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The role of cellular senescence in female reproductive aging and the potential for senotherapeutic interventions

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BACKGROUND: Advanced maternal age is associated with decreased oocyte quantity and quality as well as uterine and placental dysfunctions. These changes lead to infertility, pregnancy complications and birth defects in the offspring. As the mean age of giving birth is increasing worldwide, prevention of age-associated infertility and pregnancy complications, along with the more frequent use of ART, become extremely important. Currently, significant research is being conducted to unravel the mechanisms underlying female reproductive aging. Among the potential mechanisms involved, recent evidence has suggested a contributing role for cellular senescence, a cellular state of irreversible growth arrest characterized by a hypersecretory and pro-inflammatory phenotype. Elucidating the role of senescence in female reproductive aging holds the potential for developing novel and less invasive therapeutic measures to prevent or even reverse female reproductive aging and increase offspring wellbeing.

OBJECTIVE AND RATIONALE: The review will summarize the positive and negative implications of cellular senescence in the pathophysiology of the female reproductive organs during aging and critically explore the use of novel senotherapeutics aiming to reverse and/or eliminate their detrimental effects. The focus will be on major senescence mechanisms of the ovaries, the uterus, and the placenta, as well as the potential and risks of using senotherapies that have been discovered in recent years.

SEARCH METHODS: Data for this review were identified by searches of MEDLINE, PubMed and Google Scholar. References from relevant articles using the search terms 'Cellular Senescence', 'Aging', 'Gestational age', 'Maternal Age', 'Anti-aging', 'Uterus', 'Pregnancy', 'Fertility', 'Infertility', 'Reproduction', 'Implant', 'Senolytic', 'Senostatic', 'Senotherapy' and 'Senotherapeutic' where selected. A total of 182 articles published in English between 2005 and 2020 were included, 27 of which focus on potential senotherapies for reproductive aging. Exclusion criteria were inclusion of the terms 'male' and 'plants'.

OUTCOMES: Aging is a major determinant of reproductive wellbeing. Cellular senescence is a basic aging mechanism, which can be exploited for therapeutic interventions. Within the last decade, several new strategies for the development and repurposing of drugs targeting senescent cells have emerged, such as modulators of the anti-inflammatory response, oxidative stress, DNA damage, and mitochondria and protein dysfunctions. Several studies of female reproductive aging and senotherapies have been discussed that show promising results for future interventions.

WIDER IMPLICATIONS: In most countries of the Organization for Economic Co-operation and Development, the average age at which women give birth is above 30 years. Currently, in countries such as the Netherlands, Australia, Spain, Finland, Germany and the UK, birth rates among 30- to 34-year-olds are now higher than in any other age groups. This review will provide new knowledge and scientific advancement on the senescence mechanisms during female reproductive aging, and benefit fundamental and clinical scientists and professionals in the areas of reproduction, cancer, immunobiology and fibrosis.

Key words: ovarian aging / cellular senescence / senotherapy / atresia / aging / cell signaling / cytokines / ART / DNA damage / senolytic drugs

Introduction

Cellular senescence is a stress response aimed at inhibiting the proliferation of aged or damaged cells, ultimately leading to a state of permanent growth arrest (Calcinotto *et al.*, 2019). Cellular senescence can have both beneficial and detrimental effects for the organism (He and Sharpless, 2017; Calcinotto *et al.*, 2019) and was originally recognized as a consequence of cellular replication as organisms age (Hayflick and Moorhead, 1961). At each cell division, the chromosome ends, areas which are called telomeres, become shorter. Once they reach a critical point, telomeres activate the DNA damage response (DDR) pathway, resulting in a p53-dependent cell cycle arrest (Herbig *et al.*, 2004). In addition to replication, various unfavorable intrinsic or extrinsic stimuli that provoke premature cellular senescence have been identified. Such stimuli include irreparable DNA damage, activation of oncogenes or inactivation of tumor suppressors, oxidative stress (OS), chemotherapy, mitochondrial dysfunction or changes in epigenetics. Furthermore, secondary senescence can be induced through paracrine signaling molecules by a primary senescent cell (Hernandez-Segura *et al.*, 2018).

Depending on the insult and cell type, the p53/p21 or p16Ink4a/Rb tumor suppressor networks are activated, leading to sustained suppression of genes needed for normal continuation of the cell cycle (Pignolo *et al.*, 2020). Besides cell cycle arrest, other characteristics of senescent cells include an enlarged and flattened morphology *in vitro*, persistent DDR activation, upregulation of cell cycle inhibitors, accumulation of senescence-associated heterochromatic foci, increased senescence-associated β-galactosidase (SA-β-gal) staining and secretome alterations (Hernandez-Segura *et al.*, 2018; Paez-Ribes *et al.*, 2019).

Despite being growth arrested, senescent cells display metabolic activity and secrete various factors, such as inflammatory cytokines, chemokines, growth factors and matrix metalloproteinases. This pro-inflammatory and pro-apoptotic paracrine activity of the senescent cell is referred to as the senescence-associated secretory phenotype (SASP). The production of SASP factors can further reinforce senescence, and the nuclear factor-κB (NF-κB) is an important transcription factor involved in SASP regulation as well as promotion of senescence in collaboration with p53 (Chien *et al.*, 2011). As an organism acquires senescent cells, it acquires the phenotype and morbidities associated with aging, such as cardiovascular disease and cancer (Rea *et al.*, 2018).

Since senescent cells steadily accumulate during biological aging, contributing to many diseases, the antagonistic pleiotropy theory has been used to explain the evolutionary purpose behind it. According to this hypothesis, genes that contribute to fertility and survival in youth are favored through natural selection despite their deleterious effects later in life. For instance, senescence-induced growth arrest is beneficial during youth as it suppresses tumorigenesis and contributes to survival at the fertile stage of life. However, accumulation of senescent cells with advanced age increases frailty and morbidity. Therefore, from an

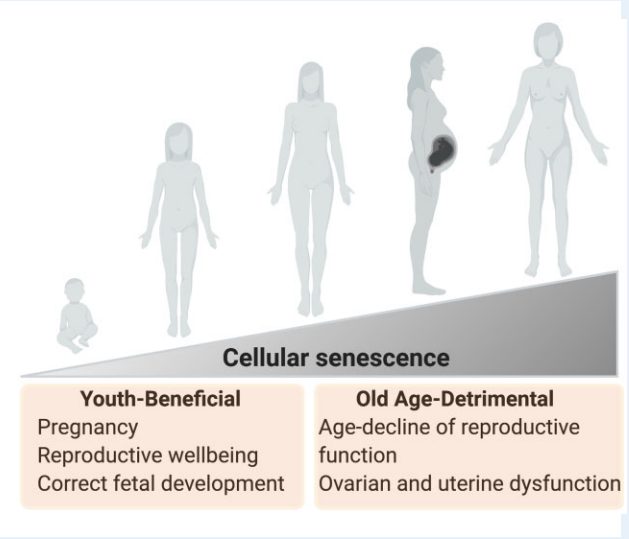

Figure 1. Reproductive senescence timeline. Senescent cells accumulate during aging and pathologies. Senescence has beneficial effects during youth to ensure proper embryo development and parturition but has a detrimental impact on uterine, placental and ovarian function later in life.

evolutionary point of view, progressive deterioration of the body as we age is worth the trade-off for the benefits of enhanced fertility in early life (Mitteldorf, 2019) (Fig. 1).

