Élisa Fonseca

ANTI-AGING EFFECT OF UROLITHIN A ON BOVINE OOCYTES

VOLUME 1

Dissertação no âmbito do Mestrado em Investigação Biomédica orientada pela Professora Doutora Ana Teresa Moreira Almeida Santos e Professora Doutora Rosa Maria Lino Neto Pereira e apresentada à Faculdade de Medicina da Universidade de Coimbra.

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Resumo

O declínio da capacidade reprodutiva feminina é uma das primeiras funções fisiológicas adversamente afetadas pelo envelhecimento. Este declínio é o resultado da diminuição da reserva e da qualidade dos oócitos. Embora os mecanismos subjacentes ao envelhecimento do gâmeta feminino não sejam claros, as alterações epigenéticas e a disfunção mitocondrial podem estar relacionadas com o declínio da qualidade do oócito. A Urolitina A (UA) é um metabolito natural com efeitos pró-apoptóticos e antioxidantes, que foi identificado por prevenir a acumulação de mitocôndrias disfuncionais durante o envelhecimento em diferentes células, por indução da mitofagia.

Este estudo teve como objetivo testar a hipótese de que o envelhecimento do gâmeta feminino altera o potencial de desenvolvimento dos complexos cumulus-oócitos (COCs) e a expressão das células da granulosa (GCs) de genes importantes relacionados com a competência reprodutiva. Um outro objetivo foi avaliar o efeito da UA em COCs e GCs nomeadamente na expressão dos genes *NFE2L2*, *NQO1* e *mt-ND5*, bem como na qualidade dos oócitos.

Foram avaliados a progressão da maturação nuclear, o potencial de membrana mitocondrial (MMP), a taxa de consumo de oxigénio (OCR) e a competência de desenvolvimento de oócitos fisiologicamente maturados e envelhecidos *in vitro* obtidos de fêmeas pré-púberes e adultas, suplementados ou não com UA. Além disso, a quantidade de mRNA de vários genes (*NFE2L2*, *NQO1* e *mt-ND5*) e o número de cópias de DNA do *mt-ND5* foram quantificados em GCs em cultura de fêmeas pré-púberes e adultas, suplementadas ou não com UA.

O nosso estudo confirmou o efeito prejudicial do envelhecimento de oócitos na progressão da maturação nuclear, MMP, competência para o desenvolvimento e níveis de expressão génica. O tratamento com UA durante a maturação *in vitro* aumentou ($P \leq 0,05$) a taxa de maturação e a subsequente capacidade de desenvolvimento dos oócitos envelhecidos. Também foi identificado um efeito positivo ($P \leq 0,05$) da UA na maturação fisiológica, MMP e desenvolvimento embrionário. Além disso, num teste preliminar foi identificado um aumento na

OCR (mais de duas vezes) em COCs tratados com UA em comparação com o controlo. A UA também interferiu no perfil de expressão dos genes *NFE2L2* e *NQO1* de GCs em cultura.

Os nossos resultados demonstraram que a suplementação de UA durante a maturação é uma forma eficaz de prevenir o envelhecimento de oócitos e incrementar o desenvolvimento embrionário bovino subsequente. Assim a UA pode ter uma potencial aplicação no controlo do envelhecimento de oócitos em outras espécies, incluindo os humanos, especificamente na manutenção da qualidade de oócitos e melhorando a taxa de sucesso das tecnologias de reprodução assistida.

Palavras-chave

- Envelhecimento do oócito
- Disfunção mitocondrial
- Urolitina A
- Nrf2
- Saúde reprodutiva

Abstract

Decline in female reproductive capacity is one of the first physiological functions adversely affected by aging. This decline is a result of decreased oocyte reserve and quality. Although the mechanisms underlying oocyte aging are unclear, epigenetic changes and mitochondrial dysfunction may be related to the decline of oocyte quality. Urolithin A (UA), a natural metabolite with pro-apoptotic and antioxidant effects, has been identified to prevent the accumulation of dysfunctional mitochondria with age in different cells, by inducing mitophagy.

This study aims to test the hypothesis that oocyte aging alters cumulus-oocyte-complexes (COCs) developmental potential and granulosa cells (GCs) expression of important genes related to reproductive competence. A further objective was to test the effect of UA on COCs and GCs evaluating its effect on *NFE2L2, NQO1*, and *mt-ND5* genes expression as well as on oocyte quality.

Nuclear maturation progression, mitochondrial membrane potential (MMP), oxygen consumption rate (OCR) and developmental competence of physiologically mature and *in vitro* aged oocytes obtained from prepubertal and adult females, supplemented or not with UA were assessed. Additionally, the amount of mRNA of several genes (*NFE2L2, NQO1*, and *mt-ND5*) and the number of *mt-ND5* DNA copies were quantified in cultured GCs from prepubertal and adult females, supplemented or not with UA.

Our study confirmed the harmful effect of oocyte aging on the nuclear maturation progression, MMP, developmental competence and gene expression levels. UA treatment during *in vitro* maturation enhanced ($P \leq 0.05$) the maturation rate and subsequent developmental capacity of aged oocytes. A positive effect ($P \leq 0.05$) of UA on physiological maturation, MMP and embryonic development was also identified. Additionally, an increase in the basal OCR (more than two-folds) of UA treated COCs compared to control was identified in a preliminary test. UA also interfered on the expression profile of *NFE2L2* and *NQO1* genes in GCs cultures.

Taken together, our findings demonstrate that UA supplementation is an effective way to prevent oocyte aging and improve the subsequent bovine embryonic development. UA may have a potential application in the control of oocyte aging in other species, including humans, specifically to maintain oocyte quality and improve the success rate of human assisted reproductive technologies.

Key-words

- Oocyte aging
- Mitochondrial dysfunction
- Urolithin A
- Nrf2
- Reproductive health

Index

Image Index

Table Index

Abbreviations

•OH - Hydroxyl radical

AGEs - Advanced glycation end products

AI - Anaphase-I

ARE - Antioxidant response element

ART - Assisted reproductive technology

ATP - Adenosine triphosphate

BSA - Bovine serum albumin

Ca^{2+} - Calcium

CCI - Condensing chromosomes I

CCII - Condensing chromosomes II

CCs - Cumulus cells

cDNA - Complementary DNA

COCs - Cumulus-oocyte complexes

COX2 - Cytochrome c oxidase subunit II

COX3 - Cytochrome c oxidase subunit III

Dnmt - DNA methyltransferase

Drp1 - Dynamin-related protein 1

ETC - Electron transport chain

ETs - Ellagitannins

FBS - Fetal bovine serum

FDA - Food and Drug Administration

FSH - Follicle stimulating hormone

GCLC - Glutamate-cysteine ligase Catalytic Subunit

GCs - Granulosa cells

GSH - Glutathione

GV - Germinal vesicle

H_2O_2 - Hydrogen peroxide

HMOX1 - Heme oxygenasse-1

IVF - *In vitro* fertilization

JC-1 - 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide

Keap1 - Kelch-like ECH-associated protein 1

MII - Metaphase-II

MMP - Mitochondrial membrane potential

MT-ATP6 - ATP synthase 6

mtDNA - Mitochondrial DNA

mt-ND - Mitochondrially encoded NADH dehydrogenase

NADH - Nicotinamide adenine dinucleotide reduced

***NFE2L2* or NRF2** - Nuclear factor-E2-releated factor 2

NF-*K*b - Factor nuclear factor kappa B

NQO1 - NAD(P)H:quinone-oxidoreductase-1

$O2^-$ - Superoxide

OCR - Oxygen consumption rate

OXPHOS - Oxidative phosphorylation

PBS - Phosphate buffered saline

PINK-1 - Putative kinase protein 1

RAGE - Receptor for advanced glycation end products

ROS - Reactive oxygen species

RT-qPCR - Quantitative Reverse-transcription polymerase chain reaction

SAC - Spindle assembly checkpoint

Sdha - Succinate dehydrogenase complex flavoprotein subunit A

SOD - Superoxide dismutase

TI - Telophase-I

TRAMP - Transgenic Adenocarcinoma of Mouse Prostate

UA - Urolithin A

UB - Urolithin B

UC - Urolithin C

UD - Urolithin D

Chapter 1 – Introduction

1.1. Maternal age and oocyte competence

In the last decades, the proportion of women delaying motherhood has progressively increased, especially in industrialized countries (Keurst, Ter, Boivin and Gameiro, 2016). In fact, the latest Portuguese data obtained by PORDATA showed an increase in the average age of women giving birth to the first child, since the 80's. While in 1980 the mean age of women experiencing childbearing for the first time was 23.6 years, in 2019, the mean age has increased to 30.5 years (Figure 1, Data from INE, PORDATA, 2020). Nowadays, changes in women's lifestyles resulted in different behaviors associated with female reproduction, such as the delay of pregnancy until their thirties, when reproductive difficulties begin. This increasing trend is related to better contraceptive methods, social, personal, career and education pressures (Keurst, Ter, Boivin and Gameiro, 2016; Reviewed in Fritz and Jindal, 2018), and also to the wrong belief that assisted reproductive technology (ART) can reverse the natural decline of fertility associated with aging (O'Brien and Wingfield, 2019). The use of these techniques and their growing popularity associated with the misconception that women can manipulate the biological clock regardless of age, has given false hopes that ART will be successful until menopause (Wyndham, Figueira and Patrizio, 2012).

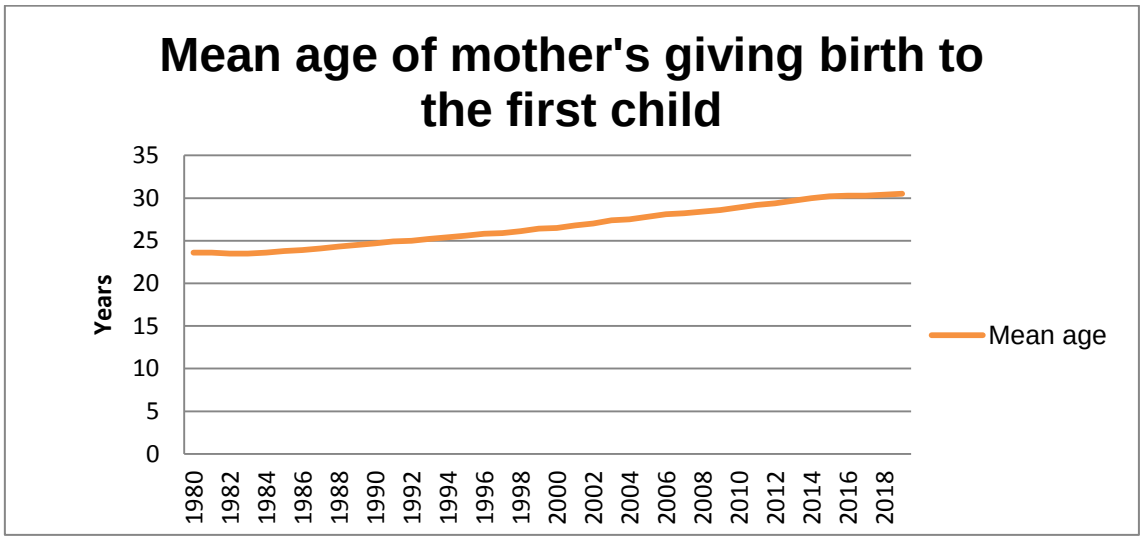

Figure 1 - Mean age of mother's giving birth to the first child since 1980 until 2019 in Portugal (Data from INE, PORDATA, 2020).

In reproductive health, the success of reproduction is closely associated to the age at which women attempt to conceive. It is well-documented that the oocyte aging process is strongly related to the decline of fecundity and fertility. This decline is a result of decreased oocyte quantity and quality as well. (Rienzi, Vajta and Ubaldi, 2011; Igarashi, Takahashi and Nagase, 2015). Women above 35 years old who attempt to conceive are more likely to have complications during pregnancy, being considered patients with advanced maternal age (Pinheiro *et al.*, 2019). Indeed, a study focused on the intrinsic fertility rate of women without hormonal manipulation, suggested that the decline of fertility starts at the age of 35 years old (Silber *et al.*, 2017). Few changes in women's fertility were observed until 34 years old. Nevertheless, the natural fertility of human oocytes is very low, even under the age of 34, with an average of 3.8 oocytes required to make a baby, which corresponds to a rate of 26% of a live baby per oocyte (Silber *et al.*, 2017). The data also showed that approximately 10% of fertility is lost every year between 34 and 42 years old, and around 42 years old the remaining oocyte fertility is about 1% (Silber *et al.*, 2017). Additionally, the incidence of miscarriage and chromosomal abnormalities rise on the opposite trend of fertility (Reviewed in Cimadomo *et al.*, 2018). A study evaluating over 15000 embryo trophectoderm biopsies, with chromosomal screening, showed an increased rate in embryo aneuploidy at ages between 35 and 37 years old. However between 43 and 45 years old, the embryo aneuploidy rate drastically increases, approaching 90%. (Franasiak *et al.*, 2014).

Oocytes can be subjected to two types of aging. The reproductive maternal aging, also known as pre-ovulatory aging, is due to the exposure to an aged ovarian microenvironment before ovulation. This process leads to an age-related quality decline of oocytes (Zhang *et al.*, 2017). After ovulation, mature human oocytes only have 24h as an optimal span for fertilization to occur. If not fertilized during the best fertilization period, the unfertilized oocyte remains in the oviduct (*in vivo* post-ovulatory oocyte aging) or in the *in vitro* culture medium (*in vitro* post-ovulatory oocyte aging) and these oocytes undergo a time-dependent degradation process (Zhou *et al.*, 2019b). In ART, oocytes are

frequently subjected to post-ovulatory aging, which leads to chromosomal abnormalities, increased miscarriage, low fertilization and poor embryonic development, as well as observed in pre-ovulatory aging oocytes (Reviewed in Igarashi, Takahashi and Nagase, 2015; Zhang *et al.*, 2019).

Currently, several dysfunctions have been associated to a decreasing capacity of women to get pregnant while aging. Besides the progressive reduction of ovarian reserves associated with aging, dysfunctions in several processes such as energy balance, physiological pathways, metabolism, chromosomal aberrations, and epigenetics, are all factors that compromise the quality of oocytes and embryo development. However, the molecular and biochemical mechanisms underlying the age-related decline in fertility and oocyte competence remain unclear. (Santonocito *et al.*, 2013; Reviewed in Capalbo *et al.*, 2017).

1.2. Oocyte microenvironment

At birth, most of the primordial follicles are already formed, constituting the women oocyte pool. The follicles contain primary oocytes arrested in prophase-I of the meiotic division that remain quiescent until their recruitment to complete oogenesis (McReynolds *et al.*, 2012; Liu, 2016). This recruitment may last around 50 years, and it is a steady process metabolically activated by complex bidirectional signaling between the oocyte and the surrounding somatic cells, the granulosa cells (GCs) (Tatone *et al.*, 2006).

Cumulus cells (CCs) are a differentiate type of GCs, that are at the vicinity of the oocyte, which together form the cumulus-oocyte complexes (COCs) (Liu *et al.*, 2017). These two types of cells undergo bidirectional communications through two mechanisms, gap junctions and paracrine signals (Lourenço *et al.*, 2014). The oocyte and the CCs communicate intercellularly via cytoplasmic projections through the zona pellucida with gap junctions, which are membrane channels constituted by connexins, coupling the two cell types (Read, Willhelm and Dyce, 2018). In the follicle, CCs contact with each other's through gap junctions composed by connexin 43 and also connects with the oocyte mostly

through connexin 37. This intercellular communication allows the transfer of small molecules (ions, small metabolites and signaling molecules), which are crucial for follicular development, oocyte maturation and competence acquisition (Simon, Chen and Jackson, 2006). Oocytes regulate the differentiation and expansion of CCs. In turn, the energy required by the oocyte through the metabolism of glucose and pyruvate is insured by the surrounding CCs (Al-Edani *et al.*, 2014)**.** On the other hand, the paracrine signaling mechanism is essential during oogenesis playing a critical role for follicular growth and maturation (Gilchrist, Lane and Thompson, 2008; Coticchio *et al.*, 2015). This cell-to-cell communication occurs through specific receptors and signaling pathways and is modulated by endocrine factors, such as follicle stimulating hormone (FSH) (Yoon *et al.*, 2015). This type of communication between the oocyte and CCs, allows meiotic resumption, nuclear and cytoplasmic maturation and also transcriptional activity (Reviewed in Broi *et al.*, 2018).

