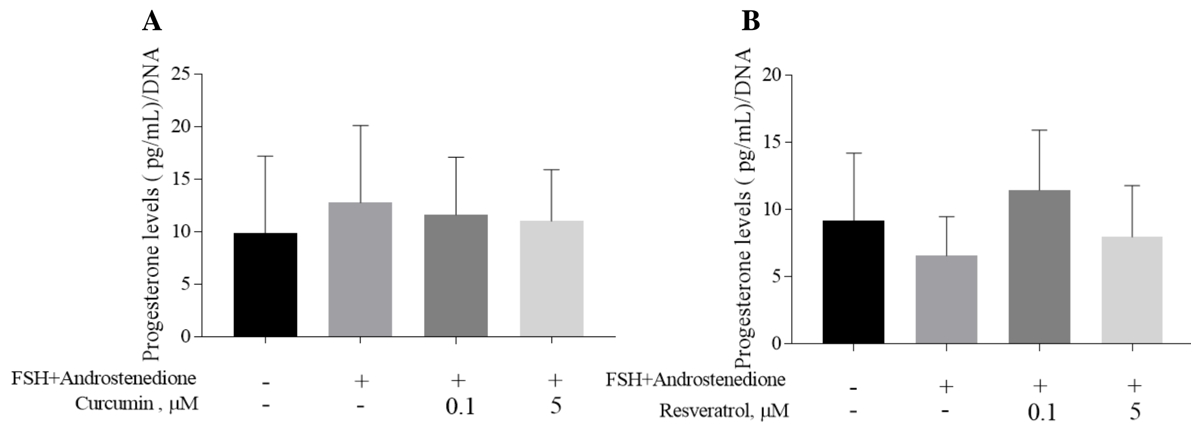

Figure 3.22- **Effects of CUR and RES in the secretion of progesterone by hGC after 72 hours of treatment**. Significant differences between control and treated cells are denoted as * (p<0.05), ** (p<0.01), *** (p<0.001) and **** (p<0.0001).

Transcription levels of StAR, CYP11A1, 3β-HSD and CYP19A1 were measured by qPCR, using cDNA samples from primary hGC treated with CUR and RES for 72 hours.

Evaluation of mRNA levels reveals that CUR at 0.1 μM have no statistical effects in StAR, CYP11A1 and CYP19A1. However, there is a significant decrease on 3β-HSD levels when compared to cells treated with FSH and androstenedione alone (figure 3.23).