However, human social changes are inclined to develop independently of evolution. Owing to better access to reliable contraceptives in the last decades, there has been a higher number of women attempting to conceive at an age when fecundability is notably decreased (Crawford and Steiner, 2015). Especially in developed countries, academic pressure, desire for financial stability and social factors have resulted in a progressive delay in childbearing since the 1970s (Wennberg *et al.*, 2016; OECD, 2018). In most countries of the Organization for Economic Co-operation and Development, the average age at which women give birth is above 30 years. In fact, in countries such as the Netherlands, Australia, Spain, Finland, Germany and the UK, birth rates among 30- to 34-year-olds are now higher than in any other age group (Steiner and Jukic, 2016). According to different cohort studies, the decline in female fecundability begins around the late 20s to early 30s and accelerates after the mid-30s, especially among nulliparous women (Howe *et al.*, 1985; Rothman *et al.*, 2013; Eijkemans *et al.*, 2014). By the age of 40 years, almost half of women are infertile, meaning unable to become pregnant within 1 year while having sexual intercourse without contraception (Leridon, 2004). In addition to infertility, postponing reproduction can result in an increased risk of pregnancy-related complications, which further decrease any chances of success (LeAn *et al.*, 2017). Several molecular mechanisms are associated with reproductive senescence and will be discussed in this review (Table 1).

ART have been incorporated as an alternative solution for the declining birth rate caused by advanced maternal age. Nevertheless, ART cannot reverse female reproductive aging. As such, even when using IVF a successful pregnancy is compromised by age. After the age of 30 years, the chances of a successful IVF cycle decrease by around 1.5% per year (Ziebe *et al.*, 2001). Oocyte quality gradually decreases as a woman gets older, which poses an increased risk for chromosomal abnormalities and miscarriage (Frederiksen *et al.*, 2018; Pasquariello *et al.*, 2019). In case a donor oocyte is used, impaired placentation and uterine vascular endothelial damage mainly related to immunologic cues and possible uterine aging can result in pregnancy complications such as intrauterine growth restriction (IUGR) and preeclampsia (Sultana *et al.*, 2018). Importantly, access to ART is dependent on various ethical and financial factors that are highly variable between countries (Asplund, 2020; Calhaz-Jorge *et al.*, 2020).

In order to improve implantation and pregnancy outcomes in the modern female population, research has been carried out regarding the prospects of novel senotherapies to prevent, or even reverse, female reproductive aging. In this review, we will focus on aging of the ovaries, the uterus, and the placenta, as well as the potential and risks of senotherapies that have been discovered in recent years.

Female (in)fertility in the 21st century and the aging reproductive system

Ovarian senescence

Age-related infertility has several causes, of which the most significant ones relate to defects at the level of the oocytes. Oocyte quality and quantity are the main limiting factors in female reproductive success (Cimadomo *et al.*, 2018). Ovarian senescence can be part of normal biological aging, where environmental as well as genetic factors influence its onset and determine the age of menopause (Daan and Fauser, 2015; Chow and Mahalingaiah, 2016; Moslehi *et al.*, 2017). In certain genetic disorders, autoimmune diseases or after chemo- or radiotherapy for cancer treatment, an untimely decline in functional ovarian reserve can occur, named primary ovarian insufficiency (POI) (Sukur *et al.*, 2014; Laven, 2016; Zhang *et al.*, 2019a,b). POI is defined as the cessation of ovarian function before the age of 40 years, and, in addition to infertility, it can have many devastating consequences for quality of life (Gargus *et al.*, 2018; Tsiligiannis *et al.*, 2019).

Oocyte quantity

Women are born with a limited number of oocytes. Around gestation weeks 16–20, female fetal ovaries have as many as 7 million oocytes, each surrounded by a layer of granulosa cells, making up primordial follicles. This peak in primordial follicle count during gestation declines non-linearly over time, with 1–2 million follicles remaining at birth. By the onset of the first menarche, the number of primordial follicles is down to 400 000–500 000, of which only ~400 oocytes eventually will be ovulated during reproductive life. This means that 99.9% of follicles will be wasted. When reaching menopause at the mean age of 51 years, fewer than 1000 primordial follicles are left, which no longer sustains ovulation (Baker, 1963; Crawford and Steiner, 2015).

Mathematical models, but not experimental validations, show that ovarian reserve depletion during the fertile years appears to accelerate with age, especially around the early 30s, mainly because of increased oocyte atresia (Faddy *et al.*, 1992). The age of menopause can vary significantly between individuals, mostly depending on genetic factors.

Table I List of molecular mechanisms of ovarian senescence.

Mechanisms	Functional activity	Target related to senescence	Reference
AGEs	Reduces oocyte developmental competence Impair vascularization, induces hypoxia and reduces glucose intake	RAGE, NF-κB	Pertynska-Marczewska and Diamanti-Kandarakis (2017), Tatone *et al.* (2008), Clark and Valente (2004), and Rovillain *et al.* (2011)
DNA damage/genetic background of menopause	Impairs oocyte quality, lowers fecundity	ATM, p53, p21	Zhang *et al.* (2015), Titus *et al.* (2013, 2015), Vermeij *et al.* (2016), Laven *et al.* (2016) and Milanese *et al.* (2019)
Mitochondrial and protein dysfunction	Reduced energy production	Endoplasmic reticulum stress response-mTOR	Lin *et al.* (2019) and Pluquet *et al.* (2015).
Proinflammatory cytokine and inflammaging	Diminish embryo receptivity, weaken immune tolerance, induces fibrosis, impairs ovulation and ovarian wound healing	SASP	Borghesan *et al.* (2020), Shirasuna and Iwata (2017), Briley *et al.* (2016), Mara *et al* (2020)
Oxidative stress	Induces abnormal follicular development, endometriosis, PCOS, infertility	NLPR3, SASP, NF-κB	Agarwal *et al.* (2012), Sasaki *et al.* (2019), Rea *et al.* (2018), Avila *et al.* (2016), and Danilovich *et al.* (2002)
Telomere shortening	Genomic instability, abnormalities in gametes	p53, TERT, Terc	Herbig *et al.* (2004), Kalmbach (2013), Keefe (2020), and Kosebent *et al.* (2020)

AGE, advance glycation product. ATM, ataxia telangiectasia mutated. NLPR3, inflammasome stress sensitive nod like receptor 3. PCOS, polycystic ovary syndrome. RAGE, receptor for advance glycation end product. SASP, senescence associated secretory phenotype. Terc, telomerase RNA component. TERT, telomeroase reverse transcriptase. mTOR, mammalian target of rapamycin.

Of the non-genetic risk factors, cigarette smoking is the main factor proven to lower the age of menopause by about 3 years, limiting reproductive potential (Sun *et al.*, 2012).

Oocyte quality

In addition to the decrease in follicle number, oocyte quality also diminishes with increasing maternal age. The gradual deterioration in oocyte competence begins around the third decade of a woman's life. This coincides with a decrease in oocyte count and partly explains the decline in fertility long before the onset of menopause, along with the abnormal endocrine profile found in reproductively older women (Broekmans *et al.*, 2009; Santos *et al.*, 2015). On a cellular level, lower fertility is mainly caused by the age-associated increased incidence of meiotic non-disjunction during oogenesis, which results in aneuploidies and a higher risk of unsuccessful pregnancy outcomes (Huber and Fieder, 2018).

Main molecular mechanisms of ovarian senescence

The molecular basis for the deterioration of oocyte quality and fertility with age is multifactorial and not fully known. Some known causes will be discussed (Fig. 2 and Table I).

Advanced glycation end products

According to the carbonyl stress theory, the age-associated decline in oocyte quality and fertility is related to the formation and accumulation of proinflammatory advanced glycation end products (AGEs) in the ovaries (Tatone *et al.*, 2011). Accumulation of AGEs in the ovarian microenvironment reduces oocyte developmental competence by promoting protein damage, OS reactions and inflammation (Pertynska-Marczewska and Diamanti-Kandarakis, 2017). AGEs are the end products of non-enzymatic glycation reactions in the body. Additionally, they can be caused by exogenous sources, such as diet and smoking (Merhi, 2014; Kellow *et al.*, 2018).

