Cellular and molecular aspects of ovarian follicle ageing

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It is well established that age-related decline of the biological capacity of a woman to reproduce is primarily related to the poor developmental potential of her gametes. This renders female ageing the most significant determinant of success in IVF. Starting with a reference picture of the main molecular and cellular failures of aged oocytes, granulosa cells and follicular microenvironment, this review focuses on age-related biochemical mechanisms underlying these changes. According to the most relevant concept of ageing, age-associated malformation results from physiological accumulation of irreparable damage to biomolecules as an unavoidable side effect of normal metabolism. More than a decade after the free radical theory of ovarian ageing, biological and clinical research supporting the involvement of oxidative injuries in follicle ageing is discussed. Looking for the aetiology of oxidative stress, we consider the effect of ageing on ovarian and follicular vascularization. Then, we propose a potential role of advanced glycation end-products known to be involved in the physiological ageing of most tissues and organs. We conclude that future investigation of age-related molecular damage in the different ovarian components will be imperative in order to evaluate the possibility to save or rescue the developmental potential of aged oocytes.

Key words: antioxidants; female infertility; follicle development; ovarian function; oxidative stress

Introduction

The reproductive organs of the human female exhibit a rate of ageing that is much faster than that of the other body systems. As reported by studies on natural, non-contraceptive populations, the biological capacity of a woman to reproduce, after a peak of efficiency in the early 20s, fails thereafter in a manner that is universal throughout mammalian species (Wood, 1989). The gradual loss of fertility becomes more dramatic in the late 30s, in spite of ovulatory cycles, ending in menopause at a mean age of 50–51 years (te Velde and Pearson, 2002). The same picture emerges from IVF studies where female age is undoubtedly the most significant factor influencing clinical outcome. According to a recent American report based on >120 000 assisted reproduction procedures, live birth rate per embryo transfer dropped from 43.2% in women <35 years old to 15.1% in women aged 41–42 years and 5.9% in women ≥42 years old (Wright et al., 2006). From an evolutionary point of view, the clock regulating female reproductive lifespan is set up in order to save women from the risks associated with pregnancy and birth delivery in advanced age and to maximize the length of time during which they can bear children (Cohen, 2004). Nevertheless, because of the increasing postponement of the choice of childbearing, populations of most industrialized societies are affected by subfertility related to female ageing (te Velde and Pearson, 2002; Baird et al., 2005). This has become an issue of careful consideration in the diagnosis and treatment of couple infertility, and interest in the possible factors underlying age-related decline of ovarian functions has increased. It is well established, indeed, that although neuroendocrine and uterine factors may reduce fertility with age, the close temporal relationship between the loss of female reproductive potential and changes in the ovarian follicle pool claims that the dominant regulator of reproductive ageing is the ovary (te Velde and Pearson, 2002). This is confirmed by the observation that age-related decline in female fertility can be overcome by oocyte donation from younger women (Sauer et al., 1990).

Ovarian functional decline with ageing has been so far extensively characterized in terms of gradual depletion of ovarian...
folllicles and reduced ability to produce oocytes competent for fertilization and further development (Ottolenghi et al., 2004; Broekmans et al., 2007). The size of the ovarian follicle pool is set up early in life when the ovary is populated by $\sim 7 \times 10^6$ oogonia as early as the fourth month of pregnancy (Byskov, 1986). By entering the prophase of the first meiotic division between 8 and 13 weeks of development, oogonia are transformed in primary oocytes and become surrounded by one layer of flat granulosa cells forming the pool of primordial follicles (Pepling, 2006). At this stage, the oocyte, which has passed through crucial steps such as DNA replication, homologous chromosome pairing and chromosome recombination, becomes arrested in prophase of the first meiotic division in the so-called dictyate phase. At birth, $\sim 1 \times 10^6$ of primordial follicles are present, a number which will decrease during childhood reaching the value of only $\sim 300 \,000$ at menarche (Faddy et al., 1992). According to recent findings, the loss of follicles by atresia after birth would be counteracted by the formation of new primordial follicles from germ-line stem cells (Johnson et al., 2004), a condition still under debate since it was not confirmed by other investigators (Bristol-Gould et al., 2006; Liu et al., 2007). Throughout life, follicles leave the resting pool to enter the growing pool on a regular basis and pass through subsequent developmental stages under the influence of stage-specific subset of intra-ovarian regulators and endocrine factors (e.g. growth factors, cytokines and gonadal steroids; Gilchrist et al., 2004; Pangas, 2007). At various developmental stages, follicles behave differently in response to factors promoting follicular cell proliferation, growth, differentiation and apoptosis and very few reach ovulation (Tilly et al., 1991; Jiang et al., 2003; Craig et al., 2007). As a result, the oocyte/follicle pool declines exponentially with age, with a marked increase in the rate of disappearance from age 37–38 years onwards. When the menopause is reached, the supply is reduced to a thousand or less follicles, a number insufficient to sustain the cyclic hormonal process necessary for menstruation (Faddy et al., 1992).

Thus, based on the above observations the analysis of the molecular and cellular aspects of follicle ageing would require careful consideration of some main points. First, oocytes and granulosa cells of primordial follicles might remain in a ‘resting’ phase for a long time, thus behaving as post-mitotic cells which can be required to start growing after 10–50 years. Second, both primordial and growing follicles become exposed to environmental factors related to the ageing of the ovarian somatic compartment. Third, the development of a competent oocyte intimately depends on the cross-talk between all compartments in the ovary. In spite of these peculiar aspects, the understanding of causal factors for follicle ageing requires the consideration of the theories on ageing mechanisms based on research on tissues and organs other than the ovary. Although it is generally accepted that ageing is a result of both inborn and environmental factors (Hamet and Tremblay, 2003), most of the numerous theories of ageing share the concept that age-associated malfunction results from physiological accumulation of irreparable damage to biomolecules as an unavoidable side effect of normal metabolism and underline the importance of the capability of defensive repair (Yin and Chen, 2005). In this context, the most relevant theory for ovarian ageing, first proposed by Tarin (1995), implies a reduced ability of oocytes and granulosa cells to counteract reactive oxygen species (ROS), which are among the most important physiological inducers of cellular injury associated with ageing (Harman, 1956, 2006).