The successful development of oocytes requires intercellular communication in the ovarian follicular microenvironment. These mechanisms allow the oocyte to acquire the necessary competence to complete meiosis and undergo consequent development after fertilization (Al-Edani *et al.*, 2014). Due to the importance of these intercellular communications, studies performed in CCs are considered as a non-invasive approach, which allows a better understanding of the complex relation between oocyte quality and developmental potential and the surrounding somatic cells. Besides, Lourenço and colleagues (2014) showed that simple parameters could be monitored in human CCs, thus constituting a useful tool to determine oocyte quality. Their results showed that the oocytes which were more prone to mature and to be fertilized were surrounded by more CCs and had higher caspase activity in those cells, when compared to degenerated and unfertilized oocytes, respectively (Lourenço *et al.*, 2014). Additionally, it has been demonstrated that mitochondrial DNA (mtDNA) copy number in GCs could predict the oocyte developmental potential, presenting higher copies in those originating embryos with greater probability to implant (Ogino *et al.*, 2016; Taugourdeau *et al.*, 2019).

The impairment of GCs during female reproductive aging was shown to negatively affect the oocyte competence, which leads to a reduced fecundity (Hurwitz *et al.*, 2010). This may be explained by the accumulation of damage in the cells during the quiescent phase or to disruption in the crosstalk within the ovarian microenvironment. It has been proposed that both oocytes and GCs during this quiescent period may be exposed to different factors leading to ovary aging, such as the accumulation of damage by oxidative stress and errors or deletion in the mtDNA (Tatone *et al.*, 2006; Hammond *et al.*, 2016). Mitochondria have been extensively reported as important organelles which directly impact in the ovarian microenvironment alteration with aging (Liu *et al.*, 2017). Some studies in human and cattle GCs have demonstrated correlations between the age-related decline in oocyte quality and mitochondrial dysfunction, such as increased mtDNA damage (Hammond *et al.*, 2016) and decreased mtDNA copies (Boucret *et al.*, 2015; Liu *et al.*, 2017), mitochondrial membrane potential (MMP), adenosine triphosphate (ATP) level and expression of ATP as well as reduced proliferation and telomerase activity, and shortened telomeres in related genes (Liu *et al.*, 2017). Additionally, in mouse ovary, the expression of genes that encode for antioxidants, which are crucial for maintaining ovarian function and fertility have also shown to be differentially altered according to maternal age (Lim and Luderer, 2011).

At an ultrastructural level, Tatone and colleagues (2006) demonstrated that GCs from older patients showed defective mitochondria and more lipid droplets, compared with younger patients. These GCs from older patients suffered oxidative stress injuries due to reduced defenses against reactive oxygen species (ROS) (Tatone *et al.*, 2006). Furthermore, the proteomic profile of GCs of young and older women (McReynolds *et al.*, 2012), pigs (Hui *et al.*, 2017) and cows (Itami *et al.*, 2014) showed to be differentially expressed with advanced maternal age. Epigenetic pattern changes have also been linked to GCs aging, showing lower genomic global DNA methylation, as identified in older cows GCs (Goto *et al.*, 2013). All these results have demonstrated a reduced function of somatic cells surrounding oocytes, through several different mechanisms which contribute to an impaired follicular microenvironment.

1.3. Mitochondrial dysfunction in ovarian aging

Advanced maternal age is the most crucial factor contributing to poor fertility outcomes in women (Klimczak *et al.*, 2018). Among all the factors examined in ovarian aging, mitochondria dysfunction has been reported to play a key role, although this process is more complex than originally postulated (Yamamoto *et al.*, 2010; Babayev *et al.*, 2016). Indeed, mitochondria are involved in several processes, such as energy production, metabolism, calcium (Ca^{2+}) homeostasis, regulation of apoptosis and also in ROS generation (Forkink *et al.*, 2014; Fragouli *et al.*, 2015). Multiple mitochondria impairment events have been associated to aging, such as altered mtDNA content, disturbed function of electron transport chain (ETC) and bioenergetic imbalance, loss of mitochondrial Ca^{2+} homeostasis and altered mitochondrial dynamics (Reviewed in Ziegler, Wiley and Velarde, 2015).

Maintenance of the mtDNA copy number is a crucial process for the proper cell functionality, being that mtDNA is constantly replicated during oocyte maturation. Several studies performed in different species, have established a correlation between mtDNA content and fertility, showing that mtDNA levels in oocytes are inversely associated with maternal age (Iwata *et al.*, 2011; Kushnir *et al.*, 2012). Similarly, a study that quantified mtDNA levels of polar bodies from oocyte biopsies of women with advanced reproductive age, ranging from 38 to 45 years old, have reported the presence of lower mtDNA quantities than those from younger women (Konstantinidis *et al.*, 2014).

The insufficiency in mtDNA copy number per mitochondria, which is associated with increasing age, leads to a decreased ATP generation, since mtDNA encodes genes involved in the functioning of ETC (Simsek-Duran *et al.*, 2013). A recent study has demonstrated that disturbances in mitochondrial functions, including oxidative phosphorylation (OXPHOS) and ATP production pathway are involved in the progression of oocyte aging (Zhang *et al.*, 2019). This study performed in mouse oocytes from different ages showed that several genes expressed in germinal vesicle (GV) oocytes of 32 weeks old mice appear to be down regulated, when compared to oocytes from 5-weeks old mice (Zhang *et*

al., 2019). Most of the genes that were differentially expressed were encoded by the mitochondrial genome and involved in the functioning of ETC, such as nicotinamide adenine dinucleotide reduced (NADH) and mitochondrially encoded NADH dehydrogenase (mt-ND) protein part of the subunit of complex I (Zhang *et al.*, 2019). Among those genes, *mt-ND2, mt-ND3, mt-ND4, mt-ND4L*, *mt-ND5*, mitochondrially encoded ATP synthase 6 (*MT-ATP6*), cytochrome c oxidase subunit II (*COX2*) and subunit III (*COX3*) were down regulated (Zhang *et al.*, 2019). Additionally, Babayev and colleagues (2016) identified differentially expressed genes associated with maternal age, genes that encode for mitochondrial function, as well as lower mtDNA copy number in mouse oocytes. This data revealed an up regulation of genes responsible for ATP production in younger mouse compared to the older ones (Babayev *et al.*, 2016).

It is well known that higher ATP content is associated with high oocyte quality and embryo developmental potential, while lower ATP levels have dramatic effects in most oocyte events (Simsek-Duran *et al.*, 2013). In a study of human embryogenesis, a close relationship between intracellular ATP content and embryo development has been clearly demonstrated. Higher levels of ATP have revealed to be determinant for embryo quality, leading to increased fertility outcomes. (Blerkom, Van, Davis and Lee, 1995). Furthermore, in a post-ovulatory aged mouse model, the Ca^{2+} oscillations occurring during fertilization have been demonstrated to stimulate ATP production. The intracellular ATP content in both fresh and aged oocytes after 5 hours post-fertilization was assessed and showed higher intracellular values in fresh compared to aged oocytes. These findings suggest that aged oocytes failed to readjust the ATP content after fertilization, which leads to a loss in Ca^{2+} homeostasis and a poor developmental potential. (Igarashi *et al.*, 2005). The failure on the ATP production was also related to harmful effects concerning chromosome segregation and embryonic development (Reviewed in May-Panloup *et al.*, 2016). Zhang and co-workers (2006) showed that disruptions in mitochondrial OXPHOS in mouse oocytes, leads to a decreased in ATP levels during oxidative stress, resulting in meiotic spindle abnormalities. Indeed, some

studies have reported that the compromised function of the spindle assembly checkpoint (SAC) was related to reduce ATP production in oocytes. This reduction does not allow the release of dynein, which mediates the transport of kinetochore protein along microtubules, at a time when the SAC should be silenced. (Silva *et al.*, 2014). The proper function of SAC is compromised in both, post-ovulatory aging and oocytes from advanced maternal age, suggesting that SAC failure is a main contributor to increased incidence of chromosome abnormalities and consequently increases the incidence of aneuploidy in early embryos (Marangos *et al.*, 2015; Shimoi *et al.*, 2019).

Morphological and functional patterns changes in mitochondria have also been reported in the process of oocyte aging. In humans and rodents with increased age, mitochondria swelling, vacuolization and cristae alterations were identified (Müller-Höcker et al., 1996; Simsek-Duran et al., 2013). Mitochondrial dynamic adapting the mitochondria size and shape to the bioenergetic requirement have revealed to be crucial for cellular function maintenance. A study in oocytes from dynamin-related protein 1 (Drp1) mitochondrial fission knockout mice showed the importance of this protein during follicular maturation and ovulation, where very few oocytes have been recovered after superovulation. Oocytes from aged mice also showed a decrease in Drp1, being suggested that Drp1 might have a critical role in mice oocyte competence. (Udagawa et al., 2014). Furthermore, studies using post-ovulatory (Lord, Martin and Aitken, 2015; Liang *et al.*, 2017) and aged models have demonstrated an age related loss in the MMP, which reflects mitochondrial activity (Pasquariello et al., 2019).

1.4. Sources of Reactive Oxygen Species in the oocyte

One hallmark of the aging cell is an increase in oxidative damage caused by ROS. The increased production of free radicals has been mainly related to aging in several chronic diseases and also in reproductive biology affecting ovaries, which in turn contributes to the decline in oocyte quality (Perkins et al., 2019; Yu et al., 2019).

The etiology underlying increased ROS production in mammalian ovary associated to age is a multifactorial process (Reviewed in Peters et al., 2020). ROS can present different forms such as superoxide ($O2^{-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH), all predominantly produced in the mitochondria as by-products of the energy metabolism. Because of their high reactivity, they can interact with other molecules, and acquire electrons from nucleic acids, proteins, lipids and cell structures, such as cell membranes (Amin et al., 2014). Besides intrinsic factors, there are several additional sources of exogenous ROS generated within the intra-ovarian microenvironment (Rodrigues-Cunha *et al.*, 2016), influenced by the women's lifestyle (Reviewed in Peters et al., 2020) or produced during the *in vitro* culture processes that can contribute to cellular aging (Sovernigo *et al.*, 2017). An age-associated increase in the amount of advanced glycation end products (AGEs) in the ovary was related to ovarian aging. AGEs can promote the generation of ROS by binding to receptors for advanced glycation end products (RAGE) that in turn triggers the activation of NAD(P)H oxidase, and the transcription factor nuclear factor kappa B (NF-*κ*B) (Takeo *et al.*, 2017; Reviewed in Mengden, Klamt and Smitz, 2020; Reviewed in Peters et al., 2020). Furthermore, COCs can also be exposed to other potential sources of ROS in ART. This occurs during *in vitro* fertilization (IVF) procedures, since COCs are in culture, subjected to variations in oxygen pressure, exposure to visible light wavelengths, pH and composition of culture media (Marques *et al.*, 2018; Reviewed in Mengden, Klamt and Smitz, 2020).

Paradoxically, mitochondria are highly susceptible to ROS, which can lead to multiple damages within this organelle. Indeed, ROS can induce damages in mtDNA that encode ETC components, due to a lack of histone protection, antioxidant defenses and efficient mechanisms of DNA repair (Hammond *et al.*, 2016). According to these facts, it has been demonstrated an age related decrease expression of some genes that encode for ETC, such as Succinate dehydrogenase complex flavoprotein subunit A (Sdha) (Hamatani *et al.*, 2004) and coenzyme Q10 (Ben-Meir *et al.*, 2015). High generation of free radicals has also been reported to have negative impact on oocytes, which results in

increased apoptosis, embryo fragmentation (Lord, Martin and Aitken, 2015), changes in the expression of transcription factors involved in early embryo development (Leite *et al.*, 2017; Wang *et al.*, 2017), lipid accumulation (Sudano *et al.*, 2011), reduction in the efficacy of lysosomal degradation (Reviewed in Peters *et al.*, 2020) and alteration in epigenetic patterns (Cui *et al.*, 2011), contributing all together to poor oocytes maturation and consequent low embryo development, quality and viability.

During women aging, oocytes experience an increase in ROS generation and exposure, as well as an associated decrease in the efficiency of mechanisms to neutralize ROS through antioxidant defenses, contributing to an increased level of oxidative stress (Mukherjee *et al.*, 2014; Reviewed in Mihalas *et al.*, 2017). This misbalance provoked by age does not only happen in the oocyte, but it is also characteristic of the intra-ovarian environment, both adversely affecting the oocyte quality and subsequent fertilization process (Lim and Luderer, 2011). Indeed, this antioxidant deregulation has been detected in aged human GCs (Tatone *et al.*, 2006), mouse GCs (Lin *et al.*, 2020), bovine GCs (Itami *et al.*, 2014), and human follicular fluid (Terao *et al.*, 2019).

1.5. Antioxidants defense mechanisms

Antioxidant mechanisms exist in all organisms and play a vital role in the regulation of oxidative environments and in the maintenance of the oxidant/antioxidant balance (Amin et al., 2014). Several strategies have been used to protect oocytes against oxidative stress, including the supplementation of compounds with antioxidant activity and the activation of mechanisms to enhance the expression of cytoprotective genes in order to reduce ROS production (Truong and Gardner, 2017; Akino *et al.*, 2019; Cavallari *et al.*, 2019). Both approaches revealed to be effective in protecting the oocytes from the deleterious effects of oxidative stress, resulting in an improved oocyte quality, ovarian function (Akino *et al.*, 2018; Soto-Heras *et al.*, 2018; Yu *et al.*, 2019) and a delay in the oocytes aging process (Song *et al.*, 2016; Yao *et al.*, 2018; Jiang *et al.*, 2019).

Analyses of the pathways affected during altered culture conditions identified the nuclear factor-E2-related factor 2 gene (*NFE2L2*, also known as Nrf2) pathway as the dominant response cascade activated by oxidative stress (Gad *et al.*, 2012). This pathway is the cellular defense mechanism against ROS that cells have developed to cope with deleterious effects of oxidative stress (Ma *et al.*, 2018). Nrf2 is a transcription factor that regulates about 250 genes involved in cellular homeostasis, such as antioxidant proteins, detoxifying enzymes and several cytoprotective proteins (Reviewed in O'Connell and Hayes, 2015). Under normal conditions, Nrf2 is negatively regulated by Kelch-like ECH-associated protein 1 (Keap1) and sequestered in the cytoplasm binding to it. Keap1 in combination with Cullin-3 and RING-box protein 1 forms a complex that enables the Nrf2 degradation through the proteasome, in a process mediated by ubiquitin (Figure 2). When exposed to oxidants, Nrf2 is released due to the oxidation of the cysteine residues within Keap1, allowing its translocation and accumulation in the nucleus (Su *et al.*, 2015). In the nucleus, Nrf2 binds to small Maf macromolecules, forming a complex that targets specific DNA sequences, named antioxidant response elements (ARE), which lead to transcriptional activation of cytoprotective genes, such as NAD(P)H:quinone-oxidoreductase-1 (*NQO1*), heme oxygenasse-1 (*HMOX1*), and glutamate-cysteine ligase catalytic subunit (*GCLC,* Sindan *et al.*, 2018; Cheng *et al.*, 2019).

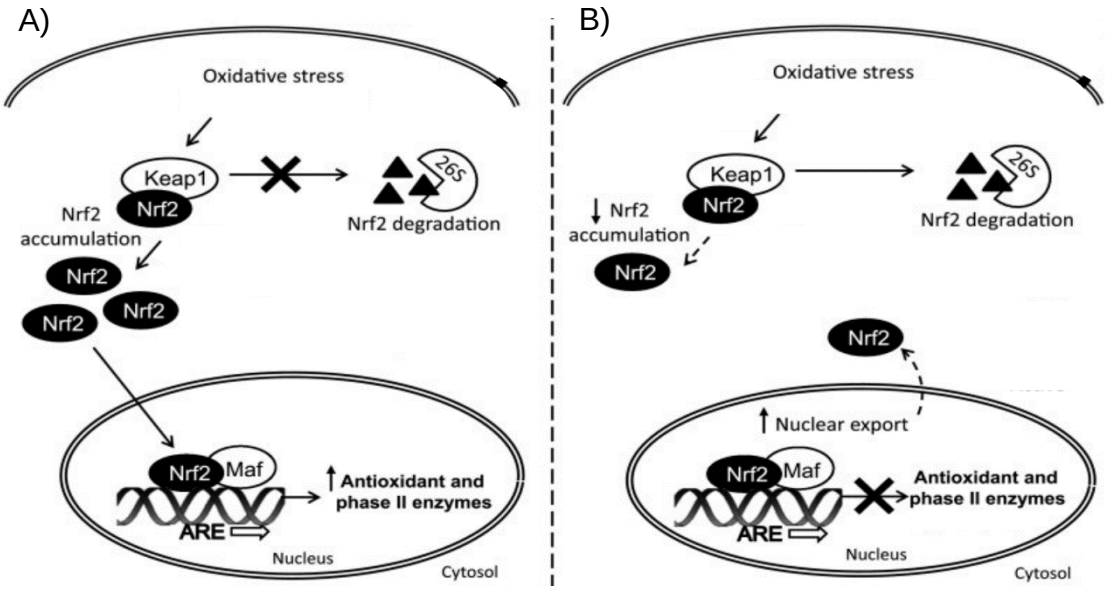

Figure 2 - Regulation of Nuclear factor-E2-related factor 2-Kelch-like ECH-associated protein 1 (Nrf2-Keap1) pathway. A) Oxidative modification in Keap1 promotes Nrf2 accumulation and

translocation into the nucleus, where Nrf2 form a complex with Maf macromolecules and binds to antioxidant response elements (ARE) in the promoter regions of various detoxification, antioxidant and anti-inflammatory genes; B) Under baseline conditions Nrf2 is sequestered by Keap1 for subsequent ubiquitination and proteosomal degradation of Nrf2 (Adapted from Cheng et al., 2013).