AGEs accumulate in the oocyte environment during normal aging and a systematic review has shown a potential acceleration of this process in diabetes and polycystic ovary syndrome (PCOS) (Merhi, 2014). AGEs are hypothesized to account for a variety of characteristics of ovarian aging, including impaired vascularization, hypoxia and reduced glucose intake by granulosa cells (Tatone *et al.*, 2008; Pertynska-Marczewska and Diamanti-Kandarakis, 2017). Granulosa cells adjacent to the oocyte differentiate into cumulus cells, which enable bidirectional communication with the developing oocyte. Additionally, in response to the LH surge, granulosa cells can produce inflammatory and tissue remodeling factors, which are needed during maturation and ovulation and can serve as biomarkers of oocyte developmental competence. Finally, granulosa and cumulus cells can act together to

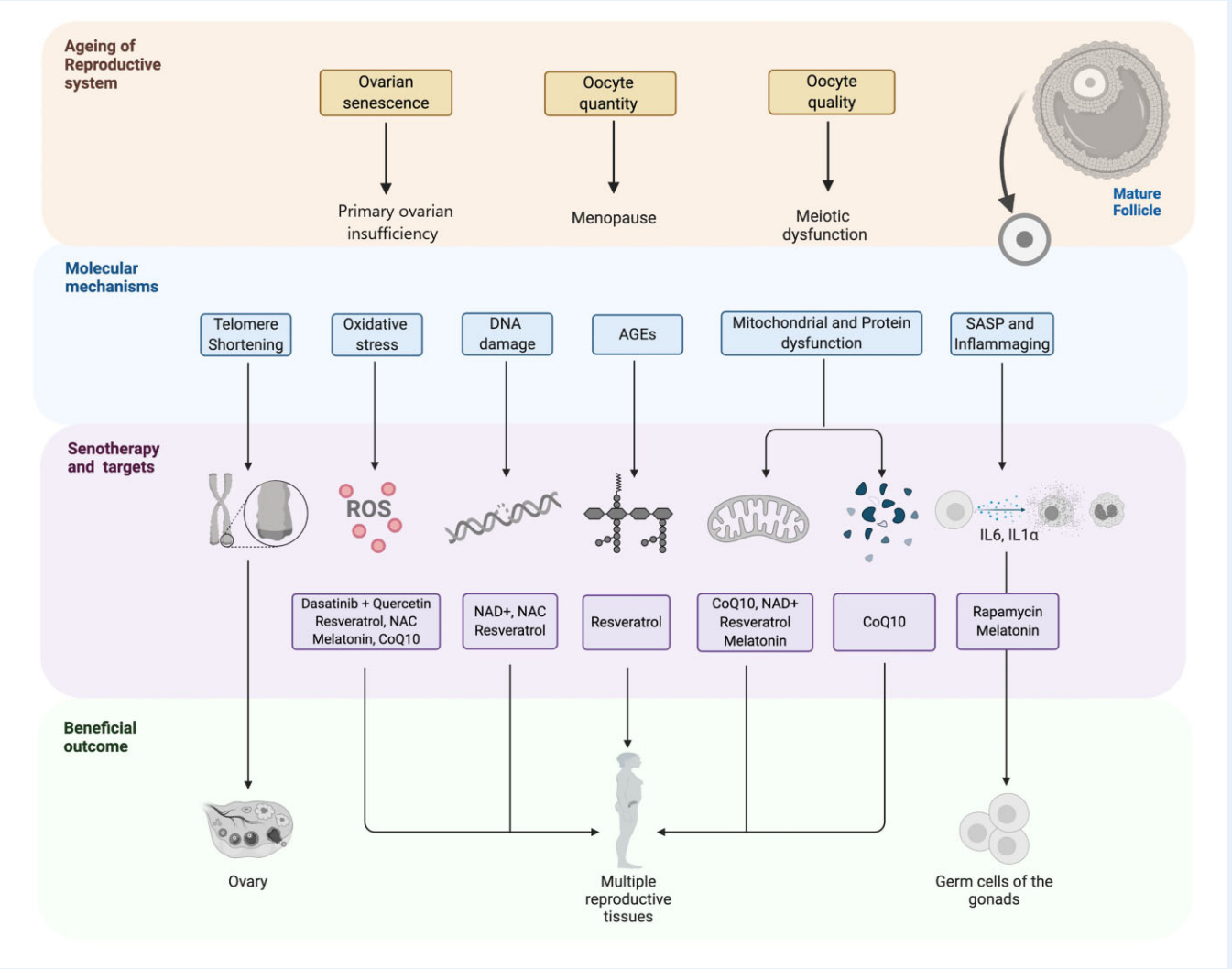

Figure 2. Molecular pathways and targets of reproductive senescence. Female reproductive structures undergoing aging and pathological processes associated with stress and damage-induced senescence are depicted. Molecular mechanisms triggering cellular senescence (indicated in blue boxes) are listed in addition to the senotherapeutics that can eliminate senescent cells and/or alleviate their detrimental effects, providing beneficial effects to multiple reproductive tissues (indicated in green boxes). ROS, reactive oxygen species; AGEs, advanced glycation end products; SASP, senescence associated secretory phenotype; NAD+, nicotinamide adenine dinucleotide; NAC, N-acetyl-L-cysteine; CoQ10, coenzyme Q10.

facilitate oocyte release and enable oocyte fertilization (Robker *et al.*, 2018). Theca cells form a case of connective tissue around the granulosa cells. They also have an important role in folliculogenesis as they synthesize endocrine regulatory factors, such as androgens and growth-regulatory factors, facilitate chemical signaling between granulosa cells and oocytes, and provide structural integrity during follicular development. This ensures the appropriate environment for optimal oocyte maturation (Young and McNeilly, 2010). Detrimental effects of AGEs on fertility are based on to their promotion of protein damage, OS reactions and inflammation in the ovaries, all of which are factors associated with the induction of cellular senescence. Most of these toxic effects are mediated by specific AGE receptors called RAGE, which are expressed on the surface of ovarian granulosa-lutein cells as well as other cells throughout the body (Pertynska-Marczewska and Diamanti-Kandarakis, 2017). The binding of AGEs with RAGE activates NADPH oxidase (Nox) and NF-κB, promoting a vicious cycle of OS and inflammation in the cell, which eventually contributes to senescence (Clark and Valente, 2004; Rovillain *et al.*, 2011). OS is, conversely, a key factor in AGE production as it is necessary in the last step of advanced glycation. Accumulation of AGEs can thus be considered both a source of OS and its consequence (Giacco and Brownlee, 2010).

Oxidative stress

According to the free radical theory of aging, reactive oxygen species (ROS) cause OS and damage to cells, which, over time, contributes to structural and functional changes that are distinctive for cellular senescence and aging (Pomatto and Davies, 2018). Consistent with this, many studies have demonstrated an increase in ROS levels in

mammalian ovaries with age, and excess OS has been suggested to play a key role in the pathogenesis of age-related infertility in females (Agarwal *et al.*, 2012; Sasaki *et al.*, 2019).

Aged oocytes have a decreased production of antioxidants, which then cannot sufficiently counteract the negative effects of OS. Interestingly, conditions of reduced antioxidant status, such as endometriosis and PCOS, are also related to abnormal follicular development and infertility (Avila *et al.*, 2016). The increase of OS in ovarian granulosa cells has been associated with abnormal follicular function through the reduced expression of the FSH receptor (FSHR) and disturbances in the FSHR signaling pathway. This can contribute to the poor response to FSH that has been established in females of advanced age (Danilovich *et al.*, 2002; Avila *et al.*, 2016).