In this paper, we first review knowledge from humans and mouse model concerning the main age-related features of follicles/oocyte and ovarian microenvironment and their relationship with the reduced developmental potential of gametes developed in advanced reproductive age. Then, searching for molecular mechanisms underlying these changes, we discuss the achievements of biological research supporting the involvement of ‘oxidative stress’ in ovarian ageing including the hypothesis of a potential role of AGEs (advanced glycation end-products), factors playing a main role in the physiological ageing of tissue and organs (Yin and Brunk, 1995; Baynes, 2001).

**Ageing of the ovarian follicle pool**

Although the rate of follicle disappearance has been extensively investigated as reported by Faddy et al. (1992), age-related cellular and molecular aspects of the follicle pool are still poorly defined. It is widely accepted that apoptosis is the driving force behind follicle loss with ageing (Tilly, 2001), a condition suggesting the occurrence of specific age-related alterations in the oocyte and granulosa cells. However, the scarce knowledge of cellular changes which characterize follicle ageing complicates the understanding of the relationship between the decline of follicle quantity and their quality. Indeed, most of the data supporting the concept of reduced follicle quality with ageing have been achieved through the evaluation of the mature oocyte (see below) and granulosa cells obtained from periovulatory follicles after gonadotropin stimulation. Observations on luteinizing granulosa cells show that in women aged $>38$ years these cells are less numerous (Seifer et al., 1996a), produce less steroids (Pellicer et al., 1994) and glycoproteins (Seifer et al., 1996b), contain higher levels of mitochondrial DNA (mtDNA) deletions (Seifer et al., 2002), damaged mitochondria (Fig. 1) and exhibit a reduced expression of antioxidant enzymes (Tatone et al., 2006a; see below) compared with younger women. The role of the somatic compartment in the regulation of follicle ageing has been outlined by studies revealing the ability of aged cumulus cells to facilitate the activation of the death program in oocytes of aged females by providing specific factors such as ceramide, a sphingolipid second messenger involved in cellular senescence (Perez and Tilly, 1997; Perez et al., 2005).

Nevertheless, since previous stages of follicular development are poorly investigated, it remains unknown when and how age-related changes arise. Alterations in the ovarian immature follicles have been reported by deBruin et al. (2004) who thoroughly characterized the morphological appearance of the pool of primordial and primary follicles in ovarian biopsies of healthy women with a mean age of 40.8 years by evaluating the level of follicle atresia. Atresia is accompanied by specific ultrastructural, morphological changes in oocytes and granulosa cells which can be revealed by means of a morphometric analysis at the ultrastructure level (deBruin et al., 2002). According to deBruin et al. (2004) and in contrast to previous findings (Gougeon and Chainy, 1987), the resting follicle pool did not change in size with respect to other follicle stages but undergoes specific age-related cellular changes which include increased cytoplasmic vacuolization,
of follicle quantity, serum levels of inhibin B and E2 decrease, reproductive ageing (Burger et al., 2002). However, FSH increase occurs late in the calendar of events of sacrifice of final oocyte quality (te Velde and Pearson, 2002). This endocrine factor predominantly expressed by granulosa cells of growing non-selected follicles, is considered a sensitive marker of ovarian reserve and, probably, of the early phase of ageing (de Vet et al., 2002), since it reflects the size of the primordial growing follicle pool (Kevenaar et al., 2006). Although a recent study reported a positive correlation between follicular fluid AMH concentrations and embryo implantation rates (Fanchin et al., 2007), its relationship with the qualitative aspects of follicle ageing remains to be clarified.

Ovarian follicle ageing

As described in section Introduction, the primordial oocytes must be ready to start growing at various time points of their adult life for up to 50 years by synthesizing several RNAs and proteins, and by a 100-fold increase of the number of mtDNA copies (Shoubridge and Wai, 2007). At that time, these primordial oocytes with a diameter of 15–20 μm grow to become fully grown oocytes of 70–150 μm, depending on species (Bachvarova et al., 1985). Mitochondria play a primary role in cellular energetic decreased mitochondrial fraction associated with high density of the matrix, and dilatation of smooth endoplasmatic reticulum and Golgi complex in the resting oocytes as well as a reduced number of mitochondria in granulosa cells. Based on the observation that the overall quality score which reflects atretic changes remains unchanged, the authors conclude that morphological changes that occur with ageing are different from those related to atresia and take them as evidence of potential metabolic alterations which may impair the production of competent oocytes or induce atresia when follicles start growing. Age-alterations in the starting point of the growth phase have been recently revealed by a morphometric analysis of the smallest ovarian follicles. Mainly relying on the observation of an increased number of granulosa cells in transitory and primary follicles, the authors conclude that in older women follicles seem to enter precociously the growing phase when compared with the younger counterparts (Westergaard et al., 2007). This condition may explain both the qualitative and quantitative aspects of follicle ageing.