Some studies have demonstrated the deleterious effect of deficient Nrf2, associating it with an increased prevalence of age-related diseases, namely skeletal muscle loss (Ahn *et al.*, 2018) and cardiovascular disorders (Shanmugam *et al.*, 2017), combined with shortened life expectancy. The same has been identified in reproductive biology. Indeed, Nrf2 protein was revealed to be predominantly expressed in GCs, as well as in the secondary and antral follicles. An age-dependent decrease of Nrf2 expression in both oocytes and GCs from aged mice was also shown (Ma *et al.*, 2018; Sindan *et al.*, 2018). Moreover, Lim and co-workers (2015) demonstrated that ovarian concentrations of glutathione (GSH) and protein levels of GCLC were reduced in Nrf2 -/- mice females, compared to Nrf2 +/+ females (Lim *et al.*, 2015). These authors also studied the impact of Nrf2 lacking in the ovarian reserve associated to aging. No differences have been found in 35 day old mice between Nrf2-/- and Nrf2+/+ females, but Nrf2-/- females of middle age (10-12 month old) revealed significantly fewer healthy primordial follicles compared to Nrf2+/+ females. These results show that Nrf2 deficiency in mice females has a negative impact on ovarian reserve, leading to a decline in the number of follicles, consistent with accelerated ovarian aging. Besides the implication of Nrf2 in the aging process, Ma and co-workers (2018) also reported the Nrf2 importance in some biological functions during oocyte maturation. A reduction in the first polar body extrusion, combined with spindle defects and chromosome misalignment in oocytes from Nrf2-knockdown females was identified, indicating that Nrf2 is essential to a proper meiotic division (Ma *et al.*, 2018).

Additionally, Lewis and colleagues (2015) found that the regulation of the Nrf2 signaling pathway was closely related to species longevity and health span. The up regulation of this pathway in human GCs and mouse ovaries revealed to be a good strategy to elevate levels of antioxidants and consequently decrease the oxidative stress damage. These results corroborate the importance of the Nrf2

regulation in ovary aging in order to obtain better pregnancy outcomes in aged woman, and restore the age-related fertility decline (Akino *et al.*, 2018, 2019).

1.6. Epigenetic changes in aging oocytes

Epigenetic is the study that explains hereditable alterations in the expression of DNA without any alteration in the DNA sequence (Marshall and Rivera, 2018). DNA methylation is a biochemical process in which occurs the addition of a methyl group on the 5-carbon of cytosines, catalysed by DNA methyltransferases (Dnmts) to form 5-methyl cytosine. Generally, the DNA is methylated at regions with high frequency of cytosine followed by guanine, named CpG islands that correspond to 1-2% of the genome (Reviewed in Menezo *et al.*, 2016). This epigenetic mechanism is often related to transcriptional repression, since 60% of methylated genes within promoters enriched with CpG islands are silenced, due to the lack of recruitment of proteins such as RNA polymerase II (Reviewed in Silva-Palacios *et al.*, 2018). Moreover, reversing epigenetic patterns seems to have a great potential for novel therapeutic development approaches in order to delay aging and associated diseases (Pal and Tyler, 2016).

Recent studies showed that GCs and aged oocytes have further identified age-related epigenetic marks. During ageing, the DNA methylation pattern of these cells changed, detecting decreased Dnmts activity. (Iwata *et al.*, 2011; Uysal and Ozturk, 2020). Yue and colleagues (2012) studied the genome-wide DNA methylation in mice oocytes during maternal aging and found a significant lower DNA methylation in the group of old mice (35-40 weeks old) when compared with the young group (6-8 weeks old). It has also been observed a significantly lower level of DNA methylation in the 2-cell, 4-cell, 8-cell and morula embryos originated in the older group. In addition, the expression of Dnmt showed a decrease, both at transcript levels of Dnmt1s, Dnmt1o, Dnmt3L (Hamatani *et al.*, 2004), Dnmt3a, Dnmt3b (Battaglia *et al.*, 2016) and proteins of Dnmt1, Dnmt3a, Dnmt3b and Dnmt3L (Yue *et al.*, 2012) in MII oocytes from the aged

group (42-45 weeks old, 36 weeks old and 35-40 weeks old, respectively) compared to the young group.

Maternal age also show to influence the Dnmt location inside the oocyte. In *in vitro* GV and MII oocytes from young mice females (7-8 weeks old), Dnmt3a and Dnmt3b have revealed to be located near the chromosomes, during both stages. On the contrary, in oocytes from older mice females (40-47 weeks old) both proteins were located in the cytoplasm. Although an alteration in the localization of Dnmt3a and Dnmt3b have been related with aging, Dnmt1 itself does not seem to be affected by age. These results suggest that the cytoplasmic-to-nuclear trafficking of Dnmt is affected by maternal aging, preventing both proteins to access the chromosomes during meiosis, which may lead to aberrant methylation, female infertility and ovarian aging (Zhang *et al.*, 2011; Uysal and Ozturk, 2020).

As previously mentioned, the Nrf2 signaling pathway is the major regulator of cytoprotective responses to oxidative and electrophilic stress, playing an important role in oocyte maturation, embryo development and foetal and placental growth. Recent reports revealed that epigenetic modifications play also a critical role in several pathologies, such as cancer (Zhou *et al.*, 2019a), neurodegenerative diseases (Zhao, Zhang and Chang, 2018), cardiovascular disorders (Yan et al., 2018) and others age related diseases (Periyasamy and Shinohara, 2017). According to these studies, evidences indicate that the Nrf2 pathway have a crucial role and can be epigenetically regulated and also provide a potential target to treat and prevent diseases.

For instance, Yu and colleagues (2010) found that the expression of Nrf2 was regulated by different DNA methylation patterns in several prostate tumors of Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) cell lines. Indeed, it has been showed a hypermethylation of the first 5 CpG sites within the Nrf2 promoter in this tumorigenic prostate cell line but not in normal prostatic tissue. This hipermethylation was correlated to the suppression of the Nrf2 expression as well as to the induced NQO1 gene expression. Furthermore, it has been observed that the hypermetylated tumorigenic prostate cell line when treated

with DNMT inhibitor 5-aza and HDAC inhibitor TSA could restore Nrf2 expression and NQO1 induction. Similar results have been identified in another study that reported a hypermethylation on three CpG sites of the Nrf2 promoter in human prostate cancer (Khor *et al.*, 2014). However, in gestational diabetic fetal umbilical vein endothelial cells, no significant differences in CpG methylation in the Nrf2 promoter was found compared with normal cells (Cheng *et al.*, 2013). Indeed, although a myriad of recent studies has revealed the broad range of contributions of the Nrf2-Keap1 system to physiological and pathological processes (Matsumaru and Motohashi, 2020), its functions during gametic and embryonic development are still open for further investigation. For instance, in bovine GCs, endogenous and exogenous epigenetic post-transcriptional Nrf2 modulation (through MicroRNAs), mediated oxidative stress response with potential implications for ovarian function (Khadrawy *et al.*, 2019). Moreover, the repercussion of epigenetic regulation of Nrf2-Keap1 signaling in female fertility and maternal aging remains to be deciphered.

1.7. Urolithin A

Natural products have historically been an extremely productive source for new medicines and continue to deliver a great variety of structural templates for drug discovery and development, including in the reproductive field (Krause, 2013). The pomegranate fruit is a rich source of polyphenol compounds as ellagitannins (ETs) and ellagic acid, with antioxidant properties (Kim, Lee and Kim, 2020). These natural compounds can also be found in other fruits and nuts, such as strawberries, raspberries, blackberries, muscadine grapes, walnuts, hazelnuts, acorns, chestnuts and pecans (Tomás-Barberán *et al.*, 2014; Heilman *et al.*, 2017). The beneficial properties of the ingestion of these foods seem to be due to the conversion of ETs on metabolites. ETs are initially hydrolyzed, leading to the release of ellagic acid in the gut, which is further converted by the microflora into Urolithins through the loss of one of its two lactones and by successive removal of hydroxyl groups (García-Villalba *et al.*, 2019). Urolithins have been found in several forms as redundant metabolites, including Urolithin A (UA), Urolithin B (UB), Urolithin C (UC) and Urolithin D

(UD). Among these, UA (Figure 3) is the most common form in humans (Kang *et al.*, 2016). However, the efficiency of the metabolism of ETs appears to be variable across individuals, who have showed lower or higher or even none amounts of Urolithins in the organism (Tomás-Barberán *et al.*, 2014). Indeed, only 1 in 3 people are carriers of the right microbiota capable of producing the final Urolithin metabolites with maximum efficiency (García-Villalba *et al.*, 2017).

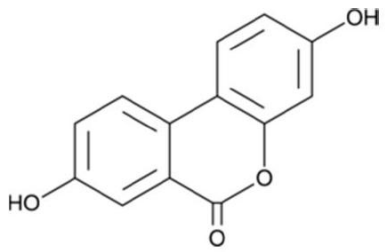

Figure 3 - Structure of Urolithin A (3,8-dihydroxyurolithin, Cásedas *et al.*, 2020).

Due to the decline of age-associated mitochondria function in several pathologies, a considerable interest in therapeutic approaches that enhance mitochondrial function through the stimulation of its biogenesis and respiratory chain has been demonstrated. However just a few promoters of mitophagy, the selective degradation and removal of damaged mitochondria by autophagy, have been described yet. A few years ago, UA received the GRAS (generally recognized as safe) designation from the Food and Drug Administration (FDA), after being tested in a series of toxicological tests, as well as subchronic exposure in rodents models during 90 days (Heilman *et al.*, 2017; Harrigan *et al.*, 2018; Andreux *et al.*, 2019). More recently, UA has been tested for the first time in a human clinical trial, being regularly consumed orally by sedentary elderly patients, which has shown that UA induces a molecular signature, in both the plasma and skeletal muscle, improving mitochondrial and cellular health (Andreux *et al.*, 2019).

In a study conducted by Ryu and co-workers (2016), UA has been identified as a first-in-class food metabolite with the capacity to prevent the accumulation of dysfunctional mitochondria with age, by inducing mitophagy and also

maintaining mitochondrial biogenesis and respiratory capacity. Furthermore, UA treatment of the nematode *Caenorhabditis elegans* at advanced aged has showed signs of improved mobility and also extended lifespan. Similarly, in rodents, UA improved endurance capacity in old mouse model presenting age related decline of muscle function, as well as in young rats. There are numerous other studies that have reported the health benefits of this end-metabolite in different pathologies, both in vivo and vitro models. UA have proved to be a promising therapeutic drug preventing some cancers, such as colorectal and prostate cancer (Liu *et al.*, 2019; Mohammed Saleem, Albassam and Selim, 2020), and also possess anti-inflammatory (Gong *et al.*, 2019), anti-obesity (Kang *et al.*, 2016) and antioxidant (Cásedas *et al.*, 2020) properties, additionally to the referred anti-aging effects (Liu *et al.*, 2019). However, there have been no reports to our knowledge of the effects of UA on the reproductive field.

Two recent studies have highlighted the effects of the Nrf2-Keap1 pathway activation, through the UA supplementation. The first study carried out by Liu and colleagues (2019), demonstrated the potential action of UA as an anti-aging agent to treat senescent human skin fibroblast. The results showed that UA enhanced antioxidant capacity through activation of the Nrf2-Keap1 pathway, leading to a significant increase in the mRNA expression of ARE genes (*SOD* - Superoxide dismutase, *NQO1, GCLC* and *HMOX1*), which resulted in significant reduced ROS levels. Another study concluded that UA can be used for the treatment of inflammatory bowel diseases through the enhancement of gut barrier function. It was shown that UA significantly increased the expression of *HMOX1*, a Nrf2 target gene, through the activation of Nrf2, promoting the up regulation of epithelial tight junction proteins. (Singh *et al.*, 2019).

Several studies have been assessed with the goal of delaying ovarian aging and improving oocytes quality and the resulting female fertility outcomes. Due to the contribution of oxidative stress to the ovarian aging process, as well as mitochondrial dysfunction, supplementation with antioxidants has appeared as a promising therapy. As shown above, UA present several roles, namely

antioxidant proprieties, anti-aging effect, as well as in supporting cellular health through the induction of mitophagy. In addition to these important roles, UA can also contribute to eliminate free radicals through the stimulation of the Nrf2 signaling pathway, modulating the expression of antioxidants. Therefore, *in vitro* UA supplementation may constitute an upcoming antioxidant therapy with the potential to prevent infertility motivated by ovarian aging.

Chapter 2 – Aims

The aim of this study was to test the hypothesis that oocyte aging could alter cumulus-oocyte-complexes (COCs) developmental potential and granulosa cells (GCs) expression of important genes related to reproductive competence. Oocyte quality (nuclear maturation stages, mitochondrial membrane potential and oxygen consumption) and developmental potential (embryo rates and quality) were related with GCs expression. A further objective was to test the anti-aging effect of Urolithin A (UA) on COCs and GCs evaluating its effect on the expression level of genes involved in the Nrf2 signaling pathway as well as on oocyte quality.

Research on human reproduction is limited due to ethical restrictions and difficulties in obtaining precious human oocytes, being therefore necessary to use other suitable models to evaluate aging-related changes in human fertility. Most aging studies have been conducted in the mouse model. However in the present study, bovine COCs were chosen to implement a new model for the female gamete aging because they share more similar characteristics with human female reproduction. Indeed, the cow is also a mono-ovular species, which present several physiological characteristics, including ovarian function, follicular wave emergence, follicle size, oocyte maturation, regulation and progression of pre-implantation embryonic development, and gestation length that are similar to women. Moreover cows also have an extended reproductive period, presenting an analogy between 13 years old cows, wherein 50% are still fertile, and women in their late thirties and early forties. In addition, about 95.7% of human genes can be mapped in the genome of bovine model, making cows an attractive specie to model human reproductive aging (Hammond *et al.*, 2016; Mattern *et al.*, 2016).

Chapter 3 – Materials and Methods

3. Experimental Design

To investigate the effect of aging on the alteration of oocyte quality and the potential anti-aging effect of Urolithin A (UA), a model using COCs collected from prepubertal and adult cows submitted to *in vitro* aging (30h of maturation) or to the physiological maturation (22h) processes were applied.

Firstly, a previous assay to determine the concentration of UA that should be used during the COCs maturation process was performed based on a dose-response study in 4 sessions. Since UA has never been tested in oocytes, previous doses successfully applied for prevention and mitigation of some cancers and to demonstrate the anti-aging effect of UA in different cell lines were used (Ryu *et al.*, 2016; Liu *et al.*, 2019; Singh *et al.*, 2019). COCs obtained from prepubertal and mature adult cows (n=978) were selected and then randomly divided into five groups to test different doses of UA: control, 1, 10, 25, and 50 µM during physiological *in vitro* maturation. After the maturation period, some oocytes (n=154) were stained with 1% aceto-lacmoid to determine the chromosomal configuration and maturation stages. The remaining matured oocytes were submitted to *in vitro* insemination with frozen/thawed semen. Presumptive zygotes were cultured and cleavage and blastocyst rates were determined at day 2 and day 7 of culture, respectively. Based on the obtained results, namely the absence of harmful effects and the promotion of maturation and blastocyst development, the concentration of 1 µM of UA was selected.