DNA damage

DNA damage results in DNA repair, senescence or apoptosis of the cell, depending on the extent of the damage (Hernandez-Segura *et al.*, 2018). With regard to the oocyte, it is particularly important that there are efficient strategies to identify and repair DNA damage in order to avoid premature aging and oocyte loss, or alternatively to prevent transmission of genetic mutations to the offspring (Stringer *et al.*, 2018). Intact DNA repair mechanisms are essential to maintain metabolic balance and delay aging phenotypes (Vermeij *et al.*, 2016; Milanese *et al.*, 2019), and genome-wide association studies have shown that genome instability is a likely contributor to menopause, further providing evidence that ovarian aging is tightly linked to the ability to repair DNA damage (Laven *et al.*, 2016). In oocytes, DNA double-strand breaks (DSBs) occur continuously, and the diminished quality of oocytes with age is largely attributable to the impairment of DNA repair mechanisms (Zhang *et al.*, 2015). In older individuals, genes responsible for the ataxia telangiectasia mutated (ATM) and RAD3-related (ATR) protein kinase-mediated DNA DSB repair pathway, such as RAD51 and breast cancer gene (BRCA) 1, are downregulated. The expression of ATM in ovaries and oocytes is drastically reduced after the age of 36 years, which is in accordance with the lower fecundity at that age. For BRCA1, this decline occurs around a decade earlier (Titus *et al.*, 2013). Women with BRCA1 mutations have also been shown to have an earlier onset of menopause and a reduced follicular reserve, confirming a direct correlation to fertility (Turan and Oktay, 2020). In addition, BRCA1 deficiency has been associated with deficient meiotic spindle assembly and aneuploid embryos (Xiong *et al.*, 2008). The cause of the diminished expression of BRCA1 and other ATM-mediated DNA DSB repair genes with increasing age is not fully known, but it has been speculated that epigenetic silencing of repair genes as a result of faulty methylation could play a role (Titus *et al.*, 2015).

Telomere shortening

The telomere theory of reproductive aging is one of the latest explanations for the age-related decline in fertility in women (Kalmbach *et al.*, 2015). Telomere shortening causes senescence via a p53-dependent cell cycle arrest (Herbig *et al.*, 2004).

In slow-dividing cells, such as oocytes, a significant cause of telomere attrition is the accumulation of ROS (Yamada-Fukunaga *et al.*, 2013). ROS have been proposed to oxidize the guanine-rich telomeric DNA as well as proteins necessary for telomere maintenance (Singh *et al.*, 2019). Sufficient telomere length has been shown to be pivotal for accurate chromosomal alignment as well as adequate function of the meiotic spindle during meiosis, both of which prevent aneuploidy in embryos (Kalmbach *et al.*, 2013). Additionally, telomere attrition has been suggested to be involved in embryonic fragmentation and developmental arrest (Keefe, 2020).

Another cause of telomere shortening in ovaries is the loss of telomerase activity. Telomerase activity is present in adult ovarian cells, such as germ cells, stem cells and granulosa cells. However, it is low or almost unmeasurable in mature oocytes (Kosebent *et al.*, 2018). Telomerase has two subunits: telomerase reverse transcriptase (TERT) and telomerase RNA component (Terc). Both subunits are normally expressed in oocytes and granulosa cells. In mouse models, Terc has been shown to gradually decrease in the later stages of ovarian aging, contributing to telomere shortening. Lower TERT activity has been associated with lower oestradiol levels in older test subjects, but TERT gene expression in mice has been shown to gradually decrease in younger groups and, surprisingly, increase in the reproductively aged group (Kosebent *et al.*, 2020).

Mitochondrial and protein dysfunction

Autophagy is a cellular system for degradation and recycling of unused or damaged cell organelles and proteins, and it is essential for basal homeostasis (Chun and Kim, 2018). It has been proposed that the decline in autophagy with aging is related to activation of the kinase mammalian target of rapamycin (mTOR), which inhibits autophagy and helps to regulate the SASP in senescent cells (Guo and Yu, 2019). Recently, it has been speculated that increased age could also cause changes in the methylation of autophagy-related genes (Li *et al.*, 2020). Altered methylation of genes associated with autophagy can lead to an increased number of defective proteins and mitochondria in the cell owing to improper elimination.

In ovaries, oxidative damage is known to accumulate in the oocyte during its long period of quiescence before folliculogenesis (May-Panloup *et al.*, 2016). Reduced energy production in oocyte mitochondria has been considered as an important factor behind age-related chromosomal errors, as higher ATP levels in human oocytes have been correlated with positive IVF outcomes (Van Blerkom *et al.*, 1995).

Protein synthesis is essential for adequate oocyte development. The endoplasmic reticulum (ER) plays an important role in providing the ovary with enough correctly folded proteins to meet the challenging demands of oocyte maturation. The age-related increase in cellular OS can disturb protein folding, causing prolonged ER stress in ovaries. This initiates the unfolded protein response (UPR), which can help restore proteostasis or, in the case of severely stressed aged oocytes, can lead to cellular apoptosis (Lin *et al.*, 2019). Little is yet known about the effects of UPR on induction of the senescent cell-cycle arrest pathway, but some interconnections have been observed (Pluquet *et al.*, 2015).

Proinflammatory cytokine and inflammaging

Inflammaging is a phenomenon characterized by a low-grade but progressive inflammatory state, partially associated with the SASP. The accumulation of senescent cells with aging leads to an

inflammatory cascade, which engages the NF-κB, IL-1α, transforming growth factor (TGF)-β and IL-6 pathways (Rea et al., 2018). In immune and reproductive cells, inflammaging has been proposed to have a negative effect on fertility outcomes mostly a result of weakened immune tolerance (Shirasuna and Iwata, 2017). For instance, the function of inducible regulatory T cells (Treg cells) is thought to decrease with age, resulting in inappropriate regulation of inflammatory responses and cytokine production (Jagger et al., 2014). During pregnancy, this translates to a diminished embryo receptivity and excessive inflammation, which leads to inadequate embryo implantation and eventual pregnancy failure (Shirasuna and Iwata, 2017). The effects of immune tolerance on other stages of human reproduction are not yet fully known.

The oocyte microenvironment is altered with reproductive aging, which has been shown to promote ovulatory dysfunction (Meldrum et al., 2016). In a mouse study, genes involved in immune cell recruitment were increasingly expressed in older animals, and a population of multinucleated macrophages was detected in their ovarian stroma (Briley et al., 2016). These unique macrophage cells secrete cytokines, chemokines and growth factors, which contribute to inflammation and ovarian fibrosis. The pro-inflammatory cytokine IL-6 is thought to be the main mediator of age-associated ovarian fibrosis. Correspondingly, ovaries from older mice become stiffer with age (Briley et al., 2016). Ovarian fibrosis contributes to impaired ovulation and ovarian wound healing (Mara et al., 2020).

Apoptotic pathways

Apoptosis is a type of programmed cell death that plays an essential role during organismal development. Apoptosis is found in ovarian follicles at both fetal and adult stages (Hsueh et al., 1994). In the fetus, apoptosis mainly occurs in the oocytes, whereas in adult life primarily in granulosa cells of secondary and antral follicles (De Pol et al., 1998). Apoptosis arguably contributes to a reduction in the total number of oocytes and follicles (Tilly, 1996), and absence of a major pro-apoptotic factor, BAX, has been shown to extend maintenance of primordial follicles, and to extend ovarian lifespan and fertility (Perez et al., 1999, 2007). However, the biological role of apoptosis in ovarian senescence remains controversial. In a mouse model, overexpression of the anti-apoptotic protein BCL-2 increases the number of primordial follicles at birth, but this effect is not maintained in adult stages (Flaws et al., 2001). Moreover, a study in *Caenorhabditis elegans* has shown that apoptosis is essential to maintain oocyte quality (Andux and Ellis, 2008).

Effects of intrinsic senescence on uterus and placenta

The rapid decline in reproductive outcome with advanced age is largely attributable to the increased number of meiotic non-disjunction in the oocyte (Cimadomo et al., 2018). Many of these chromosomal anomalies lead to spontaneous abortion during the first trimester. However, several age-associated pregnancy complications and developmental defects in the offspring present themselves only later during pregnancy and occur in the absence of karyotypic errors in the newborn. Such complications have been thought to originate in the aging uterus, which has been a research topic of interest in recent years (Woods et al., 2017).

IUGR, pre-eclampsia, premature delivery and stillbirth are pregnancy complications, and their incidence and severity increase with advanced maternal age (Norwitz, 2006). In mouse models, the number of live births is significantly deceased in older females, despite little to no reduction in oocyte count and an unaltered number of early implantation sites (Woods et al., 2017). As for birth defects, the risk of congenital heart disease is greater in children of older mothers even in the absence of chromosomal abnormalities in the oocyte (Schulkey et al., 2015). Therefore, it can be speculated that birth defects could be associated with abnormal placental development rather than diminished oocyte quality. In support of this, transferring a deficient embryo to a wild-type younger placenta in mice is sufficient to avert congenital heart problems (Hemberger and Cross, 2001).