Strong evidence for a close relationship between these two aspects of ovarian ageing was given by the observation of increased rates of aneuploidy in mature oocytes from mice subjected to unilateral ovariectomy (Brook et al., 1984). According to the most relevant hypothesis, the increase in serum FSH levels, reported to occur in the early follicular phase during reproductive ageing, may accelerate final depletion of the follicle reserve (Richardson and Nelson, 1990) thus rescuing ovarian follicles that would otherwise be excluded from selection, at the sacrifice of final oocyte quality (te Velde and Pearson, 2002). However, FSH increase occurs late in the calendar of events of reproductive ageing (Burger et al., 1999) when, with the decline of follicle quantity, serum levels of inhibin B and E2 decrease, thus not explaining the hypothesized decline of follicle quality associated with age. Nevertheless, according to recent results in transgenic mice with rising serum FSH levels, elevated levels of this gonadotropin reduce the percentage of healthy fertilized oocytes and target other stages of the reproductive process without marked changes in ovarian reserve (McTavish et al., 2007), thereby suggesting a complex role of this hormone in the ovarian ageing process. Searching for factors changing with ageing and not involved in the pituitary–gonadal axis, most studies recently focused on the anti-Mullerian hormone (AMH) (Visser et al., 2006 and references therein). This endocrine factor predominantly expressed by granulosa cells of growing non-selected follicles, is considered a sensitive marker of ovarian reserve and, probably, of the early phase of ageing (de Vet et al., 2002), since it reflects the size of the primordial growing follicle pool (Kevenaar et al., 2006). Although a recent study reported a positive correlation between follicular fluid AMH concentrations and embryo implantation rates (Fanchin et al., 2007), its relationship with the qualitative aspects of follicle ageing remains to be clarified.

The unhealthy status of the aged oocyte

Because of the well-established increase with age of human oocyte aneuploidy (Kuliev et al., 2005; Pellestor et al., 2005), most of the research on reproductive ageing emphasized the importance of chromosomal abnormalities in the reduced developmental potential of female gametes. Afterwards, the finding of other age-related nuclear morphological changes such as chromosome decondensation and chromosome misalignment associated with anomalies in the meiotic spindle (Battaglia et al., 1996; Liu and Keefe, 2002) has led progressively to the concept that oocyte aneuploidy represents the most evident effect of a highly compromised cellular machinery and has raised the hypothesis of ooplasmic ageing. The research carried out in recent years has confirmed and expanded this concept giving rise to a picture where oocytes which became fully grown and matured in advanced reproductive age have reduced chances of achieving a level of nuclear and cytoplasmic maturation suitable for sustaining fertilization and embryo development. The unhealthy status of the old oocyte, along with its elevated apoptotic potential (Fujino et al., 1996; Tatone et al., 2006b), is primarily evidenced by an altered pattern of gene expression, a condition which mainly represents the homeostatic alteration of this cell. Recent observations in both mouse and human oocytes have shown that ageing might impair the accumulation of maternal RNAs required for oocyte-specific processes and normal metabolism, or presumably stored for later use during early embryonic development prior to the activation of embryonic genome (Hamatami et al., 2004; Steuerwald et al., 2007). The differentially expressed genes include those involved in mitochondrial function and oxidative stress as well as in a variety of major functional categories including cell cycle regulation, cytoskeletal structure, energy pathways, transcription control and stress responses.

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metabolism, homeostasis and death. They possess their own multi-copy genome, which is maternally transmitted. Mitochondria are directly involved at several levels in the reproductive process since their functional status influences the quality of oocytes and contributes to the process of fertilization and embryonic development (Dumollard et al., 2007). Thus, it is not surprising that microarray analysis of transcripts in mature oocytes that represent the cumulative results of the transcripational activity during follicular growth has revealed prominent age-related changes in the expression of genes involved in ‘mitochondrial function’ (Hamatami et al., 2004; Steuerwald et al., 2007). Genes encoded in the mitochondrial genome and involved in mitochondrial electron transport chain seem to be more highly expressed in old oocytes, whereas genes encoded in the nuclear genome but related to ‘energy pathways’ and mitochondrial function were found more highly expressed in young oocytes. These observations are in accordance with the finding that ageing oocytes contain less ATP (Van Blerkom et al., 1998) and have mitochondria with morphological, genetic and functional flaws including a lower electrical potential at the inner mitochondrial membrane (Wilding et al., 2001) and higher levels of mtDNA point mutations and rearrangements (Keefe et al., 1995).

Although a mitochondrial basis for ooplasmic ageing has not been convincingly established, compelling evidence supports a role for these organelles in the oocyte alterations observed with female ageing. First, clustering of mitochondria in the old ooplasm has been associated with several cellular and morphological abnormalities of oocytes including chromosome scattering, chromosome decondensation, cellular fragmentation, milky or dark cytoplasm, absence of nuclear/chromosomal DNA fluorescence and presence of cellular remains enclosed by the zona pel lucida (Tarin et al., 2001). Second, aged oocytes were found more developmentally sensitive to photosensitization-based type of damage to mitochondria than pubertal oocytes (Thouas et al., 2005). Third, induction of this kind of mitochondrial damage in young oocytes consistently inhibits germinal vesicle (GV) breakdown, meiotic spindle formation, chromosomal segregation and polar body extrusion (Takeuchi et al., 2005). To further prove the role of mitochondria in nuclear events, the authors also demonstrated that fertilizability and developmental potential were improved when mitochondrial injured GVs were transfer into healthy ooplasts (Takeuchi et al., 2005).