In the first experiment carried out in 6 sessions, both COCs from prepubertal (average age= 9 months; n=660) and adult cows (average age= 39 months; n=674) were randomly divided into four groups for *in vitro* maturation (22h control and UA physiological maturation, 30h aged control and UA), totalizing 8 groups: (1) control prepubertal group, COCs from prepubertal calves were matured for 22h (n=148); (2) UA prepubertal group, COCs from prepubertal calves were matured in medium supplemented with 1 µM of UA during 22h (n=155); (3) control aged prepubertal group, COCs from prepubertal calves were aged through 30h of *in vitro* maturation (n=149); (4) UA aged prepubertal group, COCs from prepubertal calves were aged through 30h of *in vitro*

maturation in medium supplemented with 1 µM of UA (n=144); (5) control adult group, COCs from adult cows were matured for 22h (n=155); (6) UA adult group, COCs from adult cows were matured in medium supplemented with 1 µM of UA during 22h (n=129); (7) control aged adult group, COCs from adult cows were aged through 30h of *in vitro* maturation (n=148); (8) UA aged adult group, COCs from adult cows were aged through 30h of *in vitro* maturation in medium supplemented with 1 µM of UA (n=138). After the respective *in vitro* maturation times, oocytes were inseminated with thawed capacitated bull semen. Subsequently, embryonic development was assessed, evaluating the rate of cleaved and produced embryos, as well as their quality.

Also in this experiment, some COCs from each group, were retrieved to assess their nuclear maturation stage and mitochondrial membrane potential (MMP) (control prepubertal group, n=7 and 10; UA prepubertal group, n=7 and 9; control aged prepubertal group, n=10 and 9; UA aged prepubertal group, n=6 and 9; control adult group, n=16 and 15; UA adult group, n=21 and 13; control aged adult group, n=16 and 10; UA aged adult group, n=18 and 14, respectively). A preliminary test to assesses the basal mitochondrial oxygen consumption (Seahorse Xfe96 Extracellular Flux Analyzer) of COCs obtained from pre-pubertal and mature adult cows supplemented (n=30) or not with UA (n=30) during maturation were analyzed.

As the GCs play an essential role in follicular growth and oocyte development, a second experiment was performed in 5 sessions to further study the effect of age and UA on the expression of *NFE2L2*, *NQO1* and *mt-ND5*. The number of copies of *mt-ND5* gene was also evaluated. The follicular fluid aspirated from ovaries of prepubertal (mean age=10 months) and adult (mean age=62 months) cows were recovered and centrifuged to obtain GCs. These cells were cultured during 5 days in the following conditions: (1) GCs from prepubertal calves cultured in TCM199+10% serum; (2) GCs from prepubertal calves cultured in TCM199+10% serum supplemented with 1 µM of UA; (3) GCs from adult cows cultured in TCM199+10% serum; (4) GCs from adult cows cultured in TCM199+10% serum supplemented with 1µM of UA. After obtaining confluence at the 5th day, GCs were snap frozen in liquid nitrogen and later the DNA and

RNA were extracted, allowing the subsequent quantification of *NFE2L2, NQO1* and *mt-ND5* mRNA and also *mt-ND5* copy number.

3.1. Oocyte quality and development

3.1.1. Cumulus-oocyte-complexes Collection and Culture

Ovaries from prepubertal (≥10 months) and mature adult cows (≤20 months) were collected from two slaughterhouses (Santacarnes, Santarém and Ribassabores, Tomar) and transported to the laboratory in phosphate buffered saline (PBS), supplemented with 0.05 mg/mL of antibiotic (kanamycin sulfate) and 0.15% of Bovine Serum Albumin (BSA), maintained at 37°C. At the laboratory, the ovaries were washed two or three times with PBS plus kanamycin to remove other debris and then placed in glass containers. These containers were maintained in a water-bath at 37°C during all the procedure.

Ovarian follicles with 3-6 mm in diameter were punctured with a 10 mL syringe attached to a 19G-needle. The follicular fluid was collected and poured into a pre-heated conical tube at 37°C containing 2 mL of W1. The tube was left for a few minutes to allow the sedimentation of COCs from the extracted follicular fluid. Afterwards, the supernatant was discarded and the sediment containing COCs and follicular cells was transferred into a sterilized Petri dish of 60 mm of diameter. Cells were diluted with W1 to help the observation, selection, and classification of COCs using a stereomicroscope equipped with a thermal plate at 38.5°C.

COCs were selected based on their morphological characteristics. Only COCs with homogeneous cytoplasm and at least three complete and compact layers of cumulus cells (CCs) were selected, being the other ones discarded (Fonseca *et al.*, 2020). Collected COCs were randomly divided into the groups according the experimental design and placed in four-well culture plates containing maturation medium supplemented or not with UA. Maturation was accomplished in an incubator at 38.8°C, 5% CO_2 in humidified air for 22 or 30h.

3.1.2. Granulosa Cells Collection and Culture

Recovered follicular fluid was centrifuged for 10 min at 2300 rpm to obtain GCs. Afterwards, the supernatant was discarded and the pellet resuspended in 1 mL of culture medium (TCM199+10% serum) to perform another centrifugation for 5 min. The new pellet was resuspended in 1 mL of culture medium supplemented or not with 1 µM of UA according the experimental design, and was homogenized with a syringe attached to a 19G-needle, at least 30 times to detach the cells.

GCs viability was assessed by using the trypan blue dye (0.4% w/v). This method is based on the difference in membrane permeability of viable and dead cells. Whereas viable cells had intact cell membranes that exclude the tripan blue, dead cells present rupture of the plasma membrane, allowing the dye to enter into the cytoplasm. Therefore, if cells acquire a blue coloration, they are considered non-viable. The number of non-viable and viable cells was counted using a Neubauer chamber. The viability was calculated as a percentage of viable cells (Pereira *et al.*, 2009). The cells were seeded at an initial concentration of $2x10^{5}$ cells/ml and cultured for five days in a six-well culture plate at 38.8°C, 5% CO_2 and humidified atmosphere until they reached confluence. At every 48 hours, the culture medium was discharged and refreshed with a new one. For future DNA and RNA extraction, GCs were collected and washed by centrifugation at 2300 rpm for 10 min. Cell pellet was resuspended in 1 mL of PBS, immediately snap frozen in liquid nitrogen, and stored at -80°C.

3.1.3. Assessment of Nuclear Maturation

To assess the rate of oocytes that reached the Metaphase-II (MII) after 22h or 30h of *in vitro* maturation, a technique allowing the observation of chromosomes and Polar Body was applied as previously shown in Lapa *et al.* (2011).

Briefly, at the end of the *in vitro* maturation period, CCs of COCs were removed by repeated pipetting, under stereomicroscope observation. Denuded oocytes were transferred and fixed in a 35 mm dish containing a solution of acetic acid/ ethanol (1:3, v/v), and maintained at 4°C for 48h. On the day of the evaluation, fixed oocytes were placed in a Neubauer chamber and stained with 1% aceto-lacmoid. The assessment of the maturation stage of each oocyte was determined using a phase contrast microscopy (Olympus Bx 41), and oocytes were classified as follows: Germinal Vesicle (GV), Condensing Chromosomes I (CCI), Condensing Chromosomes II (CCII), Diakinesis, Anaphase-I/Telophase-I (AI/TI), and MII (Figure 4). Only oocytes with visible chromatin staining were taken into account.

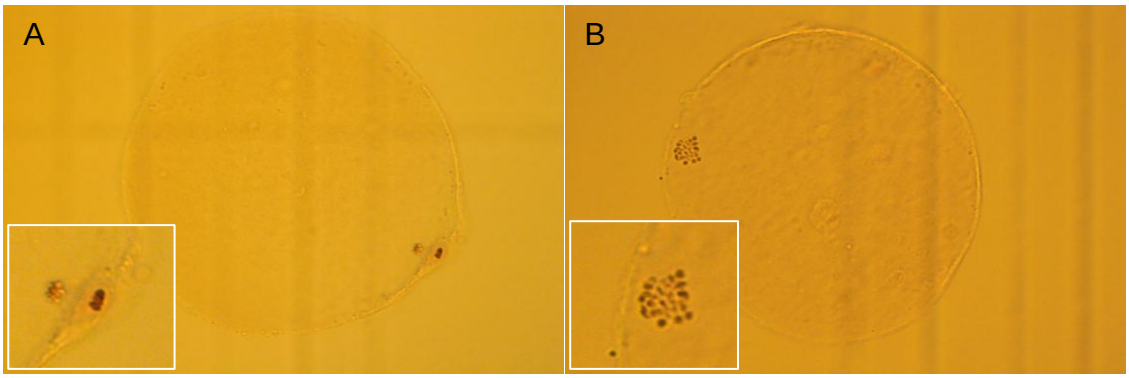

Figure 4 - Representative images of oocytes observations under a phase contrast microscopy (magnification 400x) after maturation and staining with aceto-lacmoid. A) Telophase-I: the chromosomes are grouped in two equally spread groups; B) Metaphase II: condensed and individualized chromosomes, allowing their individual reorganization.

3.1.4. Assessment of Mitochondrial Membrane Potential

The mitochondrial membrane potential (MMP) was determined using the florescent probe 5, 5´, 6, 6´-tetrachloro-1, 1´, 3, 3´-tetraethylbenzimidazolcarbocyanine iodide (JC-1, Invitrogen), as an indicator of mitochondrial activity.

JC-1 probe is a lipophilic cationic compound able to enter into the mitochondrial membrane. Once inside the mitochondria, the JC-1 accumulates as a monomer at low concentrations and yields green fluorescence, or the dye at a higher concentration starts forming complexes named J aggregates. In healthy cells,

JC-1 dye accumulates and forms J aggregates, which exhibit excitation and emission in the red spectrum. This complex formation indicates highly polarized mitochondria (mitochondrial membranes with high potential). On the opposite, in unhealthy cells, there is a lower amount of dye that enters due to low-polarized mitochondria (mitochondrial membranes with low potential). Under this condition, unhealthy cells cannot reach a concentration that allows the formation of J aggregates, so its green fluorescence is retained (Abdulhasan *et al.*, 2017).

After 22h and 30h of maturation, COCs were denuded and were incubated with 5 µg mL^{-1} of JC-1 in maturation medium for 30 min at 38.8°C and 5% CO_2 in humidified air in the dark. Then, oocytes were washed twice in PBS. Stained oocytes were transferred to a pre-heated slide glass with a small amount of PBS. They were then observed under a fluorescence microscope (Figure 5, Olympus BX51) using the blue fluorescence filter (BP 470-490, objective UPlanFI 20x/0.50).

Images were analyzed using Image J software in which the mean value of green and red fluorescence intensity in the complete oocyte was measured. Mitochondrial membrane potential was then calculated as the ratio of the red fluorescence to green fluorescence.

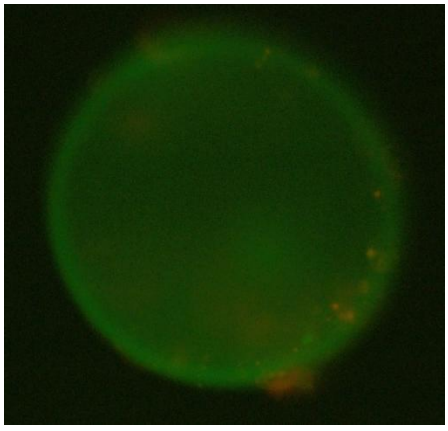

Figure 5 - Representative image of oocyte observations under a fluorescence microscope (magnification 200x) after maturation and staining with 5, 5´, 6, 6´-tetrachloro-1, 1´, 3, 3´-tetraethylbenzimidazolcarbocyanine iodide (JC-1).

3.1.5. Mitochondrial oxygen consumption rate

The measurement of basal metabolism of mitochondria oxygen consumption rate (OCR) was carried out by the Seahorse XFe96 Extracellular Flux Analyzer (Seahorse Bioscience, Germany), which provide an indicator of mitochondrial respiration in cultured cells.

Before each run, the XFe96 Extracellular Flux Analyzer was calibrated through an automated process performed by the equipment, using a calibration plate, replaced then with the study plate. Mature COCs were placed in 96-well plate in SOF medium at a density of 6 COC/100µL/well (6 wells each group). The OCR was measured using a 3 min mix and 5 min cycle. After incubation time, the basal mitochondrial OCR was measured at 38.5°C (Deus *et al.*, 2015).

3.1.6. *In vitro* Fertilization and Embryo Development

In vitro fertilization was performed in 40 µL droplets of FERT medium placed in a sterilized Petri dish of 60 mm of diameter covered by 8 mL of mineral oil. Petri dishes were previously stabilized at 38.8°C and 5% CO_2 in humidified air, at least for one hour before the transfer of 10 COCs to each droplet.

COCs were inseminated with thawed bull sperm submitted to capacitation using the Percoll gradient method. The sperm's concentration was determined in a Neubauer Chamber and added to the containing COCs droplets at a final concentration of 2×10^6 spermatozoa mL^{-1}. COCs and sperm were co-incubated for 20h at 38.8°C and 5% CO_2 in humidified air, being considered the day of *in vitro* insemination as day 0.

After 20h of co-incubation, the presumptive zygotes were transferred and washed in 100 µL droplets of SOF medium supplemented with BSA to remove all aggregated CCs. Afterwards, presumptive zygotes were transferred into a four-well culture plate, within 25 µL droplets of the same medium, covered by 700 µL of mineral oil and cultured at 38.8°C, 5% O_2, 90% N_2 and 5% CO_2 in humidified atmosphere.

On day 2 post-insemination, the embryonic cleavage rate (cleaved embryos per total inseminated oocytes) was determined. Cleaved embryos were transferred into 25 µL droplets of SOF medium supplemented with BSA and 10% of fetal bovine serum (FBS, 25 embryos each droplet), in a four-well culture plate, covered by 700 µL of mineral oil. Embryos were cultured as mentioned above for 12 days to assess the blastocyst development rate (at days 7, 9, and 12) and quality.

Embryos were also evaluated qualitatively based on their morphological integrity, according to the following criteria (Bó and Mapletoft, 2013):

- Grade 1 - Excellent/Good embryo quality: Embryos with symmetrical and spherical mass (shape, size, color, and density of individuaI cells).
- Grade 2 - Fair embryo quality: Embryos with moderate irregularities (shape, size, color, and density of individual cells), with at least 50% of intact embryonic mass.
- Grade 3 - Poor embryo quality: Embryos with major irregularities that have at least 25% of intact embryonic mass.

3.2. DNA and mRNA quantification

3.2.1. DNA extraction

After selection of the best kit, the DNA extraction of GCs was performed using High Pure PCR Template Preparation Kit (Roche), according to the manufacturer's recommendations. The choice of the Kit was directly related to the ability to obtain ultra-pure and concentrated DNA, suitable for direct use in further analyses (Supplementary data - Table 6).

3.1.1. RNA extraction

In order to select the best kit for RNA extraction of GCs several commercial kits were tested. The choice of the Kit was directly related to its ability to extract

higher amount of good quality RNA, suitable for direct use in further analyses (Supplementary data - Table 6). The RNA of GCs was extracted using PureLink™ RNA Mini Kit (Invitrogen™), according to the manufacturer's recommendations. This protocol included the use of spin columns used to isolate high-quality total RNA and DNase as treatment to remove genomic DNA from RNA.

3.1.2. DNA and RNA quantification

DNA and RNA samples were subsequently quantified and the degree of purity of the samples was analyzed by absorption spectrophotometry, using NanoDrop™ One/OneC Spectrophotometer (ThermoFisher Scientific™). The degree of purity of the DNA and RNA were calculated by determination of the ratio of optical densities determined at the wavelengths of 260nm and the 280nm (260nm/280nm), being the ideal ratio 1.8 and 2, respectively. For the quantification and determination of the purity of DNA and RNA samples, their respective elution solution in which they were dissolved, was used as a reference. The samples were then stored at -80°C. DNA and RNA concentration values obtained through spectrophotometery were used to determine the amount of nucleic acids to use in quantitative polymerase chain reaction (qPCR).

3.1.3. Complementary DNA synthesis

Synthesis of complementary DNA (cDNA) from RNA isolates were performed using the Xpert cDNA Synthesis Mastermix kit (GRiSP), according to the manufacturer's instructions. The mastermix used is an optimized reaction mix containing a balanced concentration of oligo(dT), random hexamer primers, deoxyribonucleotides (dNTPs), and RNase inhibitor.

For this work, a maximum of 500 ng of extracted RNA were used from each sample to performed cDNA synthesis that was carrying out using a

thermocycler (T100 Thermal Cycler, Bio-Rad). Finally, the resultant cDNA was stored at -20°C until use for further assays.

3.1.4. Primer design

The design of the primers (NFE2L2, NQO1 and β-actin) was performed using the Primer-Blast program. The sequences of designed primers are depicted in Table 1. Details about mt-ND5 primers were retrieved from a previous study (Endo *et al.*, 2014).