Several problems observed during pregnancy and fetal development share an age-dependent underlying pathogenesis. For example, an aged uterus has a weakened response to hormonal signals, which in turn interferes with adequate decidualization of the uterine stroma and leads to severe placentation defects (Gibson et al., 2016; Li et al., 2017).

In comparison to ovarian senescence, little attention has been given to senescence of the uterus or the placenta. Some known mechanisms leading to uterine and placental senescence, as well as their implications, are discussed below.

Morphological changes in the uterus

The uterus provides the environment for early embryonic development, implantation, placentation and fetal development. As with most tissues, several age-associated changes can be observed in the uterine tissue (Shirasuna and Iwata, 2017).

In mice, uterine aging has been associated with the development of uterine fibrosis and dilatation (Kong et al., 2012; Cavalcante et al., 2020). Uterine fibrosis is a consequence of a chronic collagen deposition process associated with prolonged and cyclical uterine exposure to oestrogen. Oestrogen activates the PI3K/AKT/mTOR signaling pathway, which contributes to senescence and fibrosis along with profibrotic microRNAs. Different microRNA families have been found to play a role in the development of fibrosis through targeting genes responsible for ovarian steroid receptors, inflammation-derived genes, and TGF-β and its receptors (Cavalcante et al., 2020). It has been postulated that fibrosis could result in the eventual dilatation of the uterus, as uterine dilatation was observed in old but not in younger mice (Cavalcante et al., 2020), although additional studies are needed to confirm whether this phenomenon exist in humans.

Endometrial decidualization and implantation

The endometrium is the innermost uterine layer and the site where implantation takes place. Additionally, the endometrium is considered to have an important role in identifying and removing defective embryos (Deryabin et al., 2020). During the implantation window, usually around Days 19–23 of the menstrual cycle, the embryo can attach to the endometrium lining of the uterine wall (Teh et al., 2016). Proper

endometrial receptivity requires interaction between the embryo and the endometrium, which involves adequate communication through cytokines, growth factors, hormones and cell adhesion molecules (Karizbodagh *et al.*, 2017).

When using ART, IVF is thought to be one of the most successful methods to combat infertility. However, it takes approximately three to four IVF cycles for a woman to become pregnant, as around 75% of embryos fail to implant in the endometrium (European IVF-Monitoring Consortium (EIM) for the European Society of Human Reproduction and Embryology (ESHRE) *et al.*, 2016; Bashiri *et al.*, 2018). This large margin of error is generally attributable to defective endometrial responsiveness and improper signaling between the fetus and the endometrium (Deryabin *et al.*, 2020).

Studies have shown that an optimal level of senescence and the accompanying cyclin-dependent kinase inhibitor p21 are necessary for normal endometrial stromal cell (ESC) decidualization (Li *et al.*, 2008; Liao *et al.*, 2015). However, the ESC decidualization process can also be negatively affected by cellular senescence (Deryabin *et al.*, 2020).

Normally, undifferentiated pre-senescent ESCs experience hormonal changes during the midluteal phase, which cause them to develop into mature and senescent decidual cells. This differentiation process is driven by the transcription factor FOXO1, which promotes cell cycle arrest and activation of decidual marker genes in an IL-8 dependent manner. The secretion of SASP factors from the senescent decidual cells improves endometrial receptivity and potentiates blastocyst implantation in the endometrium. Subsequently, IL-5-activated uterine natural killer (uNK) cells are attracted to the area by SASP to promote the removal of accumulated decidual cells (Brighton *et al.*, 2017).

In a pathological state and during aging, senescent cell clearance by uNK cells is compromised, and chronic SASP causes long-lasting sterile inflammation in the endometrium and paracrine senescence in the surrounding cells. Since uNK cells are unable to remove the increasingly accumulating senescent decidual cells, endometrial inflammation is exacerbated, eventually leading to defective functionality and receptivity, and to inaccurate embryo identification and implantation (Deryabin *et al.*, 2020).

Placental adaptability

The placenta is a temporary, highly vascularized organ that develops in the uterus during pregnancy. It is attached to the uterine wall and mediates maternal-fetal nutrient and waste exchange through the umbilical cord (Maltepe and Fisher, 2015). Owing to its temporary nature, limited to the duration of pregnancy, the placental membrane is thought to age differently than other tissues in the body (Menon *et al.*, 2016).

The placental tissue is fully developed by the 12th week of gestation and provides immune as well as mechanical protection to the developing fetus (Menon *et al.*, 2016). Once the fetus is mature and ready for delivery, placental shielding is no longer needed. Therefore, it is thought that the placenta ages faster than most other organs in the body. Indeed, elevated levels of senescence markers are found in healthy placental membranes with increased gestational age. For instance, significantly increased amounts of senescence-associated cyclin kinases p21 and p16 are found in term placentas, along with cGAMP. The presence of cGAMP is indicative of cytoplasmic DNA, which is released during senescence and can trigger a SASP (Cindrova-Davies *et al.*, 2018). In addition, the senescence markers p53, phosphorylated H2A histone family member X (γH2AX) and SA-β-gal have been detected in healthy placental syncytiotrophoblasts (Chuprin *et al.*, 2013).

The growing metabolic activity of the fetus leads to an increase in OS in the uterus, which can no longer be counteracted by its antioxidant supply. The developing fetus also causes stretching of placental membranes and uterine walls, adding to OS. OS promotes telomere shortening in placental membranes and results in a telomere-dependent p38 mitogen-activated protein kinases (MAPK)-induced senescence on the fetal side (Menon *et al.*, 2016). Fetal membranes show a decline in functional and mechanical competence at term, displaying characteristics of aging (Menon, 2016).

Several studies have proposed that senescence of fetal membrane cells at term together with the accompanying secretion of SASP and damage associated molecular patterns, such as the high mobility group box 1 protein, and cell-free fetal telomere fragments, may be the trigger for uterine myometrial activation and the initiation of parturition (Menon, 2016; Menon *et al.*, 2016). The levels of SASP-associated cytokine IL-6 along with HMGB1 are higher in fetal compartments, which suggests that inflammation may accumulate on the fetal side and spread to the maternal side, where the labor process is initiated (Menon and Taylor, 2019). However, what initially determines the extent of accumulation of senescent cells and expression of SASP factors in uncomplicated pregnancies is still unknown (Cox and Redman, 2017).

Increased complications during pregnancy

When using oocytes retrieved from a younger donor, there appears to be an age-independent probability of live birth (Hogan *et al.*, 2019). There has been increasing interest in the impact of oocyte donation on adverse obstetric and neonatal outcomes in comparison with other ART (Storgaard *et al.*, 2017). In addition, the risk for pregnancy complications, such as miscarriage, pre-eclampsia, placenta previa, stillbirth and IUGR, remains higher with advanced maternal age (Woods *et al.*, 2017). For example, a meta-analysis of six studies established stillbirth to be 65% more likely in mothers older than 35 years and twice as likely in women above 40 years, compared with women younger than 30 years (Flenady *et al.*, 2011).

Although cellular senescence is essential for natural parturition, premature placental senescence may also contribute to complications in late pregnancy, such as defective placentation and uterine vascular endothelial damage (Crawford and Steiner, 2015). Consistently, a significant reduction in placental telomere length has been found in most late-pregnancy complications when compared with uncomplicated pregnancies. In addition, a reduced expression of the telomerase subunit hTERT has been observed in older placenta (Manna *et al.*, 2019). Telomere attrition is thought to originate from hypoxia-induced OS caused by the inaccurate remodeling of the uterine spiral arteries during gestation. OS leads to DNA damage and activation of the DDR through the p53 pathway, which promotes premature senescence in placental and fetal cells (Manna *et al.*, 2019).