In addition to the finding of alterations in the expression of genes involved in cell cycle regulation, numerous evidence supports the idea that a relevant aspect of oocyte ageing is a compromised meiotic clock. These include the finding that loss of control of prophase meiotic arrest in genetically manipulated female mice increases the amount of fragmented oocytes after ovulation and provokes a premature ovarian failure in older animals (Ledent et al., 2005). Increasing evidence shows that age-related aneuploidies results from an aberrant meiosis I that, in turn, reflects an incorrect storage of molecules involved in cell cycle control during the transition from metaphase I to anaphase I. Segregation of sister chromatids or homologous chromosomes during anaphase is a key event in meiosis. Any error in this process may cause aneuploidy (Malmanche et al., 2006). To avoid these errors, cells have evolved a surveillance mechanism, the spindle checkpoint, to detect the attachment of sister chromatids or homologous chromosomes to microtubules. This spindle checkpoint is able to detect a single, unaligned chromosome in the spindle and arrest the cell cycle at metaphase to allow more time for all the chromosomes to move into the correct orientation at the spindle equator before the chromosomes separate (Malmanche et al., 2006). MAD2 is a checkpoint protein known to play a crucial role in meiosis I. Down-regulation of MAD2 was correlated with a shortened duration of meiosis I, meiotic apparatus abnormality and increased oocyte aneuploidy (Wassman et al., 2003; Homer, 2006; Zhang et al., 2006). In addition to the spindle checkpoint, aneuploidies are prevented by the action of cohesion proteins such as SMCbeta1, which maintains physical connections between sister chromatids and facilitate orderly segregation of chromosomes at both meiosis I and meiosis II (Hodges et al., 2005). The role of cohesions and checkpoint proteins in age-related aneuploidies is supported by the finding that human and mouse aged oocytes exhibit reduced amounts of transcripts for MAD2 and SMCbeta1 (Steuerwald et al., 2001; Cukurcam et al., 2007). This condition makes the aged oocyte more prone to precarious chromosome segregation during resumption of meiosis, accelerate the transition to first anaphase and to metaphase II and induce a failure in the coordination of nuclear and cytoplasmic meiotic events (Eichenlaub-Ritter, 1998; Eichenlaub-Ritter et al., 2004; Cukurcam et al., 2007). In addition to an altered meiotic control, a loss of coordination between the events leading to ovulation might be induced by a raised basal level of LH during the follicular phase, observed during a late phase of reproductive ageing (Brann and Mahesh, 2005) which could trigger meiosis resumption before the LH surge (Tarin et al., 2001).

Consistent with the above observations is the finding that oocytes ovulated by reproductively old mice undergo a precarious post-ovulatory ageing, thereby exhibiting a reduced fertilization window (Tatone et al., 2006b). According to the latter, in old oocytes cultured in vitro after ovulation, the activities of cell cycle kinases which control MII state, maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK), decreases more rapidly than in young oocytes in parallel with a more rapid increase of abnormal MII, spontaneous oocyte activation and stimulation of an apoptotic pathway. As similar effects can be induced in young oocytes by MPF and MAPK inhibitors, it is likely that the reduced ability to control MII results in the decay of positive signals targeted to anti-apoptotic factors, such as protein Bcl-2. This condition may be responsible for the increased rate of cell death which characterizes oocytes ovulated during reproductive ageing and suggest the possibility to partially preserve fertility potential of old oocytes by avoiding in vitro incubation prior to insemination during IVF procedures (Fujino et al., 1996; Tatone et al., 2006b).

An important role in age-related meiotic dysfunction may be played by telomeres, known to mediate ageing in mitotic cells. The effects of artificial shortening of telomeres in mice and the observation that aged eggs may derive from precursors which have undergone more DNA replication cycles have led to speculation that aberrant meiosis in older women may be ascribed to telomere shortening (Keefe et al., 2007).

A compromised microenvironment

Although it is likely that the long primordial follicle stage could strongly compromise oocyte differentiation, it is plausible that
age-related nuclear and cytoplasmic damage may occur in GV oocytes during the growing phase and/or in maturing oocytes under the influence of the Graafian follicle microenvironment. The first hypothesis is supported by studies on potential genetic determinants of premature ovarian failure suggesting that this form of ovarian ageing may imply alterations in the ovarian microenvironment involving paracrine regulators of follicular development such as growth differentiation factor (GDF-9), bone marrow protein (BMP-15) and the winged felix transcription factor (FOXl2) (Schmidt et al., 2004; Dixit et al., 2006; Laissue et al., 2006). The regulation of follicle growth also depends on the availability of an adequate vascular supply to provide nutrients as well as regulatory signals (Redmer and Reynolds, 1996). In this respect, it has been reported that genetically altered vascular support might have long-term effects on follicle depletion (van Asselt et al., 2003; Pripp et al., 2005; Tempfer et al., 2005; Kok et al., 2006).

Accordingly, data obtained by Ng et al. (2004) suggested that reduction of ovarian stromal blood flow with increasing age is a relatively late phenomenon being revealed by means of three-dimensional ultrasound with power Doppler only in women aged ≥41 years.

The hypothesis of negative influence of the Graafian follicle microenvironment would imply that old oocytes, at the end of growth phase, still maintain some ‘valuable genetic resources’ as first suggested by Eppig and O’Brien (1995). According to this study, old mouse oocytes seem to improve their reproductive performance when removed from pre-ovulatory follicles and matured in vitro. In this scenario, Cukurcam et al. (2007) have recently reported that the aged oocytes can be partially protected by age-related defects originating during maturation. These authors, in fact, discovered the beneficial effect exerted by the exposure of old oocytes to the follicular fluid meiosis activating sterol (FF-MAS) probably associated with recovered ability to express the cohesion protein SMC1beta and thereby prevent precarious chromatid separation. The finding that old oocytes may be helped to rescue their developmental potential is indirect evidence that the follicular microenvironment of the ageing follicle exerts a deleterious effect on the oocyte in the period preceding ovulation and encourages further research about factors or culture conditions which could improve maturation of old oocytes. In fact, although age-related changes in FF-MAS levels in follicular fluids are unknown, to our knowledge the study by Cukurcam et al. (2007) is the only one testing the potential beneficial effects of components of follicular fluids on old oocytes.