Table 1 - Sequences of primer for quantitative reverse-transcription polymerase chain reaction (RT-qPCR), designed specifically for this study.

Gene symbol	Gene ID	Primer pairs (5'-3')	qPCR Conditions
Genomic Genes			
NFE2L2	497024	F: GTCGTCGGGGAGCCTCAAAG R:ATGTCAATCAAATCCATGTCCTGCT	[Primer] 200 nM Tannealing 60°C
NQO1	519632	F: CATGGCTGTCAGAAAAGCACTG R: GGTCTGACACAGTGACCTCC	[Primer] 100 nM Tannealing 60°C
Mitochondrial Gene			
mt-ND5	_	F: ATTTACAGCAATATGCGCCC R: AAAAGGCGTGGGTACAGATG	[Primer] 75 nM Tannealing 60°C
Housekeeping Gene			
β-actin	280979	F:AGTCGGTTGGATCGAGCATT R:GCTTTTGGGAAGGCAAAGGAC	[Primer] 200 nM Tannealing 60°C

F - Forward Primer; R - Reverse Primer; [Primer] - Primer concentration; Tannealing - Annealing temperature.

3.1.5. Quantitative Reverse-transcription Polymerase Chain Reaction

The quantification of target and endogenous control genes was carried out by quantitative reverse-transcription polymerase chain reaction (RT-qPCR), in a QuantStudio 3 thermocycler (ThermoFisher Scientific), using cDNA. Endogenous control genes are usually selected from housekeeping genes, which are genes with constitutive stable expression in all cells, required for the maintenance of basic cellular functions.

The assessment of the mitochondrial DNA (mtDNA) copies number of the ND5 gene was carried out by qPCR through the previously extracted DNA, using the same equipment as for the quantification of genes.

The samples were analyzed in duplicate and one housekeeping gene was used, in order to normalize target gene expression levels. Each optimized reaction was performed, consisting of Xpert Fast SYBR Green Mastermix 2X with ROX, primer (Forward and Reverse) of each target gene, sample (cDNA/DNA) and RNase free water making up a total volume of 10 µL per reaction well. The target genes were optimized according the conditions previously described in Table 1. A negative control was also used consisting of Mastermix, primers (Forward and Reverse), RNase free water, without cDNA/DNA sample. The samples were subjected to an amplification protocol that consisted of an initial cycle at 95°C for 2 min of denaturation phase, followed by 40 denaturation cycles at 95°C for 5 s, 40 annealing cycles during 30 s at 60°C (depending on the melting temperature of primer sequences), and extension phase at 72°C for 30s and, lastly, a final extension period at 72°C for 10 minutes.

Standard curves were performed for each target gene to assess the efficiency of the reaction, being the values of the efficiency between 93.0 and 98.8%. For the gene expression quantification, the relative quantification method was used. This method of relative quantification of gene expression was carried out with the expression levels of the target genes under study, which were normalized with the housekeeping genes, by the CT comparative method. As the

amplification by RT-qPCR was performed in duplicate, the mean CT values for each gene were determined and the expression levels were calculated using the following formula:

$2^{(-\Delta Ct)}$, where $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}}$

For the quantification of the mtDNA number of copies, the relative quantification normalized against unit mass method was used. This method was carried out with the CT values of GCs treated with UA (named as test), which were normalized with control samples (currently designated as calibrator). As the *mt-ND5* copy number was assessed by qPCR and performed in duplicate, the mean CT values for the tests and calibrators samples were determined and the ratio were calculated using the following formula:

$Ratio = E^{(\Delta Ct)}$, where $\Delta Ct = Ct_{\text{calibrator}} - Ct_{\text{test}}$, and E is the efficiency.

3.2. Statistical analysis

The data from the sessions of embryo production and quality were analyzed using Proc Glimmix from SAS (Statistical Analysis Systems, SAS Inst., Inc., Cary, NC, USA), using the binary distribution and the logit as link function. The generalized linear mixed model included treatment (UA doses, aging effect and female age) as fixed effect and replicates as random effect. In addition, the means for each treatment were calculated, and comparisons between groups were performed. The JC-1 data, mRNA transcript levels and mtDNA number of copies were analyzed using the Proc Mixed of SAS with a model including treatment (aging effect, UA effect and/or female age) as fixed effect. The session was considered a random effect.

The data of chromosomal configurations were compared between groups using the exact Ficher test, in a 2 × 2 contingency table.

The analysis of results was considered statistically significant when $P \leq 0.05$.

Chapter 4 – Results

4.1. Previous assay – Dose-response study

4.1.1. Nuclear Maturation

The results obtained after microscopic observation of the oocyte maturation status during the dose-response study of UA supplemented to the maturation medium, are shown in Figure 6.

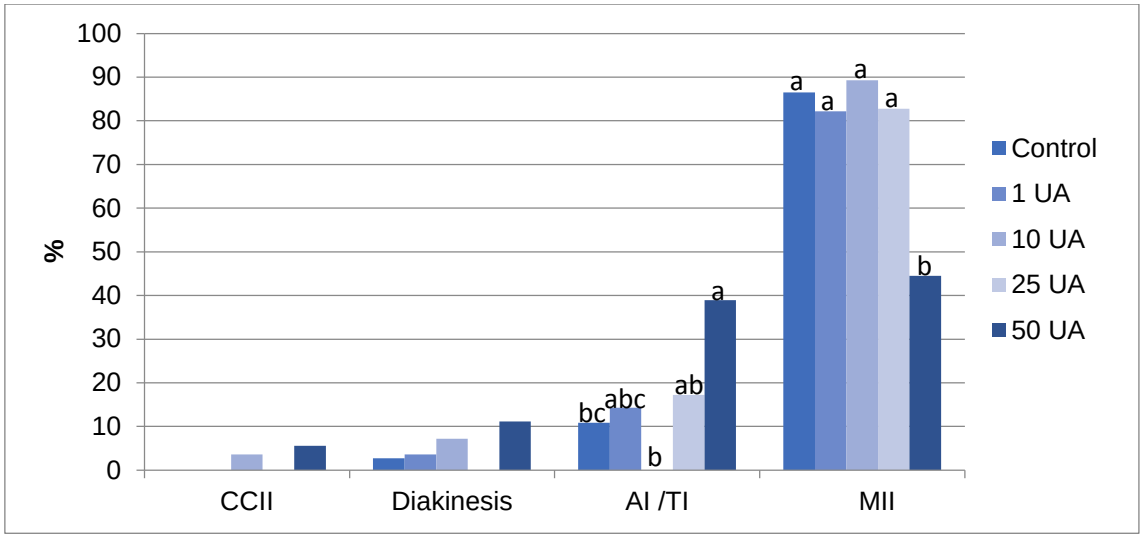

Figure 6 - Effect of Urolithin A (UA) doses supplemented to the maturation medium in the oocyte chromosomal configuration (control, 1 UA, 10 UA, 25 UA, and 50 UA, correspond to 0, 1, 10, 25 and 50 µM of UA, respectively). Different letters indicate significant differences between groups for the same stage (CCII - Condensing chromosomes II; AI/TI - Anaphase-I/ Telophase-I; MII - Metaphase-II, P≤0.05).

The supplementation of the oocyte culture medium with UA significantly influenced the nuclear maturation of oocytes. On the Anaphase-I/Telophase-I (AI/TI) phase of the maturation status, the 50 UA group (38.9%) revealed a higher rate of oocytes in this status compared to the Control (10.8%, P=0.03) and 10 UA (0.0%, P=0.0006) groups. In this phase it was also identified a higher rate in the 25 UA (17.2%, P=0.05) group compared to the 10 UA group. Moreover, a trend was found between 50 UA group and 1 UA (14.3%, P=0.08). The highest dose also showed a harmful effect, reducing the number of oocytes classified at the Metaphase-II (MII) stage (50 UA, 44.4%) when compared to control (86.5%, P=0.003), 1 UA (82.1%, P=0.01), 10 UA (89.3%, P=0.002) and 25 UA (82.8%, P=0.01) groups. No differences were observed between the remaining stages of oocyte maturation. The Germinal Vesicle (GV) and

Condensing Chromosomes I (CCI) maturation phases are not shown in the graph, as no oocytes were assessed in these categories.

4.1.2. Embryonic Development

The embryonic development evaluated through the cleavage, embryos at day 7 (D7) and hatched rates obtained in the assay performed to determine the best concentration of UA is resumed in Table 2 and Figure 7.

Table 2 - Effect of Urolithin A (UA) doses supplemented to the maturation medium on the cleavage, D7 and hatched embryo rates (4 sessions, control, 1 UA, 10 UA, 25 UA, and 50 UA, correspond to 0, 0.1, 10, 25 and 50 µM of UA, respectively).

	Oocytes	Cleavage		D7 Embryos		Hatched Embryos	
Groups	(n)	(n)	(%)	(n)	(%)	(n)	(%)
Control	161	138	69.7±4.3	20	17.1±3.9ab	14	60.0±11.0
1 UA	167	123	67.7±4.2	32	28.5±4.8a	10	31.3±8.2
10 UA	181	137	66.3±4.1	25	20.4±4.0a	10	40.0±9.8
25 UA	127	104	65.0±5.1	15	18.5±4.7a	7	33.3±12.2
50 UA	142	104	64.3±4.7	7	7.2±2.7b	2	28.6±17.1

Different letters indicate significant differences between groups (P≤0.05). Data are expressed as the mean ± SEM (Standard Error Mean). Oocytes - Inseminated oocytes. D7 Embryos - Embryos at day 7.

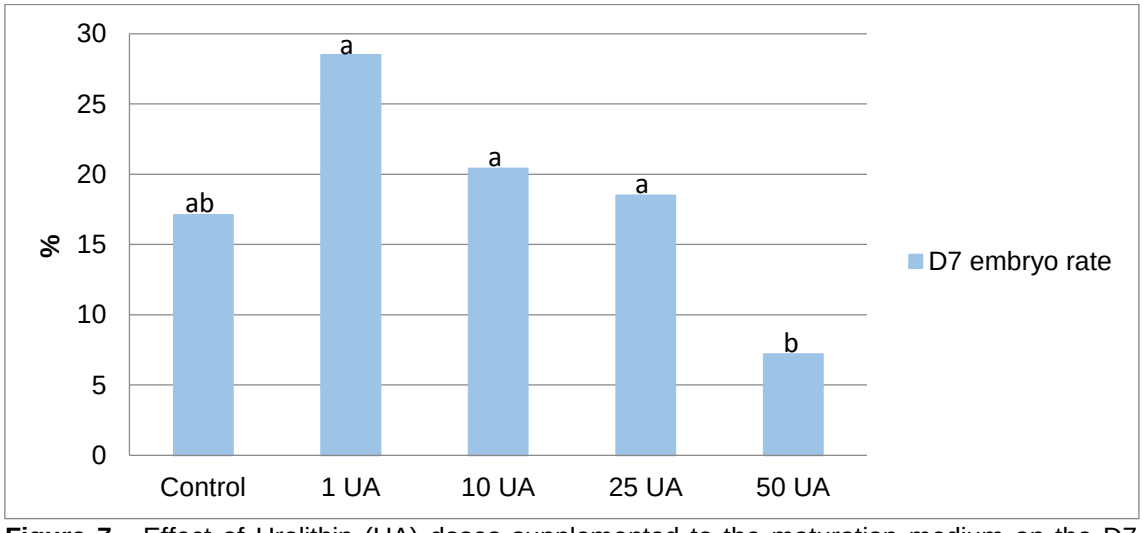

Figure 7 - Effect of Urolithin (UA) doses supplemented to the maturation medium on the D7 embryo rate (4 sessions, control, 1 UA, 10 UA, 25 UA, and 50 UA, correspond to 0, 1, 10, 25

and 50 µM of UA, respectively). Different letters indicate significant differences between groups (P≤0.05). D7 - Day 7.

The dose-response test showed no significant effect of UA concentrations on cleavage and hatched embryo rates. However, the rate of embryos produced at day 7 revealed to be influenced by the different concentrations of UA. The 50 UA group had lower rates of embryos at day 7 compared to the 1 UA (P=0.004), 10 UA (P=0.03) and 25 UA (P=0.05) groups. Although no significant differences were showed between the control and 50 UA groups, a trend (P=0.06) was identified. Moreover Day 7 embryo rates tend (P=0.07) to be higher after 1 UA dose supplementation compared to control.

4.1.3. Embryo Quality

The data showing the effect of the different doses of UA supplemented to the maturation culture medium on embryo quality are presented in Table 3.

Table 3 - Effect of Urolithin A (UA) doses on the quality of the produced embryos (control, 1 UA, 10 UA, 25 UA, and 50 UA, correspond to 0, 1, 10, 25 and 50 µM of UA, respectively). The embryo quality is classified based on morphological criteria as excellent/good (Grade 1), fair (Grade 2) and poor (Grade 3).

Groups	D7 Embryos (n)	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)
Control	26	50.0±11.1	50.0±11.2	0.0±0.0
1 UA	32	59.4±8.7	34.3±8.4	3.1±3.1
10 UA	28	44.0±9.9	52.0±10.0	4.0±3.9
25 UA	23	33.3±12.2	46.7±14.9	20.0±10.3
50 UA	6	42.8±18.7	57.1±18.7	0.0±0.0

Data are expressed as the mean ± SEM (Standard Error Mean). D7 Embryos - Embryos at day 7.

The different concentrations of UA supplemented to maturation medium did not significantly (P>0.05) influenced the quality rates of produced embryos of grade 1, 2 and 3. However, the 1 UA group obtained a rate of 68.9% of grade 1 embryos (corresponding to 20 excellent/good embryo quality), followed by the 50 UA (35.4%, n=3), control (34.6%, n=10), 10 UA (26.8%, n=10) and 25 UA group (15.4%, n=4).

Once the presented results from the previous assay showed higher rates of embryos produced at day 7, as well as a higher number of produced embryos of excellent/good quality after the supplementation of 1 µM-UA to the maturation medium this dose was chosen to proceed to the following studies.

4.2. Experiment 1

4.2.1. Nuclear Maturation

The assessment of the chromosomal maturation status according to the different treatments in the prepubertal and adult females, of the first experiment is shown in Figure 8.

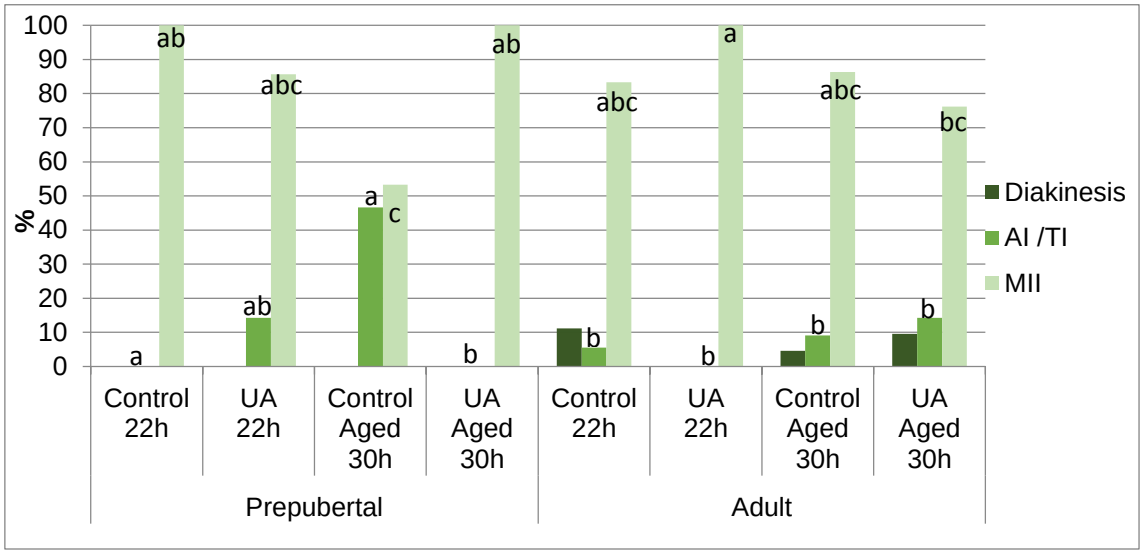

Figure 8 - Effect of the Urolithin A (UA) supplementation to the maturation medium, cumulus-oocyte-complexes (COCs) aging and female age on the oocyte chromosomal configuration (Prepubertal: Control 22h, UA 22h, Control Aged 30h, UA Aged 30h; Adult: Control 22h, UA 22h, Control Aged 30h, UA Aged 30h groups). Different letters indicate significant differences between groups for the same stage (AI/TI - Anaphase-I/Telophase-I; MII - Metaphase-II) (P≤0.05).