Efficacy of senotherapies for reproductive aging

In the last decade, different therapeutic approaches have been designed to prevent the detrimental effect of cellular senescence. The development of drugs that can either stimulate apoptosis of senescent cells, called senolytics or attenuate senescent cell action and secretion, called SASP inhibitors, senomorphics or senostatics, has been proven to be effective in the amelioration of some age-associated conditions, such as idiopathic pulmonary fibrosis, atherosclerosis and osteoarthritis, although far more testing is needed to confirm their safety and reliability in humans (Roos *et al.*, 2016; Jeon *et al.*, 2017; Justice *et al.*, 2019; Pignolo *et al.*, 2020).

Senolysis can be carried out through the administration of specific drugs that cause inhibition of pro-survival pathways characteristic of senescent cells (Soto-Gamez *et al.*, 2019). Inhibition of the anti-apoptotic proteins (Bcl-2 family) is one of the most used senolytic interventions, although its non-specificity and toxicity has prompted the discovery of more targeted therapies (Zhu *et al.*, 2017). Senolysis can alternatively be achieved through a reduction of senescent cell metabolism by blocking glycolysis, diminishing ATM, histone deacetylase and forkhead box protein O4 (FOXO4) activities, or attenuating the multifaceted PI3K/AKT pathway. Another viable strategy is to incite the immune system to stimulate clearance of senescent cells. For instance, NK cell activity against senescent cells can be enhanced, and antibodies can be created to target senescent cell receptors such as dipeptidyl peptidase-4 inhibitor, urokinase receptor and vimentin (Paez-Ribes *et al.*, 2019).

Senomorphics are molecules that disturb the senescence-associated signaling pathways or common mediators of SASP factors without causing apoptosis. For example, NF-κB and c/EBPβ are two of the major transcription mediators of SASP expression, and administration of neutralizing antibodies that interfere with them or their upstream regulators helps to diminish the detrimental pro-inflammatory effects of the SASP (Paez-Ribes *et al.*, 2019). Alternatively, neutralizing antibodies can be administered against individual SASP factors, such as IL-1α, IL-8 and IL-6, for higher specificity (von Kobbe, 2019). Other senomorphics include telomerase activators, caloric restriction mimetics and diets, sirtuin activators, mTOR inhibitors, antioxidants, autophagy activators and proteasome activators (Kim and Kim, 2019).

To date, there are no clinically feasible techniques to either maintain or reverse ovarian and uterine dysfunction associated with advanced age (Bertoldo *et al.*, 2020). However, important advances have been made in the field of senotherapy during the last years, and pre-clinical research and upcoming clinical trials display great potential for future improvements in female fertility (Table II). The majority of the studies described below are performed in organismal models, particularly in rodents.

Dasatinib and quercetin

When used in combination, senolytics can target a broader range of cellular pro-survival pathways, increasing their apoptotic impact on

Table II List of senotherapies for reproductive aging.

Drug	Functional activity	Molecular target of therapy	Reference
Dasatinib and Quercetin	Antifibrotic uterine effect, reduce accumulation of ROS, reduce decline of SIRT expression	Ephrin receptors/BCL-2 family, p53/p21/serpine, PI3K/AKT	Kirkland and Tchkonia (2017), Cavalcante *et al.* (2020) and Wang *et al.* (2018)
Rapamycin	Prolong murine ovarian lifespan, improved oocyte survival, preserve fertility	mTOR, FOXO3a	Garcia *et al.* (2019) and Sun *et al.* (2018)
NAD and precursors	Improved oocyte quality and fertility, prevent meiotic spindle anomalies	SIRT pathway	Lee *et al.* (2019), Feldman *et al.* (2012), Zhang *et al.* (2014) and Bertoldo *et al.* (2020)
Resveratrol	Improves telomerase activity and telomere length, diminished ROS formation, DNA damage, and AGE product	SIRT1	Lee *et al.* (2019) and Liu *et al.* (2013b)
Melatonin	Reduce accumulation of ROS, increase telomere length, decrease inflammation	MT1/AMPK/p53-signaling pathway, Nrf2, NF-κB regulation	Zhang *et al.* (2019a,b), Reiter *et al.* (2016), Galano *et al.* (2013) and Hardeland (2019)
Coenzyme Q10	Local reduction in ROS, oocyte quantity and quality	Mitochondria	Wang and Hekimi (2016), Özcan *et al.* (2016), Bentov *et al.* (2014)
NAC	Delayed decline in fertility, ROS scavenger	Telomere	Zhang *et al.* (2019a) and Liu *et al.* (2012)

AGE, advance glycation product. AKT, protein kinase B. AMPK, AMP-activated protein kinase. BCL-2, B-cell lymphoma-2. FOXO3, forkhead box O3. MT1, metallothionein 1A. NAC, N-acetyl-L-cysteine. NAD, nicotinamide adenine dinucleotide. Nrf2, nuclear factor erythroid 2-realted factor 2. PI3K, phoshoinositide 3 -kinase. ROS, reactive oxygen species. SIRT, sirtuin. mTOR, mammalian target of rapamycin.

senescent cell subpopulations (Pignolo *et al.*, 2020). Recently, the individual and synergistic senolytic effect of dasatinib (D) and quercetin (Q) has been studied extensively. Dasatinib is a selective tyrosine kinase receptor inhibitor that interferes with dependence receptor, Src family kinase and tyrosine kinase cellular apoptosis resistance networks (Kirkland and Tchkonia, 2017). It has been used clinically in the treatment of chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia (Keating, 2017). Quercetin is a plant-derived bioflavonoid with antioxidant and anti-inflammatory properties that targets the BCL-2 family, p53/p21/serpine and PI3K/AKT anti-apoptotic pathways (Kirkland and Tchkonia, 2017).

Individually, dasatinib targets senescent primary human preadipocytes and quercetin targets senescent endothelial cells and mesenchymal stem cells. However, in combination (D + Q), these drugs are effective in eliminating several other subtypes of senescent cells (Pignolo *et al.*, 2020). In humans, two studies using intermittent D + Q senolytic therapy have been conducted, and they have resulted in significant improvements in lung and kidney pathologies while displaying reduced levels of senescence biomarkers (Hickson *et al.*, 2019; Justice *et al.*, 2019).

In the murine uterus, intermittent oral administration of a D + Q combination resulted in an upregulation of p53 and a downregulation of the profibrotic microRNA miR34a, which suggest a potential antifibrotic uterine effect, although this was not detected *in vivo* (Cavalcante *et al.*, 2020). In addition, D + Q administration did not result in changes in uterine dilation of aged mice. Moreover, no changes were found in uterine levels of the fibrosis-associated PI3K/AKT1/mTOR signaling pathway or the phosphatase and tensin homolog (PTEN) protein, which inhibits PI3K. However, this may be due to dose-dependent effects of D + Q and a shorter duration of the study (Cavalcante *et al.*, 2020). Additional research is necessary to better establish the effect of D + Q therapy on the PI3K/AKT1/mTOR pathway in aged fibrotic uterine tissue.

Single treatment with quercetin has been shown to reduce the age-associated accumulation of ROS and morphological changes in cultured murine oocytes. In addition, quercetin improved abnormalities in oocyte meiotic spindle assembly and postponed age-associated decline of sirtuin (SIRT) expression, which has been correlated with deterioration in oocyte quality (Wang *et al.*, 2017). Another mouse study has demonstrated that a low dose of quercetin can increase the antioxidant potential of the ovary through improved expression of oxidative-stress-associated genes, such as superoxide dismutase (SOD)-1, catalase and glutathione synthetase, which are known to decrease with age, although significant changes in ovarian morphology and function were not established *in vivo* (Wang *et al.*, 2018).

Studies on dasatinib and its individual effects on the ovaries have mainly related to treatment of ovarian cancer and will not be discussed in this review. In addition, studies on the aging-associated ovarian effects of D + Q combination treatment are yet to be conducted.

It is important to note that elimination of senescent cells in the uterus poses risks for errors in physiological events, such as decidualization and embryo implantation, which rely on the effect of senescent cells and the local proinflammatory environment they generate (Deryabin *et al.*, 2020). Timing, dosage and potential adverse effects of senolytic therapy should therefore be carefully considered for optimal results.