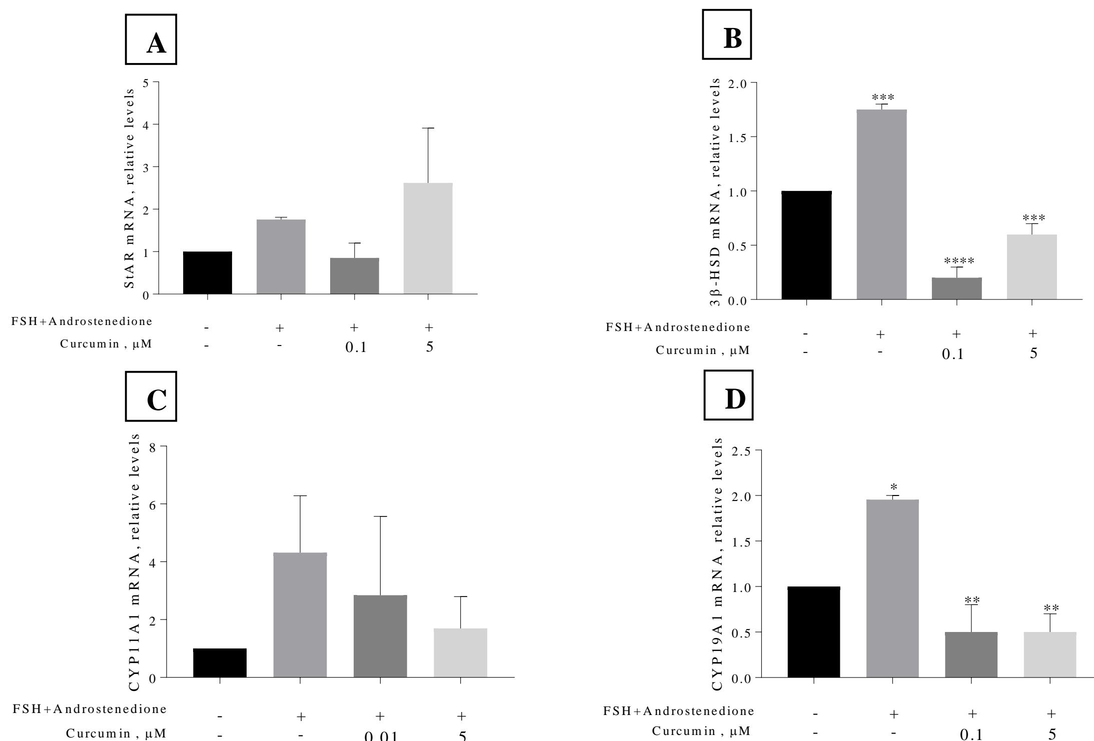

Figure 3.23 - **Effects of CUR on the transcriptional levels of StAR, CYP11A1, 3β-HSD and CYP19A1 after 72h of treatment, on hGC**. Significant differences between cells co-treated with FSH and androstenedione and cells treated with FSH, androstenedione and CUR are denoted as * (p<0.05), ** (p<0.01), *** (p<0.001) and **** (p<0.0001).

In contrast, results following RES treatment suggest a decrease in mRNA levels of StAR, CYP11A1, 3β-HSD and CYP19A1 at 0.1 and 5 μM (figure 3.24).

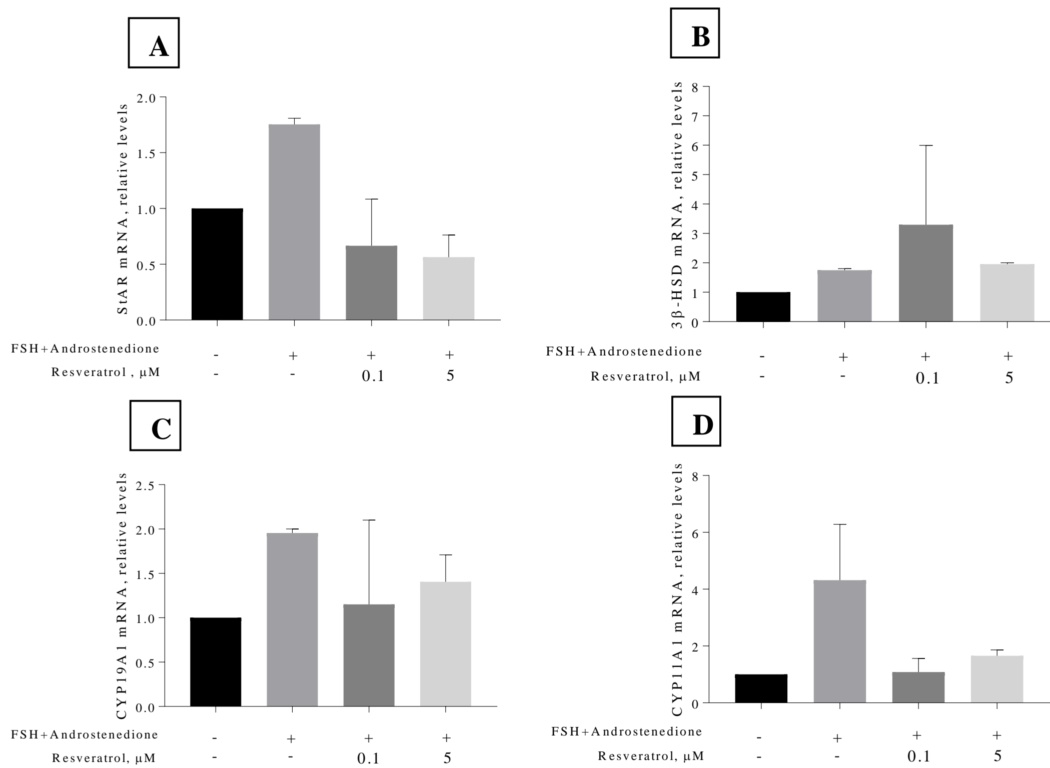

Figure 3.24- **Effects of RES on the transcriptional levels of StAR, CYP11A1, 3β-HSD and CYP19A1 after 72h of treatment, on hGC**. Significant differences between cells co-treated with FSH and androstenedione and cells treated with FSH, androstenedione and CUR are denoted as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and **** ($p<0.0001$).

Chapter 4: Discussion and Conclusion

Female fertility is largely determined by the quality of the oocyte as mirrored by its ability to be fertilized and give rise to a healthy embryo [37]. GC are known to be critical for ensuring oocyte maturation and maintaining normal hormone levels [38]. There is a bidirectional communication between the oocyte and GC through the movement of small molecules across follicle gap junctions channels [39]. Defects in both type of cells can lead to female infertility and may lead concerned women to a growing use of natural supplements whose uptake has been advertised to improve fertility. Therefore, it seems appropriate to study the effects of natural supplements sold as fertility promoters, as they often balance beneficial and detrimental effects on different types of cells. This study aims to consider the effects of two potent natural antioxidant and anti-inflammatory compounds, CUR and RES, on GC main functions.