A large number of these factors, being positively correlated to a successful IVF outcome, have been proposed to reflect the healthy status of the oocyte, such as insulin-like growth factor (IGF-II), IGF binding protein (IGFBP-3), IGFBP-4 (Wang et al., 2006), BMP-15 (Wu et al., 2007) lactoferrin (Yanaihara et al., 2007) and AMH (Fanchin et al., 2007), but very little or no data exists on their age-dependent changes (Klein et al., 2000) and/or on their potential effects on in vitro maturation of old oocytes.

It was suggested that an important environmental factor responsible for oocyte senescence might be represented by a reduced oxygen supply to the leading follicle, a condition dependent on a compromised perifollicular vascularization (Gaulden, 1992; Van Blerkom, 1996). Indeed, modifications found in old MII oocytes, such as spindle and chromosome abnormalities, were reported to resemble those occurring in young oocytes obtained from Graafian follicles with reduced perifollicular vascularization and oxygen content (Van Blerkom, 1996; Van Blerkom et al., 1997). In contrast to primordial and preantral follicles which derive their blood supply from the stromal vessels, growing follicles clearly depend on a sufficient ingrowth of capillaries into the theca (Gaulden, 1992). It is well known that dominant follicles have not only a more vascular theca, but also an increased uptake of serum gonadotrophins compared with other follicles (Redmer and Reynold, 1996). In a prospective study based on pulsed Doppler ultrasonographic analysis, Huey et al. (1999) found that oocytes deriving from follicles with optimal vascularization and oxygen content (≥3%) had higher fertilization and developmental potential. Furthermore, studies of perifollicular vascularity before oocyte aspiration by transvaginal power Doppler ultrasonography, which enables a sensitive analysis of the microvessels surrounding each follicle, reported a positive correlation between high-grade vascularity and improved outcome during IVF cycles (Bhal et al., 1999, 2001).

To the best of our knowledge, however, the question as to whether advancing age is associated with decreasing ovarian follicular blood flow has been so far poorly investigated. Recently, Costello et al. (2006) described, for the first time, a significant negative correlation between age and ovarian perifollicular blood flow, which was only observed very late in the follicular phase of ovarian stimulation. Although the authors caution against the validity of their results because of the potential introduction of measurement bias, support to their finding is the observation of increased levels of vascular endothelial growth factor (VEGF) in the follicular fluid from ageing women (Friedman et al., 1997; Klein et al., 2000; Artini et al., 2003). Transcriptional up-regulation of VEGF is involved in the cellular adaptation to hypoxia under control of hypoxia-inducible factor 1, a transcription factor activated by low oxygen tension (Wang et al., 1995) to prevent depletion of oxygen at anoxic levels and subsequent cell death (Bell et al., 2005; Chandel and Budinger, 2007). This growth factor plays a central role in the regulation of angiogenic processes in the ovary (Artini et al., 2003) and in the growth of the ovarian follicle (Artini et al., 1998), where granulosa and theca cells are the main producers of VEGF in response to gonadotrophin (Lam and Haines, 2005).

Although the cause for potential age-related decline in ovarian follicle vascularity remains unknown, the presence of elevated levels of VEGF along with reduced blood flow in the follicular environment of aged ovaries suggests that, in an attempt to compensate for hypoxia, granulosa and theca cells increase the synthesis of VEGF which nevertheless fails in completing the adaptive response. This could be ascribed to a low responsiveness of endothelial cells consequent to possible defective signalling pathways or to an increased distance between the perifollicular bed and the wall of the growing follicle in relation to age (Gaulden, 1992).

Although the effect of ageing on the vascular dynamics in the ovary needs to be elucidated, further insights into this issue may be obtained from the evaluation of potential oxidative stress markers in the aged follicles. As a consequence of a reduced blood supply, the aged maturing follicle may suffer from a decreased uptake of nutritional and regulative molecules as well as from a condition of oxidative stress related to a reduced oxygen supply.
Age-related ovarian molecular injury: the oxidative damage hypothesis

Oxidative stress in ageing

As previously recalled, research on ageing mechanisms is mainly focusing on the concept of spontaneous damage accumulation during normal cellular metabolism (Yin and Chen, 2005). Under this hypothesis, biological reactions underlying ageing are believed to occur spontaneously and to give rise to cellular injuries with a certain universality (Baynes, 2001). According to these observations, the most widely recognized biological reaction leading to ageing is the modification of different kinds of molecules caused by oxidative stress (Harman, 1956; Sohal, 2002).