Independently of the other studied factors, oocyte aging and female age provoked a significant harmful effect on maturation. Higher rates of MII phase were identified in control oocytes compared to aged ones (22h=93.2% vs. 30h=77.6%, P=0.02). Moreover a delay on maturation progression was identified on aged gametes and prepubertal females with more oocytes at AI/TI phase (22h=3.4% vs. 30h=17.9%, P=0.01 and prepubertal=21.1% vs. adult=6.8%, P=0.03).

Aging and the UA supplementation to the maturation medium significantly influenced the chromosomal configuration of oocytes from prepubertal and adult females (P<0.05, Figure 8). On the AI/TI phase of the maturation status, higher rates of oocytes were found in this stage in the prepubertal control aged 30h group (46.7%) when compared to prepubertal control 22h (0.0%, P=0.05) and UA 30h (0%, P=0.02) and both adult 22h (control, 5.6%, P=0.01 and UA, 0.0%, P=0.0002) and 30h (control aged, 9.1%, P=0.02) groups. A trend was also identified between prepubertal control aged 30h and adult UA aged 30h (14.3%, P=0.0581) groups and between adult UA 22h and adult UA aged 30h (P=0.0769).

Regarding the MII phase corresponding to a complete nuclear maturation, significant differences were also found between groups (Figure 8). Lower rates of oocytes on the MII stage were found in the prepubertal control aged 30h (53.3%) compared to the prepubertal control 22h (100%, P=0.05), prepubertal UA aged 30h (100%, P=0.02) and adult UA 22h (100%, P=0.0002) and control aged 30h (86%, P=0.056). Besides, the adult group supplemented with UA, showed higher rates of matured oocytes when matured during 22h, compared to the adult UA aged 30h of maturation (76.2%, P=0.01), control aged 30h (86.4%, P=0.08) and control 22h (83.3%, P=0.057).

No differences were observed between the remaining stages of oocyte maturation. The GV, CCI and condensing chromosomes II (CCII) maturation phases are not shown in the graph, as no oocytes were assessed in these categories.

4.2.2. Mitochondrial Membrane Potential

In order to analyze the involvement of mitochondrial dysfunction in female aged oocytes and the effect of UA, the Mitochondrial Membrane Potential (MMP) was assessed in the different groups.

Independently of the other studied factors, oocyte aging had a significant harmful effect on MMP assessed as the ratio of the measured red and green fluorescence (22h ratio=0.54 vs. 30h ratio=0.47, P=0.002).

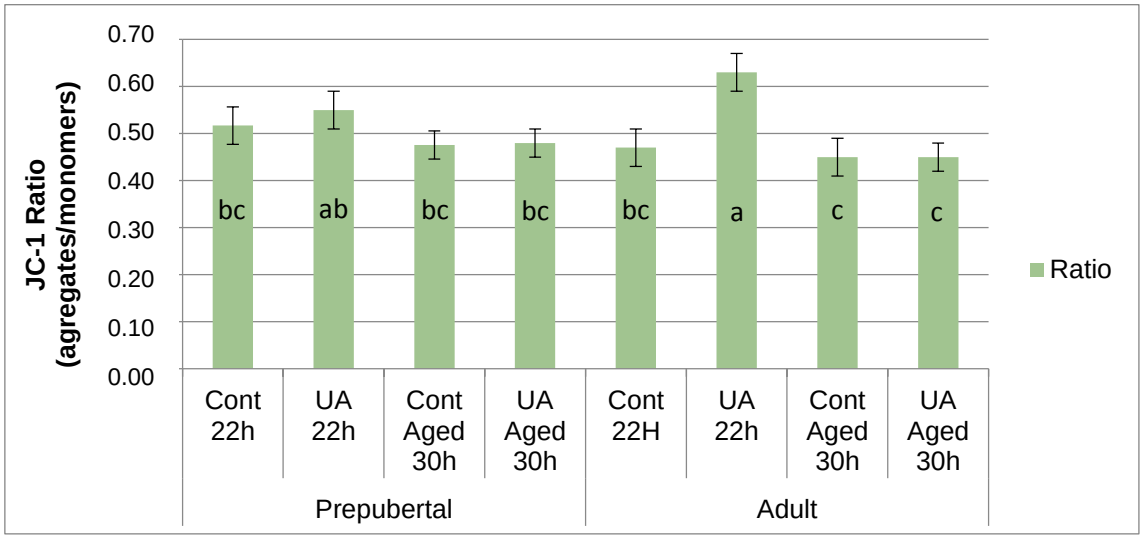

Figure 9 - Effect of the Urolithin A (UA) supplementation to the maturation medium, cumulus-oocyte-complexes (COCs) aging and female age on mitochondrial membrane potential (MMP). The assessment of mitochondrial activity was performed using the average of the ratios (aggregate/monomers) for each oocyte analyzed in each group (Prepubertal: Control 22h, UA 22h, Control Aged 30h, UA Aged 30h; Adult: Control 22h, UA 22h, Control Aged 30h, UA Aged 30h groups). Different letters indicate significant differences between groups (P≤0.05). Data are expressed as the mean ratios ± SEM (Standard Error Mean).

The combination of the female age, aging effect and the suplementation with UA during maturation significantly (P=0.007) influenced the MMP (Figure 9). As observed in Figure 9, higher rates of MMP were obtained when the prepubertal (ratio=0.55±0.04) and adult (ratio=0.63±0.04) oocytes were matured during 22h with UA supplementation, compared with the adult aged 30h control (ratio=0.45±0.04, P=0.04 and P=0.0007, respectively) and UA (ratio=0.45±0.03, P=0.03 and P=0.0003, respectively). Moreover, a significant increase in JC-1 aggregate/monomers ratio, which indicates a significant increase in MMP, was

observed in adult UA 22h group when compared with adult control 22h (ratio=0.47±0.04, P=0.0031), prepubertal 22h control (ratio=0.52±0.04, P=0.03), aged 30h control (ratio=0.48±0.03, P=0.003) and UA (ratio=0.47±0.03, P=0.001). A trend was also identified between prepubertal UA 22h and prepubertal UA aged 30h (P=0.07).

4.2.3. Mitochondrial oxygen consumption rate

A preliminary test was carried out using a pool of oocytes from both prepubertal and adult females, in order to evaluate the effect of UA on the basal oxygen consumption rate (OCR).

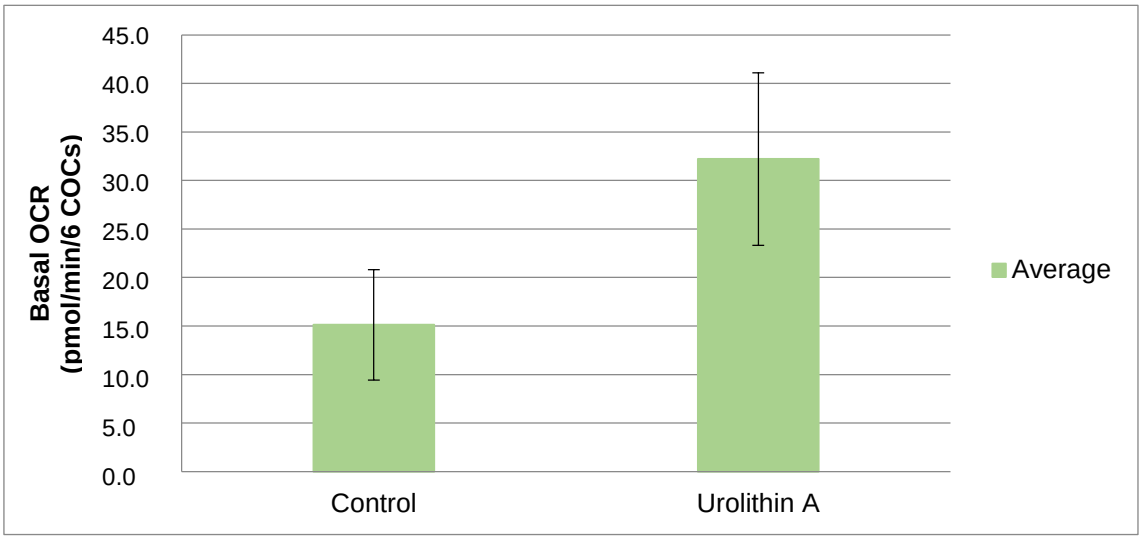

Figure 10 - Preliminary test of the effects of the Urolithin A (UA) supplementation into the maturation medium on mitochondrial oxygen consumption rate (OCR), analyzed by Seahorse XF96 Extracellular Flux Analyzer. The preliminary test of OCR was assessed through a pool of cumulus-oocyte-complexes (COCs) from prepubertal and adult females, divided by 6 in each well. The averages of the 6 wells were then determined. Data are expressed as the mean ratios ± SD (Standard Deviation).

Although no statistics have been performed due to the limited number of tests to assess basal mitochondrial OCR, our preliminary results demonstrated that the supplementation of UA in the culture medium improved the basal OCR (Figure 10). Indeed, UA (32.2 pmol/min/6 COCs) had increased more than two-folds the basal OCR when compared with the control (15.1 pmol/min/6 COCs) group.

4.2.4. Embryonic Development

The embryonic development evaluated through the cleavage, D7 and hatched embryo rates, obtained with the different treatments (aging and UA supplementation) applied to the prepubertal and adult females COCs during maturation, is resumed in Table 4, Figures 11 and 12.

Independently of the other studied factors, oocyte aging had a significant harmful effect on cleavage (22h=79.5% vs. 30h=68.6%, P=0.0001) and D7 embryo rates (22h=18.6% vs. 30h=12.1%, P=0.01). The UA supplementation also shown to improve the D7 embryo rates (control= 11.1% vs. UA=20.0%, P=0.0009).

Table 4 - Effect of the Urolithin A (UA) supplementation to the maturation medium, cumulus-oocyte-complexes (COCs) aging and female age on the cleavage, D7 and hatched embryo rates (6 sessions, Prepubertal: Control 22h, UA 22h, Control Aged 30h, UA Aged 30h; Adult: Control 22h, UA 22h, Control Aged 30h, UA Aged 30h groups).

Groups		Oocytes	Cleavage		D7 Embryos		Hatched Embryos	
		(n)	(n)	(%)	(n)	(%)	(n)	(%)
Preubertal	Cont 22h	148	110	74.7±3.6ab	16	13.8±3.3bc	4	20.5±10.2
	UA 22h	155	125	80.6±3.2a	26	20.5±3.6ab	14	56.7±11.3
	Cont Aged 30h	149	99	66.9±3.9b	8	7.7±2.6c	4	47.4±19.4
	UA Aged 30h	144	97	67.2±4.0b	19	18.9±4.0ab	3	8.9±6.4
Adult	Cont 22h	155	120	81.4±3.2a	19	15.9±3.4bc	7	34.7±12.0
	UA 22h	129	111	80.9±3.4a	30	26.8±4.3a	12	34.8±9.6
	Cont Aged 30h	148	112	73.0±3.6ab	9	7.7±2.6c	5	35.0±16.5
	UA Aged 30h	138	84	66.5±4.2b	14	15.9±4.0bc	5	22.8±10.7

Different letters indicate significant differences between groups (P≤.05). Data are expressed as the mean ± SEM (Standard Error Mean). Oocytes - Inseminated oocytes. D7 Embryos - Embryos at day 7.

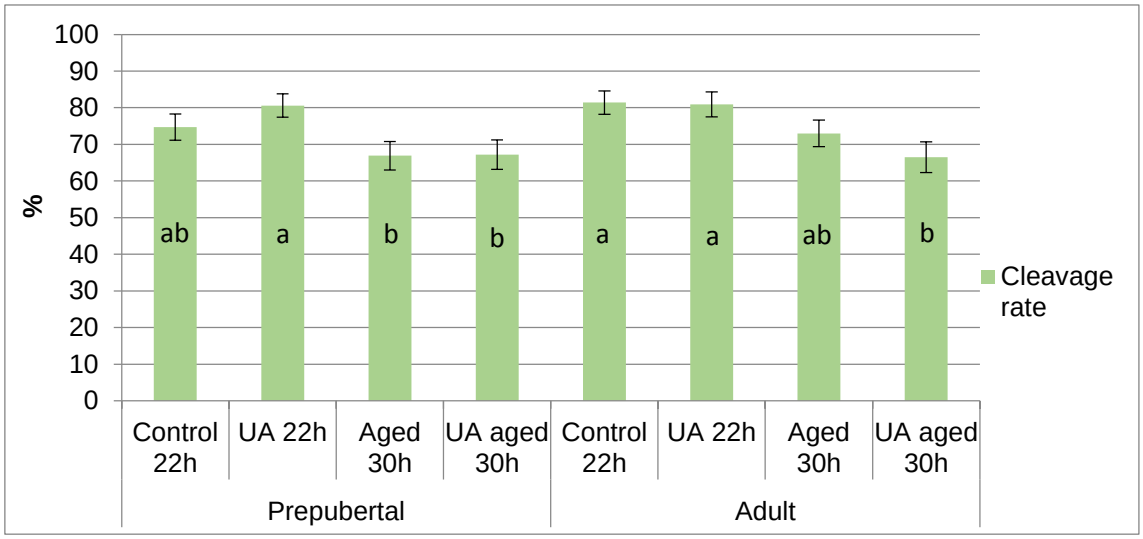

Figure 11 - Effect of Urolithin (UA) supplementation to the maturation medium, cumulus-oocyte-complexes (COCs) aging and female age on the cleavage rate (6 sessions, Prepubertal: Control 22h, UA 22h, Control Aged 30h, UA Aged 30h; Adult: Control 22h, UA 22h, Control Aged 30h, UA Aged 30h groups). Different letters indicate significant differences between groups (P≤0.05). Data are expressed as the mean ± SEM (Standard Error Mean).

The combination of the female age, aging effect and the suplementation with UA during maturation significantly (P=0.01) influenced the embryonic development, namely the cleavage and D7 embryo rates (Table 4). Higher rates of cleavage were achieved when the adult oocytes were matured during 22h with or whithout UA supplementation (adult cont 22h and adult UA 22h groups) and prepubertal oocytes matured during 22h with UA supplementation (prepubertal UA 22h group), compared to adult UA aged 30h (P≤0.01), prepubertal aged 30h control (P≤0.02) and UA (P≤0.01) groups (Figure 11). A trend (P=0.09) was also identified between the adult control 22h and the adult control aged 30h groups.

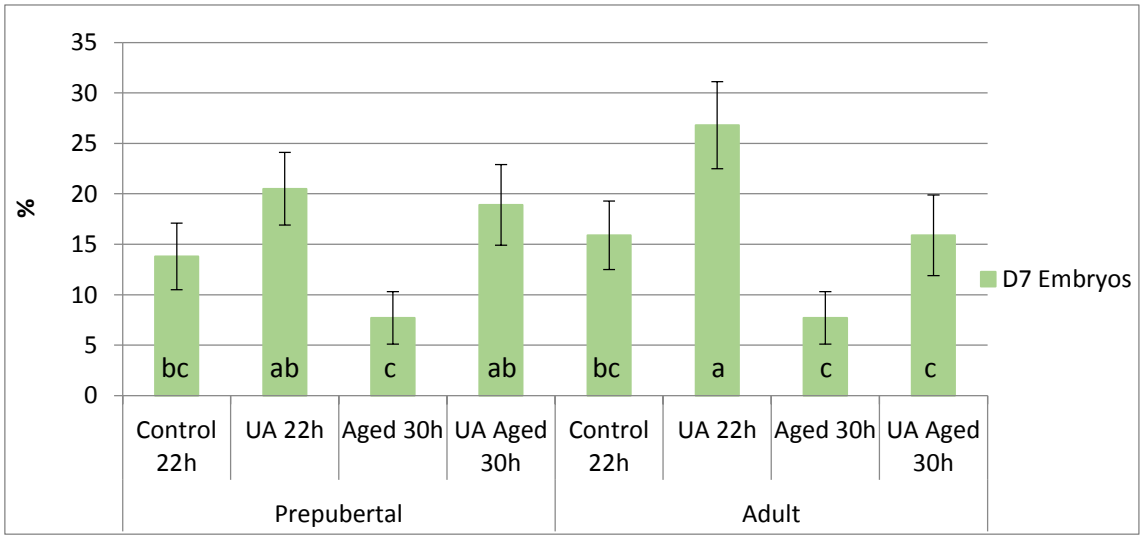

Figure 12 - Effect of Urolithin (UA) supplementation to the maturation medium, cumulus-oocyte-complexes (COCs) aging and female age on the hatched embryo rates (6 sessions, Prepubertal: Control 22h, UA 22h, Control Aged 30h, UA Aged 30h; Adult: Control 22h, UA 22h, Control Aged 30h, UA Aged 30h groups). D7 Embryos - Embryos at day 7. Different letters indicate significant differences between groups (P≤0.05). Data are expressed as the mean ± SEM (Standard Error Mean).