Rapamycin

Rapamycin is an immune suppressant and caloric restriction mimetic, which functions pharmacologically as an inhibitor of the protein kinase mTOR (Garcia *et al.*, 2019). In pre-clinical models, rapamycin has been shown to reduce OS, prolong health span and even reverse age-associated conditions (Bitto *et al.*, 2016; Zaseck *et al.*, 2016; An *et al.*, 2017; Nacarelli *et al.*, 2018).

Short-term treatment with rapamycin has recently been shown to prolong murine ovarian lifespan regardless of age at initiation, displaying improved pregnancy rates and healthier offspring in reproductively aged test subjects (Dou *et al.*, 2017). Rapamycin inhibits the activation of the initial follicle through regulation of the mTOR and sirtuin signaling pathways, preservation of the ovarian primordial follicular reserve and delay of menopause (Zhang *et al.*, 2019a,b). These changes have been correlated with increased ovarian FOXO3a gene expression and decreased phosphorylation of the FOXO3a protein, which, when retained in its non-phosphorylated form, maintains primordial follicles in their inactive state, thus preserving fertility (Garcia *et al.*, 2019). Additionally, general improvements have been observed in oocyte quality and in the ovarian microenvironment, including increased numbers of morphologically normal oocytes and decreased expression of proinflammatory interleukins during treatment (Dou *et al.*, 2017). Rapamycin treatment is known to promote autophagy, which concomitantly represses apoptosis of germ cells during the formation of the primordial follicle pool, resulting in improved oocyte survival (Sun *et al.*, 2018).

Potential adverse effects during rapamycin treatment include a reduction of ovarian size, irregular menstrual cycles and suppression of follicle development. However, these effects appear to fade after cessation of treatment and, for that reason, it is important that rapamycin treatment is kept short term and is eventually discontinued (Dou *et al.*, 2017). Further studies are still required to ensure its safety before possible translation to human trials.

NAD and precursors

Nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR) are precursors of the metabolic cofactor nicotinamide adenine dinucleotide (NAD+/NADH), which shows decreased availability with advanced age. NADH is an essential redox cofactor and enzyme substrate that mediates energy metabolism, DNA repair and gene expression (Bertoldo *et al.*, 2020). Administration of NAD+ precursors has been successfully used to prevent age-associated conditions from metabolic and neurodegenerative diseases to cancer (Mills *et al.*, 2016; Yaku *et al.*, 2018). Recently, NAD+ has been found to be essential for the activation of the sirtuin pathway and consequently the modulation of cellular senescence, and NAD+ replenishment with NAD+ precursors present significant pharmaceutical potential (Feldman *et al.*, 2012; Lee *et al.*, 2019).

In aged ovaries, NMN treatment improved oocyte quality and fertility in correlation with increased expression of the NAD+-dependent SIRT2. The deacetylase SIRT2 helps to maintain microtubule-kinetochore stability, which is needed for optimal spindle and chromosome organization in the oocytes (Qiu *et al.*, 2018). SIRT2 knockdown in cultured murine oocytes led to increased aneuploidy, but *in vivo* studies have observed opposite results, suggesting alternative mechanisms that could also influence oocyte quality (Zhang *et al.*, 2014;

Bertoldo et al., 2020). Adequate NAD+ levels are essential for optimal energy metabolism during the spindle assembly phase, which requires increased amounts of ATP. Timely administration of NMN could therefore help to prevent meiotic spindle anomalies by boosting NAD+ levels in reproductively older females (Bertoldo et al., 2020).

Different members of the sirtuin family are indicated to play a role in oocyte development, and other NAD+ precursors, such as NR, are also relevant for prevention or improvement of reproductive aging. A recent study on NR supplementation in mice demonstrated improved fertility outcomes related to adequate mitochondrial function in the ovaries (Yang et al., 2020). Additional studies could further help in understanding the exact mechanisms of fertility preservation and restoration when using NAD+ precursor treatment.

Resveratrol

Resveratrol is a natural polyphenol found in the skin of grapes, peanuts and wine with anti-inflammatory and antioxidant effects (Yang et al., 2020). Previous studies have found resveratrol to be a potent activator of the longevity-related protein SIRT1 in different types of cells (Sin et al., 2015; Cao et al., 2018; Lee et al., 2019). Recently, many studies have begun to review the impact of resveratrol in improving female fertility outcomes.

In the ovaries, resveratrol seems to prevent age-associated infertility through the enhanced expression of SIRT1, which improves telomerase activity, telomere length and longevity of the ovarian follicular reserve. Additionally, resveratrol treatment has shown to reduce the expression of p21 in aged mice when compared with the age-matched control group (Liu et al., 2013a,b). In another study, resveratrol treatment diminished methylglyoxal-induced ROS formation, DNA damage and AGE product accumulation in ovaries, minimizing local OS and improving oocyte quality (Liu et al., 2013a,b). Resveratrol further appeared to induce mitochondrial synthesis and autophagy in developing oocytes and granulosa cells, which resulted in improved oocyte competence in a study on reproductively aged cows (Sugiyama et al., 2015).

Notably, in the uterus, resveratrol treatment seems to induce detrimental anti-deciduogenic effects through disruption of the retinoic acid signaling pathway in decidualizing ESCs (Ochiai et al., 2019). Therefore, although resveratrol treatment appears promising *in vitro*, it could potentially have adverse effects in overall pregnancy outcomes by means of lower implantation rates and an increased risk of miscarriage. Additional studies are needed to confirm the optimal dosage as well as the full impact of resveratrol as a fertility drug.

Melatonin

Melatonin (N-acetyl-5-methoxy-tryptamine) is a potent antioxidant mainly produced in the pineal gland. Additionally, melatonin can be synthesized in other tissues, such as the ovary, testes and the bone marrow (Zhang et al., 2019a,b). Melatonin and its metabolites efficiently decrease OS levels by directly scavenging ROS as well as enhancing antioxidant activity in the body (Galano et al., 2013; Reiter et al., 2016). Besides antioxidation, other anti-aging properties of melatonin include modulation of mitochondrial and inflammatory activities (Hardeland, 2013). Moreover, melatonin appears to have anti-apoptotic effects through the inhibition of mitochondrial release of cytochrome c (He et al., 2016).

In ovaries, the melatonin-induced decrease in OS is caused by an upregulation of the antioxidant enzymes glutathione peroxidase (GPx) and SOD via the MT1/p53-signaling pathway (Zhang et al., 2019a,b). Alongside OS reduction, melatonin also helps to prevent ovarian senescence by increasing telomere length, upregulating SIRT pathways and decreasing inflammation (Zhang et al., 2019a,b). In recent studies, melatonin administration has been associated with increased litter size as well as improved oocyte quantity and quality in reproductively older females (Song et al., 2016; Tamura et al., 2017). Some of these developments have been attributable to upregulation of the MT1/AMPK pathway, although the SIRT2-dependent H4K16 deacetylation pathway also appears to specifically affect meiotic quality in oocytes (Zhang et al., 2019a,b; Li et al., 2020). As an immunomodulator, melatonin can either provoke the release of pro-inflammatory mediators or, in alternative circumstances, mitigate inflammation through SIRT1, Nrf2 and NF-κB regulation (Hardeland, 2019). This modulatory capacity of melatonin could prove useful in improving follicle development in aged ovaries, which are chronically affected by the SASP, although further research is needed to evaluate the exact effect on the female reproductive system.

Coenzyme Q10

Coenzyme Q10 (CoQ10; also known as ubiquinone) is a natural component of most cellular membranes in the body. In its reduced form, CoQ10 functions as an antioxidant owing to its ability to strengthen internal antioxidant systems and directly inhibit lipid peroxidation as well as protein and DNA oxidation (Littarru and Tiano, 2007; Kashka et al., 2016). Importantly, CoQ10 is also a potent stimulator of mitochondrial function and a component of the electron transport chain. As such, CoQ10 has a central and critical role in cellular energy production (Wang and Hekimi, 2016).