It is generally accepted that the decline of ageing-associated cellular respiratory functions can result in increased electron leakage and enhanced production of ROS by mitochondria, which in turn affects mtDNA and protein structure and function (Miquel et al., 1980). Within a certain concentration range, ROS regulate cellular functions and act as secondary messenger inducing stress through the activation of specific transcription factors such as NF-kB and AP-1 (Dalton et al., 1999) to uphold energy metabolism and thus rescuing the cell. When the overall balance between physiological ROS production and the total antioxidant defences becomes unbalanced, this disequilibrium may cause a wide spectrum of oxidative damages and may induce release of cytochrome C and other apoptogenic factors from cell mitochondria which eventually drives the cell to apoptosis (Orrenius et al., 2007). To cope with ROS, cells express an array of antioxidant enzymes, including the cytosolic copper/zinc superoxide dismutase (Cu/ZnSOD) and the mitochondrial Mn$^{2+}$-dependent superoxide dismutase (MnSOD) which convert superoxide anions to hydrogen peroxide, which is then transformed to water by catalase (CAT) and by glutathione S-peroxidase (GSSPx) (Wei and Lee, 2002). In the peroxidase reaction, reduced glutathione (GSH) is oxidized to GSSG (oxidized glutathione). The regeneration of GSH is, consequently, of fundamental importance for the ability of cells to challenge exposure to oxidizing metabolites. In the cell, GSSG is reduced by NADPH, through the action of glutathione reductase (GSSG-Rx). Glutathione transferases (GST) comprise a family of multifunctional enzymes that catalyze the conjugation to GSH of a large variety of electrophilic alkyllating compounds, some of which are the products of the oxidative damage of biological membranes and macromolecules (Hayes and McLellan, 1999; Amicarelli et al., 2001).

The possible increase of ROS production with ageing seems to be ascribed more to a lowering of the enzymatic antioxidant defence of the organism rather than to a decrease in the non-enzymatic ones (ascorbate, thiols and tocopherol) (Linton et al., 2001 and references therein). However, since an age-related decrease in the non-enzymatic antioxidant defences in humans has been proven to be difficult to generalize (Kreigel and Zhang, 2007 and references therein), this point remains controversial. Also the indirect proof of a beneficial effect of dietary administration of antioxidants was followed by conflicting results especially in mammals (Thomas, 2004; Bengmark, 2006; Kamel et al., 2006).

Generally speaking, the study of the role of ROS in human health and disease or its changes during ageing, is severely hampered by methodological difficulties in the validation of the level of biomarkers of oxidative stress in biological samples (Dalle-Donne et al., 2006). Indeed, most of the commonly used methods for the determination of the oxidative stress of a biological fluid, such as follicular fluid, have been criticized regarding several aspects including preparation of the sample, sensitivity and specificity of the assay, speed of the analytical method, etc. (Dalle-Donne et al., 2006).

To give just a few examples, the chemiluminescence assay using luminol for the quantification of ROS levels, mainly because of the low stability of the measured biomarkers, requires the presence of enzymatic systems or intact cells for accuracy and reproducibility (Li et al., 1998). For this reason its use on biological fluids after cell removal should be considered inappropriate or not useful (see below). Furthermore, this assay is unable to discriminate between individual oxygen or radical species and many possible interferences limit its application to biological systems (Vilim and Wilhelm, 1989; Wardman, 2007).

Another widely used assay for the determination of the oxidative stress level of a biological sample is the measurement of malonaldehyde (MDA) production as a marker of polyunsaturated fatty acid peroxidation (LPO). Again the effectiveness and reproducibility of this assay has been challenged under several technical and theoretical aspects (Del Rio et al., 2005; Dalle-Donne et al., 2006). Following these and other considerations it has been long recognized that ‘no single measurement of antioxidant status is going to be sufficient but a battery of measurements will be necessary to adequately assess oxidative stress in biological systems’ (Prior and Cao, 1999).

Oxidative damage in the ageing ovary

Oxidative stress, defined as an unbalance between oxidant and antioxidant systems, has been suggested to have a role in virtually all the steps of human reproduction, from gametogenesis to embryo implantation and development as well as in some pathologies leading to sub- or infertility in both males and females. Although several papers appeared in the last few years addressing this point, the role of oxidative stress in female infertility is not clearly understood (Agarwal et al., 2005). The effect of an increase in ROS production without modification of the antioxidant defences leading to oxidative stress has been studied, for example, in human follicular fluid in the search for a correlation with oocyte quality as judged by the in vitro fertilization outcome (i.e. embryo quality, fertilization, cleavage and pregnancy rates). The reported results are highly controversial ranging from reported beneficial effects of an higher level of ROS in follicular fluid on the IVF outcome (Attaran et al., 2000) to an opposite study by Das et al. (2006) who suggested that high levels of ROS in follicular fluid tend to decrease the fertilization potential of oocytes although they both used a luminol-based chemiluminescence assay for the determination of ROS (see below). Interestingly, but not unexpectedly, ROS level in centrifuged follicular fluid was found to be negligible thus confirming that, also in this case, their production is a cellular related event mainly due to the metabolic activity of granulosa cells (Attaran et al., 2000). Oyawooye et al. (2003) measured the baseline total antioxidant capacity (TAC) and its decline over 72 h as a marker of oxygen radical activity in follicular fluid from 63 women undergoing IVF. In this study, whereas baseline low levels of TAC seem to correlate with decreased fertilization...
potential, antioxidant consumption had no predictive value on reproductive success. In another study, Pasqualotto et al. (2004) analysed the LPO and TAC levels in the follicular fluids of 41 women undergoing IVF. They found no correlation between LPO, as measured by the MDA concentration, and TAC levels with respect to oocyte maturity, fertilization rate, cleavage and embryo quality. Intriguingly, they found a positive correlation between pregnancy rate and both LPO and TAC levels but only after adjusting their data for age since, as expected, pregnancy rate was higher in younger than in older women. Indeed, they found that both values are lower in older, non-pregnant women than in younger women who became pregnant. Not surprisingly, other authors concluded that ‘the concentration of oxidative stress markers in follicular fluid do not reflect the reproductive potential of the oocyte’ (Jozwik et al., 1999). Although an explanation for these discrepancies could be partially found in the different protocols adopted by the authors, for example, regarding the patients recruited for the study (Pasqualotto and Pasqualotto, 2007; Chaudhury et al., 2007), it is reasonable to presume that the methodological issues outlined above could strongly contribute to the inter-laboratory variability and therefore, to the overall interpretation of results. All the above observations contribute to the painting of a scenario of uncertainty and confusion regarding the real meaning of ROS production and oxidative stress in female reproduction.