As referred, another parameter that have been significantly (P=0.01) influenced by the combination of the femeale age, the suplementation with UA and COCs aging was the rate of embryos produced at day 7 (Figure 12). Exception made for the prepubertal UA 22h and UA 30h groups, the adult UA 22h group presented the highest rates of embryo at day 7 (P≤0.05). Moreover, lower D7 embryo rates were produced from aged oocytes from both adult and prepubertal females without the supplementation of UA (prepubertal and adult aged control groups) compared to those that were supplemented with UA respectively (prepubertal UA aged 30h, P=0.03 and adult UA aged 30h, P=0.09).

The different treatments did not influenced the hatched embryo rate (P>0.05).

4.2.5. Embryo Quality

The data presented in the Table 5 shown the effect of the prepubertal and adult females COCs aging and UA supplementation in the maturation culture medium on the quality of the produced embryos.

Table 5 - Effect of the Urolithin A (UA) supplementation into the maturation medium, cumulus-oocyte complexes (COCs) aging and female age on the quality of the produced embryos (Prepubertal: Control 22h, UA 22h, control Aged 30h, UA Aged 30h; Adult: Control 22h, UA 22h, control Aged 30h, UA Aged 30h groups).

	Groups	D7 Embryos (n)	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)
Prepubertal	Control 22h	16	31.2±11.8	58.5±12.9	7.3±7.2
	UA 22h	25	43.4±10.4	60.6±10.4	1.5±1.7
	Cont Aged 30h	8	41.5±16.9	44.3±17.4	0.0±0.0
	UA Aged 30h	19	20.11±9.2	45.9±11.8	33.8±12.2
Adult	Control 22h	19	41.4±11.5	45.5±11.9	10.0±6.6
	UA 22h	30	43.8±9.5	38.9±9.4	13.7±6.7
	Cont Aged 30h	9	40.7±17.1	46.3±17.5	10.0±10.0
	UA Aged 30h	14	33.9±13.0	52.0±14.0	11.2±8.3

Data are expressed as the mean ± SEM (Standard Error Mean). The embryo quality is classified based on morphological criteria as excellent/good (Grade 1), fair (Grade 2) and poor (Grade 3).

The supplementation of UA to the maturation medium did not significantly ($P>0.05$) influenced the quality rates of produced embryos of grade 1, 2 and 3 nor did the aging effect or all the studied factors together. However increased numbers of embryos of excellent/good quality were produced when the UA were supplemented mainly on oocytes matured during 22h.

4.3. Experiment 2

4.3.1. Gene Expression levels

In order to study the UA effect and maternal age influence in the Nrf2 signaling pathway, analysis of *NFE2L2* and *NQO1* genes expression levels in granulosa cells (GCs) culture from prepubertal and adult cows was assessed by RT-qPCR. Results from this assay are presented in Figures 13.

Independently of the other studied factors, female age, significantly influenced the *NFE2L2* gene expression, reflected in the higher level of *NFE2L2* transcripts in GCs from prepubertal females (mRNA levels prepubertal=0.00015 vs. adult=0.00011, P=0.048).

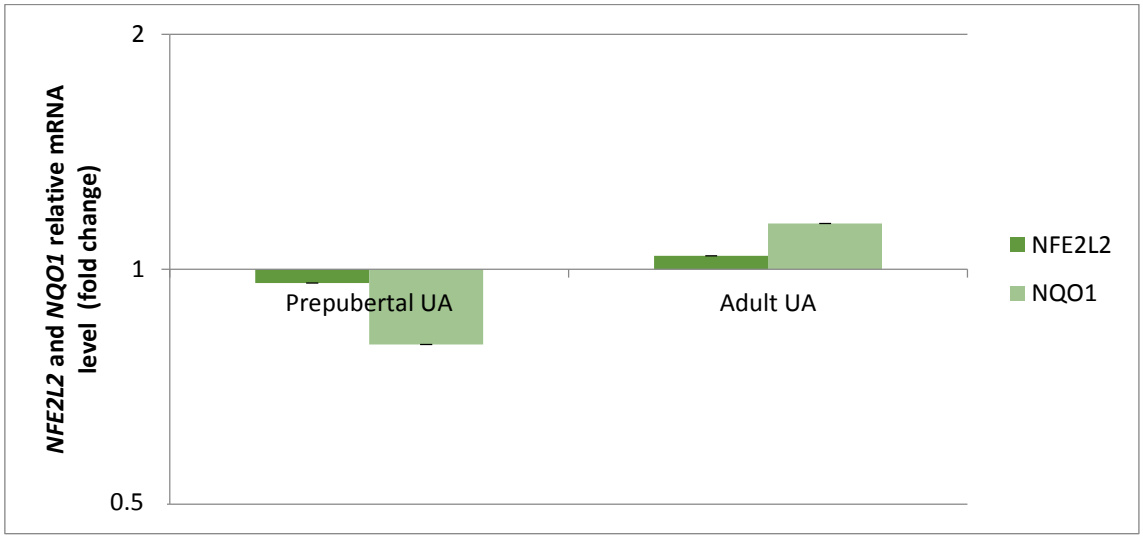

Figure 13 - Gene expression levels of *NFE2L2* and *NQO1* in Granulosa Cells (GCs) from prepubertal and adult cows (Prepubertal: Control, Urolithin A (UA); Adult: Control, UA, n=5 for each group) supplemented with UA in the medium culture. Results are normalized to the β-actin gene. mRNA levels in control GCs for *NFE2L2* and *NQO1* genes were set to 1. Data are expressed as the CT value mean ± SEM (Standard Error Mean).

The supplementation of UA to the culture medium of GCs, both independently and considering female age, did not significantly (P>0.05) influence the gene expression levels of both *NFE2L2* and *NQO1* genes. However, when the expression levels were normalized with the respective control, it was observed that the levels of transcripts of *NFE2L2* and *NQO1* decreased in UA treated prepubertal GCs but increased in the UA treated adults cells (Figure 13).

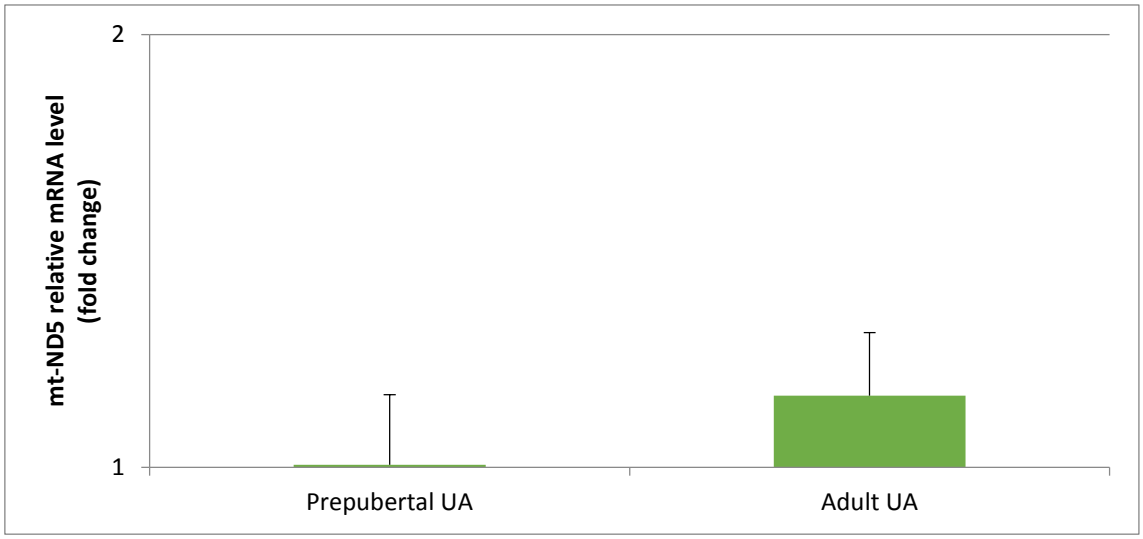

Figure 14 - Gene expression levels of *mt-ND5* in Granulosa Cells (GCs) from prepubertal and adult cows (Prepubertal: Control, Urolithin A (UA); Adult: Control, UA, n=5 for each group) supplemented with UA in the medium culture. Results are normalized to the *β-actin* gene. mRNA levels in control GCs for *mt-ND5* gene were set to 1. Data are expressed as the CT value mean ± SEM (Standard Error Mean).

The supplementation of UA to the culture medium of GCs, from prepubertal and adult females, did not significantly (P>0.05) influence the expression levels of *mt-ND5* gene. Nevertheless, when the expression levels were normalized with the respective controls, this gene expression increased in UA treated prepubertal and especially in adult GCs (Figure 14).

4.3.2. mtDNA copy number

Analysis of *mt-ND5* DNA content in GCs culture from prepubertal and adult cows, were assessed by qPCR. Results from this assay are presented in Figures 15.

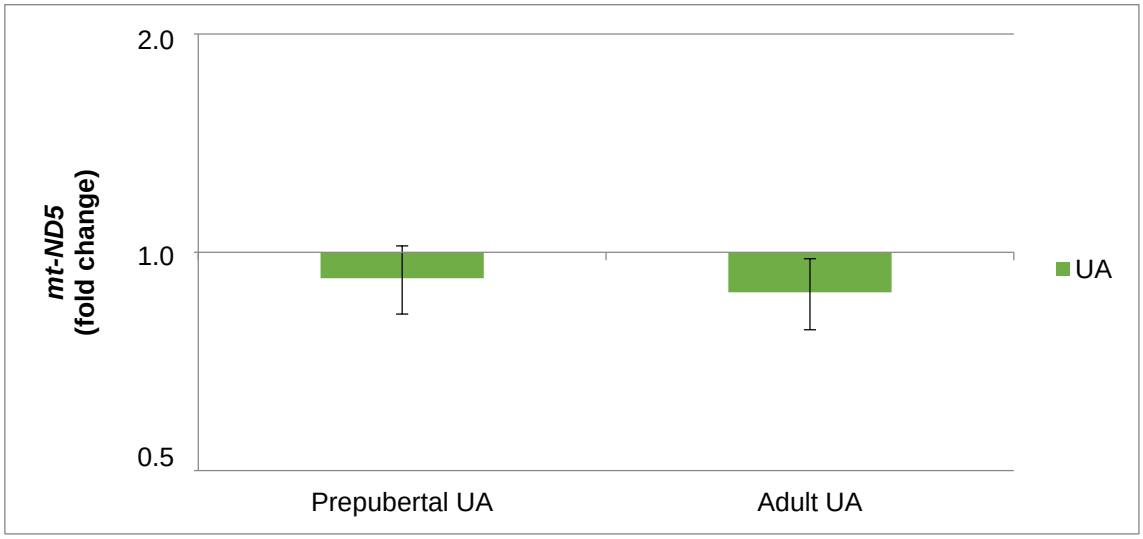

Figure 15 - *mt-ND5* copy number in Granulosa Cells (GCs) from prepubertal and adult cows (Prepubertal: Control, Urolithin A (UA); Adult: Control, UA, n=5 for each group) supplemented with UA in the medium culture, assessed by qPCR using DNA. Results are normalized to their respective GCs control samples. *mtDNA* copy numbers in control GCs for ND5 gene were set to 1. Data are expressed as the ratio of CT values mean ± SEM (Standard Error Mean).

The supplementation of UA to the culture medium of GCs, both independently and considering female age, did not significantly (P>0.05) influence the copy number of *mt-ND5* gene. However when the expression levels were normalized with the respective controls, it was observed that copy numbers decreased in both UA treated prepubertal and adult GCs (Figure 15).

Chapter 5 – Discussion

During the past decades, the number of women postponing pregnancy in industrialized countries has increased, significantly impacting their ovarian reserve and oocyte quality, as well as their fertility outcomes (Keurst, Ter, Boivin and Gameiro, 2016)**.** The changes in women reproductive behavior are mainly due to the women entrance in the workforce, a consequence of better educational conditions and contraceptive methods (Reviewed in Fritz and Jindal, 2018). By delaying childbearing into their late thirties, women are subjected to the natural limits of their own reproductive system (Wyndham, Marin Figueira and Patrizio, 2012) consequently seeing their fertility and reproductive capacity decrease with age (Liu *et al.*, 2017). In reproductive aged women, namely above 35 years, women start to experience reproductive difficulties such as the chromosomal abnormalities, organelle dysfunction, low fertilization rate and poor embryonic development (Reviewed in Cimadomo *et al.*, 2018; Igarashi, Takahashi and Nagase, 2015). Delayed childbearing associated to advanced maternal age is one of the main factors that leads women to resort to assisted reproductive technology (ART). Despite the increasing advances made in this field, the technologies applied are not yet able to restore fertility, reverting the biological clock (Igarashi, Takahashi and Nagase, 2015).

Ethical and physical restrictions hamper the study of age-associated infertility in human oocytes. Therefore several models have been used but cows have appeared as the most appropriate for these studies as clearly shown in the present work. In fact, compared to other models, cows have a longer reproductive life and show pregnancy, follicular and endocrine events similar to humans (Mattern *et al.*, 2016; Takeo *et al.*, 2017)(Hammond et al., 2016; Mattern et al., 2016)(Hammond et al., 2016; Mattern et al., 2016)(Hammond et al., 2016; Mattern et al., 2016)(Hammond et al., 2016; Mattern et al., 2016). Moreover our model for aging female gametes proved to be very efficient and useful to study different problems associated with reproductive aging and fertility impairment, currently one of most critical challenges in the world. Furthermore, the research of new upcoming antioxidant therapies with the potential to prevent infertility provoked

by ovarian aging, as studied in the present work, is equally of utmost importance.

Urolithin A (UA) is a first-in-class food metabolite which have been identified due to its capacity to prevent the accumulation of dysfunctional mitochondria with age, by inducing mitophagy and extended lifespan (Ryu et al., 2016). Presented results reported for the first time the beneficial properties of UA in ART. Since UA was never tested in the female reproduction prior to this study, doses successfully applied to test its potential effects as an anti-cancer, anti-inflammatory and anti-aging agent in different cell lines were used (Liu *et al.*, 2019; Ryu *et al.*, 2016; Singh *et al.*, 2019). In this study, a previous dose-response assay was carried out on oocytes and the concentration of 1 µM-UA was clearly identified as the most promising. Moreover it was demonstrated a harmful effect of higher doses, especially the 50 µM UA dose, which had a deleterious effect on nuclear maturation progression until Metaphase-II (MII). In addition, this effect was reflected on embryonic development impairment. Accordingly, Liu and colleagues (2019) noticed significant reduced cell viability and proliferation on the human senescent skin fibroblasts, at a UA concentration of 50 µM. Thus, suggesting that high UA doses leads to diminished cell viability increasing the number of cells arrested in the G2/M cell-cycle. On the contrary, higher rates of embryos were produced at day 7 after the supplementation of 1 µM-UA to the maturation medium when compared to the other doses.

An important predictor to assess embryo viability is its key morphological features after fertilization. Embryos can be categorized into three quality groups (Grade 1 - Excellent/Good; Grade 2 - Fair; Grade 3 - Poor) based on their morphology, as previously described (Bó and Mapletoft, 2013), reflecting their potential for development. Presented results showed a higher number of produced embryos of excellent/good quality when oocytes were matured with 1 µM of UA, being of primordial importance for ART outcomes.

To further explore the potential mechanisms of action of UA in female reproduction, a study during oocyte aging and using females with different ages

as oocyte donors was implemented. Yamamoto and co-workers (2010) reported an age-associated decline in the fertilization rate of old cows. In fact, oocytes from old cows were more prone to resume first meiotic division during maturation and often had already initiated the meiotic maturation at oocyte collection (Yamamoto *et al.*, 2010). These data suggest that oocytes from older cows have a faster nuclear maturation progression and reach the MII phase faster than oocytes from young cows. Also, Soto-Heras and colleagues (2018) have studied fertility outcomes in juvenile and adult goats, reporting a reduced embryo development in juvenile goats compared to adults, due to a lower oocyte competence. Accordingly, our study revealed that female donors age and the process of oocyte aging, significantly influence the chromosomal configuration of oocytes during maturation progress. In fact, oocytes from prepubertal cows showed a higher rate of delayed stages, such as Anaphase-I/Telophase-I (AI/TI) phases (prepubertal=21.1% vs adult=6.8%), revealing a slower progression of nuclear maturation, which may be due to lower oocyte competence as proposed by Soto-Heras (2018). Aged oocytes, also present lower rates of oocytes that have reached the MII phase during 30h (77.6%) compared to the physiological period, 22h of *in vitro* maturation (93.2%). Moreover, a positive effect has been identified with UA treatment during the physiological maturation process and also an anti-aging effect in both prepubertal and adult females. We observed that when oocytes from prepubertal cows were matured with UA during 30h higher maturation rates were obtained compared to control aged ones, as well as oocytes from adult cows matured during 22h.