According to our study, viability and cytotoxicity assays demonstrate that CUR induces a dual effect. While at lower concentrations there is a slight increase in GCs viability, whereas at higher concentrations, CUR induces necrosis since we have found that cell viability decreases and LDH release increases both in COV434 and primary hGC. Between the concentrations of 1 and 5 μM, cell viability was found to be lower without significant LDH release, which can probably indicate an apoptotic mechanism. However, Höechst staining did not clearly demonstrate a nuclear condensation or fragmentation, indicative of apoptosis, although a reduction in mitochondrial potential was observed. This was further explored by measuring caspase -3/7, -9 and PARP-1 activities. Concerning COV434 cells, an accumulation of total PARP-1 could be observed, but there was no presence of cleaved-PARP-1. The process of PARP cleavage is highly dependent on caspase activity. These results were in line with those obtained with measurements of caspase -3/7 and -9 activities, which did not present significant differences. On the contrary, primary hGC showed an increase in caspase 9 activity, suggesting that a mechanism of programmed cell death was occurring. The activities of caspases -3/7 did not change, but this may result from conditions in our model, prompting us to suggest the need for further studies, particularly regarding exposure time.

Several studies have shown that CUR has two different effects, depending on its concentration. It has been called a hormetic compound [40], since hormesis is a term given to phenomena characterized by a high-dose inhibition and low-dose stimulation [40]. On porcine granulosa cells CUR induces inhibitory effects on proliferation and evidence programmed cell death insights as caspase activation [41]. Also, Vashisht *et al* described the loss of cell viability when using CUR at 50 μM in buffalo granulosa cells, but the mechanism of cell death has not been investigated [42].

Reactive oxygen species were also analyzed, since their accumulation can lead to oxidative stress, reduction in oocyte quality and promotion of GC apoptosis [43]. Our experiments suggest that, after 72 hours, ROS basal levels of GC remain the same after incubation with the turmeric extract. Surprisingly, when exposed to a stress inducer (TBHP), lower concentrations of CUR show a protective effect on both COV434 and hGC. These outcomes emphasize the antioxidant properties of the phenolic compound and are in line with Lan Li *et al´* experiments using Zearalenone (ZEA) to increase oxidative stress in porcine granulosa cells after CUR pretreatment. Their results show that CUR is effective in reducing the cellular redox balance dysregulation in these cells [44]. Other studies using rats and bovine endothelial cells reached the same conclusion [45-47].

However, even when granulosa cells survive after CUR lower concentrations, there is no guarantee that their function has been preserved. This subject was further explored by analyzing the differences in estrogen and progesterone production and steroidogenesis key genes expression. These experiments

were performed in the presence of androstenedione, the estrogen precursor, and under stimulation of FSH. This combination induces changes in the expression of estrogen-metabolic enzymes in COV434, whereas in hGCs the expression of 3β-HSD, responsible for progesterone formation, and aromatase are significantly enhanced by FSH, as already described [48]. Nonetheless, progesterone levels remained equal to the control as we have not used a progesterone substrate. Low doses of CUR in COV434 (0.01 μM) and primary hGC (0.1 μM) showed no significant effects in estradiol secretion. However, at a higher concentration (5μM) an increase in estradiol secretion was observed. In a follow-up to these results, it was performed RT-qPCR for assessing levels of estrogen metabolic enzymes. After incubation with a low concentration of CUR, no change was found on mRNA levels of CYP11A1, CYP19A1, 3β-HSD and StAR in the cell line. However, at a concentration of 5uM, a decrease in CYP11A1, 3β -HSD and StAR levels was found, followed by a tendency to increase aromatase expression, which explains the increase in estradiol levels. Overall, at lower concentrations, CUR does not induce changes in steroidogenic regulatory enzymes, although at supraphysiological concentrations, CUR interferes with estradiol synthesis.

RES is also often used in nutritional supplements and is known as a potent antioxidant and anti-inflammatory compound. The incubation with different concentrations of RES, on COV434 cell viability, after, does not agree with the results obtained using primary cells, suggesting that primary hGC are less sensitive to this phenolic compound. Also, ROS and mitochondrial membrane potential analysis, together with morphological studies, did not indicate occurrence of apoptosis in the GC. Some studies suggest that RES is involved in autophagy, but the mechanisms underlying cell death remain unclear [49]. Preliminary data of acridine-orange staining in hGC after 48 hours, were also inconclusive. Interestingly, Ortega *et al* showed that the same RES concentrations used in this study, applied to rat ovarian granulosa cells, induce a biphasic effect on DNA synthesis, inhibiting it at higher concentrations. However, morphological studies also reached the same conclusion, as no morphological changes were noted and there were no characteristic evidences of apoptosis like cell shrinkage or chromatin condensation [22]. These findings are also corroborated by Morita *et al* [50]

The antioxidant potential of RES was also evaluated. There were no changes in ROS production using lower or higher doses of RES after 48 hours. However, in line with CUR results, this natural compound also seems to protect both granulosa cell models from stress induction. Accordingly, a study conducted by Kolesarova and collaborators using porcine granulosa cells, suggested that toxicity induced by deoxynivalenol is inhibited by RES, proposing a protective effect by this natural compound [51].