As for many other aspects of human health, the accumulation of damage exerted by increased levels of ROS is claimed to be involved in ovarian ageing (Tarin, 1995,1996). ROS play a role in the modulation of an entire spectrum of physiological reproductive functions such as oocyte maturation, ovarian steroidogenesis, corpus luteal functions and luteolysis and, as recalled above, they are involved in fertilization, embryo development and pregnancy (Agarwal et al., 2005). To ensure physiological levels of ROS, oocytes and granulosa cells in all follicular stages as well as follicular fluid are well endowed with the major antioxidant and detoxifying enzymes (El Mouattassim et al., 1999; Carbone et al., 2003). Several studies in both animal models and humans, suggest that primordial and periovulatory follicles suffer from age-related oxidative stress in association with an impairment of antioxidant enzymatic defences.

Decreased levels of GSH and GST were described in ovulated mature oocytes from aged mice (Tarin et al., 2004). Interestingly, it has been found also that administration of oral antioxidants to reproductively old mice is effective in counteracting the negative effects of female ageing on oocyte quality (Tarin et al., 2002a), whereas similar treatment negatively affects the fertility of young mice (Tarin et al., 2002b). Finally, by using the mouse model, it has been recently reported that a condition of oxidative stress in young oocytes negatively affects spindle stability by decreasing mitochondrial ATP production thereby mimicking the ageing effect (Zhang et al., 2006).

In humans, oxidative damage to the structure of oocyte and granulosa cells was described in the cohort of primordial follicles in women of advanced age (de Bruin et al., 2004). We also described age-related modifications in the antioxidant enzymatic pattern that could impair ROS scavenging efficiency in the follicular environment of periovulatory follicles. Indeed, some of us (Carbone et al., 2003) firstly reported the presence of the major antioxidant and detoxifying enzymes in human follicular fluid. According to this study, the activities of SODs, CAT and GSPPx ensure an efficient scavenging action against ROS, thus preventing them from rapidly diffusing into the oocyte, and the high level of GST activity also contributes to an efficient detoxification from ROS by-products. Moreover, the elevated levels of GSSG-Rx in this compartment efficiently supply GSH, which, besides being a cofactor essential for both GSPPx and GST activity, is also one of the most efficient non-enzymatic antioxidants. Such a pattern of enzymatic defences is significantly affected by reproductive ageing as follicular fluid from older women exhibit reduced levels of GST and CAT activities and higher level of SOD activity. Moreover, the age-dependent changes in SOD and CAT activities cause a reduction in the CAT/SOD ratio and a slight decrease in GSPPx/SOD ratio, thus suggesting a lowering of ROS scavenging efficiency with ageing (Carbone et al., 2003).

Weakening of antioxidant defences also occurs in granulosa cells where reproductive ageing has been associated with down-regulation of Cu/ZnSOD, MnSOD and CAT genes and accumulation of oxidative damage mainly involving mitochondria (Tatone et al., 2006a). The possibility that such a condition may result in increased levels of ROS is supported by the fact that in granulosa cells antioxidant enzymes play a crucial role in the scavenging of superoxide anions and hydrogen peroxide generated during the synthesis of steroid hormones.

These data support the previously mentioned hypothesis that, as for other biological systems, follicle ageing is linked to a decrease in the enzymatic antioxidant defences. To check the other two corollaries, we are also evaluating the possible age-related modifications of the non-enzymatic total antioxidant capability of human follicular fluid and the level of oxidative stress in this biological sample.

Total antioxidant capability was measured by the ferric reducing/antioxidant power assay. This method has been demonstrated to be sensitive and reproducible when compared with other colorimetric assays (Oyawoye et al., 2003; Collins, 2005). No statistically significant differences could be detected between individual follicular fluids obtained from young (24–26 years) and older (>40 years) women (unpublished data).

For the reasons described above, the level of oxidative stress of each sample was not evaluated by using chemiluminescence or colorimetric methods but by assessing the overall oxidative status of follicular fluid proteins. Indeed, since a possible increase in ROS production during ageing should lead to an accumulation of oxidized material into proteins, this could represent a useful marker of oxidative stress (Linton et al., 2001). In this context, we labelled free protein -SH groups with biotin by using MBP (3-N-maleimidopropionyl biocytin) and then analysed them by two-dimensional gel electrophoresis. Since protein cysteiny1 thiols are highly susceptible to oxidation, loss of reduced thiols has been established as one of the most sensitive and stable markers of oxidative stress (Eaton, 2006). Indeed, although oxidation of sulphydryl groups of proteins is to be considered reversible, the accumulation of oxidized molecules is thought to represent a valid marker of the oxidative stress status of biological samples (Davies et al., 1999) and their labelling has been already successfully used for the study of the modifications of the human spermatozoa during capacitation (De Lamirande and Gagnon, 2003). Our results clearly demonstrated a marked quantitative...
and especially qualitative reduction in labelled proteins from follicular fluid of older women. In particular, among others, we demonstrated that a (~56 kDa) protein is strongly labelled in follicular fluid samples from young women, whereas it is practically absent in older women. This reduction in free-SH groups with age strongly suggests that the follicular microenvironment undergoes an increase in oxidative stress with ageing (unpublished data).