In the oocyte, CoQ10 synthesis appears to decrease with age, coinciding with the decline in oocyte quality and general fertility (Ben-Meir et al., 2015). Previous research has indicated low plasma CoQ10 levels to be correlated with an increase in spontaneous abortions, although it is not clear whether this effect is related to changes in the oocyte, its environment, or the uterus (Noia et al., 1996). CoQ10 also promotes repair of dysfunctional oocyte mitochondria and reduces the ovarian expression of 8-hydroxydeoxyguanosine (8-OHdG), indicating a local reduction in OS (Özcan et al., 2016).

CoQ10 administration to aged mice has presented several positive effects in improving the age-associated decline in fertility, including prevention of follicular atresia and enhanced oocyte mitochondrial gene expression and activity, leading to an increase in oocyte quantity and quality (Ben-Meir et al., 2015). Additionally, CoQ10 supplementation resulted in an increased number of cumulus cells around the oocytes. CoQ10 treatment in younger mice with normal mitochondrial function did not show significant improvements, implying the effect of CoQ10 to specifically target age-associated mitochondrial dysfunction (Ben-Meir et al., 2015).

A randomized double-blind study conducted on a small group of reproductively older women found the frequency of oocyte aneuploidy to be 46.5% with CoQ10 treatment versus 62.8% in the control group. Clinical pregnancy also increased slightly from 26.7% to 33% with CoQ10 treatment, although neither of these results was statistically significant owing to the small scale of the study (Bentov et al.,

2014). Another clinical study on young women with diminished ovarian reserve demonstrated pre-treatment with CoQ10 to be effective in improving the ovarian response to stimulation during IVF-ICSI cycles as well as improving oocyte and embryo quality (Xu *et al.*, 2018). Such results are encouraging for the treatment of age-associated infertility using CoQ10, although further work is needed to determine the overall effect on pregnancy complications and live birth rates as well as the optimal timing and dosage of CoQ10 supplementation.

N-acetyl-L-cysteine

The antioxidant N-acetyl-L-cysteine (NAC) is not found in nature, instead, it is synthetically produced to help stimulate the synthesis of glutathione, which is the most essential naturally occurring antioxidant (Mokhtari *et al.*, 2017). NAC is a potent scavenger of free radicals, especially oxygen radicals, and it has been suggested to have beneficial effects in reducing OS-induced telomere shortening, telomere fusion and chromosomal instability in the ovaries, resulting in improvement of pregnancy rate (Zhang *et al.*, 2019a,b). A study conducted in mice has proposed NAC to prevent oxidative DNA damage and to significantly improve SIRT1 and SIRT2 expression (Liu *et al.*, 2012). However, expression levels of the apoptosis-associated genes BCL2 and BAX as well as the DNA damage repair gene *Mlh1* did not change with NAC treatment. In the study, mice administered NAC had improved quality of preimplantation oocytes, an increase in the total blastocyst cell number and increased litter sizes. Nevertheless, the larger number of used oocytes along with absence of germ cell renewal still contributed to depletion of oocytes, although at a slower pace than without NAC treatment. Overall, the fertility decline with reproductive aging was delayed with NAC treatment but not fully prevented (Liu *et al.*, 2012).

Generally, studies on the effects of NAC treatment on reproductive senescence are scarce. Some clinical trials have studied NAC efficacy in the improvement of PCOS-associated subfertility, although a systematic review assessing these studies concluded the evidence so far to be insufficient and inconsistent (Showell *et al.*, 2017). More fertility-related studies are necessary to reliably assess the effects and side-effects of NAC administration before it could potentially be used for human reproductive aging.

Metformin

Multiple epidemiological studies have documented the anti-aging properties and chemopreventative potential of metformin. Initially used for the treatment of type 2 diabetes, metformin is now a repurposed drugs with excellent safety profiles and appealing strategy for cancer prevention and treatment in an adjuvant setting. Depending on the target organ, metformin can have direct and indirect activity, both of which converge in the activation of AMP-activated protein kinase (AMPK), which inhibits the mTOR pathway (Pollak, 2012). Metformin has been shown to stimulate ovulation in patients with PCOS (Heard *et al.*, 2002).

Future perspectives

As the age of mothers attempting to conceive becomes progressively higher in developed countries, the associated increased risk of problems such as infertility, pregnancy complications and major congenital defects in the offspring can pose considerable threats to the wellbeing of the prospective mother and child (Schummers *et al.*, 2018). Since maternal age is one of the most significant limiting factors for a favorable pregnancy outcome, diminishing senescent cell burden in the female reproductive system could have notable positive effects within a society (de la Rochebrochard and Thonneau, 2002; Kenny *et al.*, 2013; Thompson, 2019). If successful, delaying reproductive aging with the use of senotherapies could help lower the costs and emotional burden of infertility and recurrent unsuccessful outcomes when using ART (Yilmaz *et al.*, 2017).

The use of senotherapies as a method of delaying or reversing age-associated infertility has been assessed in early animal studies and in a limited number of clinical studies so far (Bentov *et al.*, 2014; Jahromi *et al.*, 2017; Xu *et al.*, 2018). Positive effects have mainly been related to the prevention of follicular atresia as well as improvement of oocyte quality through OS reduction, upregulation of SIRT gene expression, mitochondrial modulation and promotion of autophagy. Most studies to date have focused on the effect of different senotherapies on the ovary, but their uterine and placental consequences should not be overlooked when assessing the overall reproductive outcome. While limiting cellular senescence in the placenta may be a potential strategy to limit pregnancy complications caused by placental senescence, this will be challenging because senescent cells may also play a role during embryo development. Finding a senolytic agent that can only target the placenta and spare embryo senescence may be difficult. Senescent cells and their proinflammatory effects have been reported to play a fundamental role in reproductive processes such as uterine endometrial decidualization, placental formation and parturition. Therefore, the removal or attenuation of senescent cells in the uterus or placenta would likely have adverse effects for overall reproductive success (Velarde and Menon, 2016; Cox and Redman, 2017; Deryabin *et al.*, 2020).

Currently, the main limitation for the use of senotherapies in human reproductive aging is targeting the appropriate senescent cells at the correct time. Firstly, the optimal frequency, dosage, and stage of initiation for senotherapy treatment needs to be established in animal models. That way, senotherapies could be adjusted according to the phase of the female reproductive cycle, and side effects on normal reproductive processes could be minimized. Secondly, a better comprehension of senescence biomarkers and the exact effects that senescent cells have on mammalian female reproduction is necessary. The fact that reproductive senescence occurs naturally across mammalian species is favorable for the development of drugs that can translate well from animal models to humans (Packer *et al.*, 1998; Sharp and Clutton-Brock, 2010; Karniski *et al.*, 2018). However, aging processes can differ to some extent among species, and these differences should be recognized for optimal treatment translation from animal models to humans. Thirdly, further studies are needed to determine the possible side effects of long-term senotherapy and the on- and off-target effects of different pharmacological agents. Since infertility does not normally lead to death, as some diseases do, side effects of senotherapies should be minimal before their use in humans can be justified. As more accurate biomarkers of cellular senescence as well as novel on- and off-target sites of senotherapy treatment are identified, it is plausible that safe and effective senotherapies could be developed to improve IVF success by promoting embryo implantation, or even fully replace ART in the future. It will

also be of interest to explore the possibility of combining senotherapies with other approaches. For example, senotherapies might improve the efficacy of ovarian transplantation, implantation of oocyte stem cells or the effect of drugs that limit ovarian fragmentation, such as inhibitors of the Hippo pathway (Akahori *et al.*, 2019; Hsueh and Kawamura, 2020), thus achieving a superior pro-rejuvenation effect.

Data availability

There are no new data associated with this article.

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L.S., M.B. and M.D. wrote the draft of the manuscript; M.B., M.V. and M.D. reviewed the manuscript; M.D. supervised the study.

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Conflict of interest

M.D. is founder, shareholder and advisor of Cleara Biotech. Cleara Biotech was not involved with the study.

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