Finally, regarding steroidogenic function, our results indicate that are no changes in estradiol and progesterone levels at lower concentrations. However, there is an increase in estradiol production at 5 μM, which is in agreement with previous reports of RES estrogenic function, in COV434 and hGC [52-54]. Concerning cell line RT-qPCR results, after cells incubation with RES, the expression of all studied genes were observed to decrease. On the other hand, on primary hGC, aromatase and 3β-HSD remained unchanged. Taken together, these results suggest that RES may decrease the steroidogenic function of GC.

In agreement with our findings, several studies in the past demonstrated inhibitory effects of RES on steroidogenesis. In rat ovarian granulosa cells, there is a decrease in aromatase expression and estrogen production, without affecting progesterone synthesis [22].In contrast, Morita *et al* study, show an increase in StAR and aromatase levels and an increase in progesterone secretion, using RES in the same cells´ type [50]. Another study using the swine granulosa cell model, demonstrated that RES analogues

may promote steroidogenesis [55]. The lack of consistent results regarding RES role in GC steroidogenesis, requires caution and further studies on its effect on GC, being this study the first to our knowledge that uses primary hGC.

In recent years, CUR and/or RES have been widely studied as they have demonstrated antioxidant, anti-inflammatory and apoptotic functions. Most of these studies are associated with brain and age-related disorders However, due to their low bioavailability, a lot of pharmaceutical technologies have been developed to encapsulate these compounds, increasing their plasma values [56]. Also, combination with other natural compounds, such as piperine, have been correlated with increased bioavailability of both compounds. Although not investigated in this study, the combination of CUR with RES was also addressed in other models, namely astrocytes, microglia, cancer cell lines and inflammation processes [11, 57, 58]. Again, the results point to some level of protection induced by both compounds.

In conclusion, this study suggests that moderate consumption of CUR or RES supplements may promote oocyte quality. However, when in presence of ADDS or piperine, the bioavailability of both compounds increase, which may negatively impact GC function, since higher concentrations of CUR and RES appear to lead to decreased cell viability and compromise steroidogenic function. To our knowledge, this is the first study that has been conducted on the effects of CUR and RES on primary hGC. Further studies are required to support our findings.

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Attachment A

TERMO DE CONSENTIMENTO INFORMADO

Projeto de investigação: *Novos mediadores lipídicos como pot6enciais biomarcadores de qualidade oocitária e embrionária.*

Eu, abaixo-assinado, fui informado de que o Projeto de Investigação acima mencionado se destina ao estudo de potenciais biomarcadores da qualidade oocitária e embrionária. Também fui informado que a investigadora responsável por este estudo é a Prof. Doutora Irene Rebelo da Faculdade de Farmácia da Universidade do Porto (irebelo@ff.up.pt).

Sei que é pretendida a colheita de líquido folicular, meio de cultura dos embriões e células da granulosa (material biológico rejeitado), tendo-me sido explicado o seu propósito e que esta colheita não coloca em risco o meu estado de saúde ou a qualidade dos meus embriões. Sei que as amostras vão ser armazenadas e posteriormente analisadas na Faculdade de Farmácia da Universidade do Porto, identificadas por um código especificamente criado para este estudo, constituído de modo a não permitir a imediata identificação do participante, sendo eliminado 5 anos após o fim do estudo.

Foi-me garantido que todos os dados de identificação dos participantes neste estudo são confidenciais e que será mantido o anonimato. Sei que posso recusar ou interromper a participação no estudo, sem qualquer tipo de penalização por este facto. Compreendi a informação que me foi dada, tive oportunidade de fazer perguntas e as minhas dúvidas foram esclarecidas.

Aceito participar de livre vontade no estudo acima mencionado e concordo que sejam efectuadas as colheitas das amostras referidas, para a realização das análises que fazem parte deste estudo. Também autorizo a divulgação dos resultados obtidos no meio científico.

Data:______/______/___________

Nome da/das/dos Participante(s) no estudo	Assinatura
______________________________________	_________________________
______________________________________	_________________________

Nome do Médico Responsável ou Nome do Investigador Responsável	Assinatura
______________________________________	_________________________