In the same context, Wiener-Megnazi et al. (2004), by using a novel thermochemiluminescence assay, reported for the first time an age-related increase in free radical activity which also correlates with a poorer IVF outcome. These authors thus suggested that a higher oxidative stress is present in follicular fluid from older women.

Although much more data and investigations are needed, these observations suggest that, as for many other biological stress, age-related ageing in the female ovary could be due to a lowering of enzymatic antioxidant defences and a contemporaneous increase in ROS production of possible multiple cellular origin such as granulosa cells apoptosis (Moffatt et al., 2002), whereas total non-enzymatic activity is not significantly affected by increasing age.

An important factor contributing to intracellular ROS levels is the oxygen tension in the extracellular environment. It is, in fact, well known that both hypoxic and hyperoxic conditions can be responsible for oxidative stress and that hypoxia can either directly cause the formation of ROS or indirectly after reoxygenation (Chandel and Budinger, 2007). Thus, as reported above, a determinant factor in the oxidative stress associated with oocyte ageing might be a condition of hypoxia due to an insufficient ingrowth of capillaries into the theca of the mature follicle. As fully-grown oocytes are characterized by an increase in oxygen-mediated metabolism (Van Blerkom, 2004), it is plausible that a reduced oxygen supply triggers a condition of oxidative stress. This hypothesis is further supported by the observation that mitochondria of granulosa cells from aged women exhibit structural damage similar to those found in other cells exposed to hypoxia (Amicarelli et al., 1999; Tatone et al., 2006a).

A new hypothesis for age-related ovarian molecular damage: potential deleterious effects of protein glycation

Proteins can be damaged both by free radicals and by glycation. Non-enzymatic protein glycosylation (glycation) leads to the formation of adducts, called AGEs, which are considered universal symptoms of ageing, adversely affecting skin, lungs, muscles, blood vessels and organ function in general (Baynes, 2001; Yin et al., 2000). AGEs cause tissue injury directly through protein cross-linking or indirectly by binding to specific receptors known as RAGE (receptor for advanced glycation end-products) present on different cell types such as endothelium, smooth muscle cells, etc. (Schmidt et al., 2000). Interestingly, AGEs are recognized as potentially strong inducers of an oxidative stress status (Baynes, 2001; Yin et al., 2001; Wen et al., 2002).

Long-lived proteins such as collagen are the molecules most vulnerable to cross-linking and AGE formation with subsequent reduction of proteolysis and tissue remodelling (Verzijl et al., 2000). The irreversible cross-linked proteins of AGEs in vessel collagen also contributes to atherosclerosis (Soldatos and Cooper, 2006), as well as to kidney failure (Bohlender et al., 2005), conditions worsened in diabetes (Goldin et al., 2006). The interaction of AGE with their receptors, the so-called RAGE, results in generation of intracellular oxidative stress and subsequent activation of the redox-sensitive transcription factors such as NF-κB (Schmidt et al., 2000; Wautier et al., 2001). Surprisingly, a recent paper reported the presence of AGEs in normal ovarian tissue obtained from women with a mean age of 28.8 ± 5.47 years (Diamanti-Kandarakis et al., 2007). By using a specific antibody, the authors observed AGE-modified proteins in a low percentage of granulosa and theca layers and luteinized granulosa cells. In contrast, RAGE was highly expressed in the ovary being present in granulosa cells, theca interna, endothelial and stromal cells. Although possible effect of reproductive ageing on the accumulation of AGEs in the ovary are still unknown, Tatone et al. (2007) observed in ovaries of reproductive old mice reduced expression and activity of enzymes for detoxifying methylglyoxal, a major precursor of AGEs (Oya et al., 1999; Ramasamy et al., 2006). Based on the above observations, the hypothesis of the involvement of AGEs in ovarian ageing is intriguing especially because the potential accumulation of these compounds in the ovary may account for compromised efficacy of vascularization and for the activation of oxidative stress response through RAGE interaction.

Final remarks and future challenge

In this review, we highlighted the main cellular and molecular aspects of ovarian follicle ageing in order to understand the factors jeopardizing the development of competent gametes during reproductive ageing.

From the literature emerges a clear picture where follicle ageing is characterized by the impairment of specific functions of oocytes and granulosa cells, along with general cellular dysfunctions typical of the ageing process in other tissues and organs, such as mitochondrial activity, energetic failure, changes in gene and protein expression profiles. It can be also speculated that in some cases the level of this cellular decline would be so elevated to make ovarian follicles and ovulated oocytes more susceptible to the activation of an apoptotic program. In contrast, when we move our attention to potential causal factors of follicle ageing the issue appears more puzzling, first because of the complex dynamic of folliculogenesis, second because of the limited data available in the literature and third because of the multifactorial agents with a possible role in this biological phenomenon. Given that gonadotropin changes are not sufficient to explain the process of follicle ageing especially because of their late occurrence, data reviewed in this study point to a mechanism mainly based on the oxidative stress theory of ageing. According to the literature, in the ageing ovary both resting and mature follicles suffer from oxidative stress although to a different extent. As summarized in Figure 2, we propose that subtle oxidative damage revealed in primordial follicles may result from their prolonged exposure to factors, such as the AGEs, which can irreversibly accumulate during reproductive lifespan, gradually affecting vascular supply and promoting a gradual increase of ROS in the ovarian microenvironment. It is in fact well known that a highly complex interplay occurs between oxidative stress and AGEs with the latter acting as a potent inducing factor of oxidative stress. When the follicle starts growth, these conditions may hamper, in both germ cells and...
saving or rescuing the developmental potential of aged oocytes. In this respect, a continuous interaction between biological and clinical research is imperative in order to develop prevention and treatment modalities for age-related subfertility.

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