In agreement with our findings, Rodrigues-Cunha and colleagues (2016) demonstrated that melatonin supplementation during the *in vitro* maturation could stimulate the meiosis resumption in bovine COCs, whereas control oocytes cultured without hormones had slower meiosis resumption rates. On the contrary, the supplementation with other antioxidants agents, such as quercetin, vitamin C or resveratrol did not present any effect on nuclear maturation rates, even when a reduced reactive oxygen species (ROS) level or increased antioxidant enzymatic levels where observed. Despite these

antioxidants did not affect the MII stage, some authors proposed that their supplementation are essential for the improvement of cytoplasmic maturation, which leads to the acquisition of competence for subsequent embryonic development (Mukherjee *et al.*, 2014; Sovernigo *et al.*, 2017). Therefore, our results showed, unlike the last results presented above, that UA could rescue oocytes from aging effects in both aged adult and prepubertal females which present lower competence, improving maturation rates.

One of the main contributors to poor fertility outcomes affecting oocyte quality is mitochondrial functions which are compromised with advanced maternal age. Many mitochondrial processes depend on the maintenance of membrane potential for the proper function of cells, including ATP production, maintenance of Ca^{2+} homeostasis, cell apoptosis and ROS generation (Goto *et al.*, 2013; Yao *et al.*, 2018). Mitochondrial membrane potential (MMP) have been widely studied in different models relating that both, aged gametes after ovulation and maternal aging, induce the loss of mitochondrial function (Liu *et al.*, 2017; Pasquariello *et al.*, 2019). Consequently, the loss of MMP was negatively reflected on oocyte and embryo development (Lord, Martin and Aitken, 2015; Liang *et al.*, 2017). According to these findings, in our study, a reduced MMP ratio was found in aged oocytes from both prepubertal and adult cows. Moreover, UA supplementation to the culture medium induced an increase on MMP of prepubertal and adult oocytes matured during 22h, reverberating in higher cleavage rates. These data are in agreement with the results found by Liang and co-workers (2017) which observed a MMP enhance when bovine oocytes were supplemented with melatonin during 22h. However, we also identified a reduction of MMP in aged oocytes supplemented with UA, denoting that UA may not be able to reverse the negative effect of aging in MMP. Discrepant results have been observed concerning the MMP after supplementation of aged oocytes with antioxidant compounds. Indeed, several authors related greater levels of MMP on oocytes after supplementation with melatonin (Liang *et al.*, 2017; Marques *et al.*, 2018) and laminarin (Yao *et al.*, 2018), whereas others have observed the opposite, a decreased MMP in aged oocytes treated with L-carnitine (Jiang *et al.*, 2019) and melatonin (Wang *et al.*,

2017). Also Ryu and colleagues (2016) showed a reduction of MMP in mice myoblasts cultured with UA. The obtained results were consistent with PTEN-induced putative kinase protein 1 (PINK-1) expression noticed to be induced in aged myoblasts treated with UA. In fact, PINK1 functions as a sensor for mitochondrial damage, imported into the inner membrane of healthy mitochondria, where it is degraded by proteolysis and maintained at low concentrations. On the opposite, in damaged mitochondria, characterized by loss of MMP, PINK-1 accumulates in the organelles due to the inhibition of its degradation and subsequently recruits Parkin from the cytosol and activates the autophagic machinery to engulf damaged mitochondria (Reviewed in Youle and Narendra, 2011). Regarding our results, further studies should be addressed to deepen the mechanism of action of UA in the oocyte mitochondria and explain the identified differential effects on aged and physiological mature oocytes.

The current classification of oocyte quality is performed through evaluation of morphological criteria. However, the evaluation of oocytes through this method only provides superficial and approximate information, remaining a limited method (Rienzi, Vajta and Ubaldi, 2011). Mitochondrial oxidative phosphorylation is the major producer of ATP in mammalian cells, being directly associated to the oxygen consumption. Indeed, oxygen consumption rate (OCR) has been reported as a potential predictor for evaluating reproductive outcomes (Muller *et al.*, 2019). Studies revealed that oocytes which present higher oxygen consumption before fertilization, present higher ability for fertilization and developmental potential (Tejera *et al.*, 2011, 2012). In our study, a preliminary test was performed to assess the effect of UA on COCs OCR. Our data, did not present statistical differences due to the limited of tests performed during the COVID pandemic. However, we observed that the UA treatment enhanced more than two-folds the basal OCR of COCs when compared to the control group. Previously Ryu and co-workers (2016) reported an induced mitophagy and an increased basal OCR in mouse skeletal muscle following UA treatment, compared with control, resulting in an overall improvement of cellular functions. In view of the promising results, more studies are needed to give a better understanding and to assess the OCR associated

with post-ovulatory aging and the influence of maternal age on oocytes treated with UA.

Intrinsic quality of oocytes has been widely reported as the main determinant of subsequent embryonic development. Accumulated evidence revealed that several cellular and molecular abnormalities occur during extended *in vitro* maturation periods as well as during *in vivo* post-ovulatory aging (Lord, Martin and Aitken, 2015). Furthermore, these abnormalities can exert relevant impacts on oocyte quality reverberating on embryo production (Rodrigues-Cunha *et al.*, 2016). To demonstrate that UA can act as an anti-aging compound and improve oocyte quality delaying oocyte aging, we investigated the developmental capacity of oocytes after *in vitro* fertilization. In our study a significant ($P \leq 0.01$) harmful effect of gamete aging on cleavage (22h=79.5% and 30h=68.6%) and day 7 embryo produced rates (22h=18.6% and 30h=12.1%) were observed. Accordingly, several authors referred that old females have an age-associated decline in reproductive capacities, reflected in lower fertility rates and poor embryo quality (Yamamoto *et al.*, 2010; Goto *et al.*, 2013). On the other hand, previous studies testing other antioxidant molecules to rescue aged oocytes have reported the beneficial effect of a few of these compounds during *in vitro* maturation on the embryo development of aged females from different species, such as bovine (Jiang *et al.*, 2019), pig (Wang *et al.*, 2017) and mice (Zhang *et al.*, 2019). For instance, L-carnitine was tested on bovine aged oocytes, but no significant differences were found on the cleavage rates. However a significant increase in the rate of zygotes developed to the blastocyst stage was identified, compared to aged oocytes without L-carnitine (Jiang *et al.*, 2019). Our results also demonstrated that UA supplementation during physiological maturation (22h) improved the cleavage rate in prepubertal and adult females. Although the cleavage rates of UA aged oocytes were not significantly different from control aged oocytes, higher D7 embryos rates were identified in UA aged oocytes from both prepubertal and adult females. These results pointed out that the anti-aging effect of UA previously identified in different cell cultures (Ryu *et al.*, 2016; Liu *et al.*, 2019) is valid for oocytes and embryos. Oocyte aging is a multifactorial process that impairs the development of the embryo and restoring

the developmental capacity of aged oocytes is an important objective that was attained in the present study.

Additionally, we also assessed the preventing role of UA in the age-related deterioration on embryo quality. Although in this study no significant differences were found on the rates of each embryo quality scored, the oocytes supplemented with UA increased the number of transferrable embryos from excellent/good quality, compared to the untreated ones. Previous studies, also had reported higher oocyte quality, leading to the improvement of blastocyst quality. Jiang and colleagues (2019) have reported that L-carnitine treatment could reduce levels of ROS and improved levels of glutathione (GSH) as well as others antioxidant enzymes, improving the quality of embryos developed from aged bovine oocytes. The differences between Jiang and colleagues (2019) results and ours may be due to the different techniques applied to assess embryo quality. The morphological evaluation of embryo quality remains a subjective method which depends on the observer's experience. In the future, more studies should be addressed using other techniques to accurately assess embryo quality.

It is widely known that oxidative stress play a crucial role in age-associated fertility. Oxidative stress is one of the major contributor for low efficiency in oocyte maturation, deterioration of oocyte quality, impairing subsequent embryo development (Song *et al.*, 2016). Intercellular communication existent between the gamete and somatic cells are crucial for the proper development of high quality oocytes. Changes in the microenvironment of aged ovaries have been reported, such as decreased antioxidant enzymatic activity leading to an impaired ROS scavenging efficiency. Furthermore, GCs from young and older females showed to have differentially expressed genes associated to antioxidant activities and maternal age (Tatone *et al.*, 2006; Al-Edani *et al.*, 2014; Hui *et al.*, 2017). Thus, it is of great importance to found mechanisms to manage oxidative stress in order to rescue oocyte from aging and to overcome infertility issues. The Nrf2-Keap1 pathway has been extensively studied and its capacity to cope with the deleterious effects of oxidative stress and exerting antioxidant proprieties clearly established (Lewis *et al.*, 2015). Numerous

publications have reported the importance of the activation of the Nrf2-Keap1 signaling pathway, showing its beneficial effect on reducing oxidative stress caused by ROS (Akino *et al.*, 2018; Liu *et al.*, 2019), and its relationship with longevity (Ma *et al.*, 2018).

To further demonstrate the potential anti-aging effect of UA on GCs through the activation of the Nrf2 signaling pathway, we assessed the mRNA expression level of *NFE2L2* and its downstream antioxidant (*NQO1*). Our results showed a significant influence of female age in the level of *NFE2L2* transcripts that decreases with age. Indeed, greater mRNA expression levels were observed in prepubertal cows than those found in adults. This is in agreement with previous studies which reported a highly expressed level of Nrf2 protein and mRNA on ovarian tissues of childbearing age mice, whereas in aged mice a lower expression was found. The same analyses were performed on human GCs, from donors aged between 22 and 49 years old and a decrease in Nrf2 with age was detected, as observed in mice. It was suggested, that decreased expression of Nrf2 may be involved in the decline of reproductive capacity of older women and its control may have important implications in delaying ovarian aging (Ma *et al.*, 2018; Sindan *et al.*, 2018). Furthermore, Akino and co-workers (2019) showed that the activation of Nrf2-Keap1 pathway through the dimethylfumarate administration could reduce ROS levels. Higher expression level of *NFE2L2* and *NQO1*, mRNA and protein, have been identify due to the Nrf2 activation, could alleviate ROS levels and lead to delayed infertility. Similar results regarding the effect of UA in senescent human skin fibroblasts were reported. When these cells were treated with UA, a significant increase in the mRNA expression of Nrf2 targeted genes, such as *SOD1, NQO1, GCLC* and *HMOX1* were reported. This increased mRNA expression level was correlated with significantly reduced ROS levels, suggesting that UA enhanced antioxidant activity through the activation of Nrf2-mediated oxidative response, indicating a strong antioxidant and anti-aging capacity of UA. (Liu *et al.*, 2019). In our study, we observed that the expression level of *NFE2L2* and *NQO1* genes was differentially affected by UA supplementation. Higher levels were identified in the GCs retrieved from adult females treated with UA, whereas in the

prepubertal females this expression was diminished. These findings suggest that *NFE2L2* may be involved in ovarian regulatory functions, probably with mechanism of action adapted to the age of the female, as reported by other authors (Ma *et al.*, 2018; Sindan *et al.*, 2018). Therefore, further studies should be addressed to confirm the reduction of ROS through the UA activation of Nrf2-Keap1 signaling pathway and subsequent improvement of produced blastocyst, as well as the UA effect on mRNA expression on post-ovulatory aged oocytes.

Besides damages of mitochondrial DNA (mtDNA) and the decline in MMP (Hammond *et al.*, 2016; Lord, Martin and Aitken, 2015), the disruption of mitochondrial gene expression have also showed to contribute to mitochondrial dysfunction with age. Zhang and colleagues (2019) reported that some genes involved in the oxidative phosphorylation (OXPHOS) were significantly downregulated in the germinal vesicle (GV) stage of oocytes from age mice, compared with those from young mice. Most of these downregulared genes encode for proteins of the subunits of the complex I, including *mt-ND2, mt-ND3, mt-ND4, mt-ND4L* and *mt-ND5*, revealing a disturbed mitochondrial respiratory chain and OXPHOS function, as oocytes aged. Despite in our study, none significant difference were found in the *mt-ND5* mRNA expression, between the GCs retrieved from prepubertal and adult cows, the UA supplementation showed a mRNA increase in these two groups. Moreover, we also assessed the mtDNA copy number, because the content of mtDNA in CCs has been positively associated with oocyte quality and embryo development. This method is considered as one of the best non-invasive approaches to evaluate the oocyte quality and its development potential(Ogino *et al.*, 2016). For instance, Taugourdeau and colleagues (2019) observed that during *in vitro* fertilization GCs that presented higher mtDNA content were closely related to successfully implanted embryos. Although in our study no significant differences were identified between the mtDNA copy number in GCs from prepubertal and adult cows, neither when treated with UA, we observed a greater number of oocytes which developed to the blastocyst stage, compared with the untreated groups. Also, Ryu and co-workers (2016) reported that the mtDNA content and protein

level from the respiratory complexes did not change in mice myoblasts supplemented with UA. Further studies should be performed to disclose the mechanism of action of UA in improving female fertility.

Chapter 6 – Conclusion

Nowadays, female reproductive aging is considered as an emerging health problem, due to the advanced age of women delaying motherhood, which reflected in poor fertility outcomes. The research for new therapeutic approaches to delay ovarian aging is of great importance to rescue aging-related alterations in ovarian function and has been widely studied in the past decades. In the past years, a marked progress has been made, through the research of anti-ovarian aging agents or alternative approaches, such as antioxidants therapies, autophagy inducers, among others. Also, current research on aging with appropriate models, have provided new insights to better understand the mechanisms underlying this phenomenon.

The results obtained in this study, confirmed the harmful effect of oocyte aging on its developmental competence. Moreover our model for aging female gametes proved to be very efficient and useful to study different problems associated with reproductive aging and consequent fertility impairment. Additionally, UA supplementation during the maturation process of aged oocytes improved the rates of maturation and produced embryos as well as it seems to ameliorate their quality. Therefore, an anti-aging effect of UA in rescuing aged gametes was identified for the first time, improving the blastocyst development, which leads to an increased number of good quality embryos able to transfer. A positive effect of UA on physiological maturation, MMP and embryonic development was also identified.

In conclusion, UA treatment provides a new therapeutic potential approach to prevent or delay gamete aging, and improve the subsequent blastocyst formation and fertility outcomes during human assisted reproductive technologies.

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Supplementary data

Tabela 6 - Results of nucleic acid extraction from different commercial kits (TRIzol™ Reagent - Invitrogen; Quick-RNA™ MiniPrep - ZYMO RESEARCH; PureLink™ RNA Mini Kit - Invitrogen; AllPrep DNA/RNA Micro Kit - QIAGEN; High Pure PCR Template Preparation Kit - Roche Life Science) in oocytes, cumulus cells (CCs) and granulosa cells (GCs). The choice of the Kit was directly related to its ability to extract higher amount of good quality RNA and DNA, suitable for direct use in further analyses.

Samples	Extraction	ng/µL	A260/A280	A260/A230	Kit
CC (of 40 oocytes)	RNA	86.4	1.48	0.18	TRIzol
GCs (1well)	RNA	37.3	1.79	0.15	TRIzol
GCs (½ well)	RNA	54.3	1.51	0.18	TRIzol
CCs (of 20 oocytes)	RNA	3.8	0.78	0.7	ZYMO RESEARCH
GCs (1 well)	RNA	63.5	2.03	1.72	ZYMO RESEARCH
GCs (½ well)	RNA	13.2	1.92	0.93	ZYMO RESEARCH
GCs (1 well)	RNA	229.3	1.93	1.34	PureLink
Oocytes (n=40)	RNA	3.7	2.58	0.01	QIAGEN
Oocytes (n=80)	RNA	2.7	1.7	0.01	QIAGEN
GCs (1well)	DNA	96.8	1.88	1.67	Roche
GCs (½ well)	DNA	44.8	1.79	1.24	Roche
Oocytes (n=40)	DNA	13.3	1.61	0.26	QIAGEN
Oocytes (n=80)	DNA	11.3	1.7	0.15	QIAGEN