Age-dependent changes in the expression of superoxide dismutases and catalase are associated with ultrastructural modifications in human granulosa cells

C.Tatone[1,2,7], M.C.Carbone[3], S.Falone[3], P.Aimola[3], A.Giardinelli[4], D.Caserta[5], R.Marci[2,6], A.Pandolfi[4], A.M.Ragnelli[3] and F.Amicarelli[3]

[1]Department of Biomedical Sciences and Technologies, [2]Center for Assisted Reproduction, [3]Department of Basic and Applied Biology, University of L'Aquila, L'Aquila, [4]Department of Biomorphology, Aging Research Center, Ce.S.I., Gabriele D'Annunzio University Foundation, Chieti-Pescara, [5]Department of Obstetrics, Gynecology and Perinatology, S. Andrea Hospital, La Sapienza University, Rome and [6]Department of Experimental Medicine, University of L'Aquila, L'Aquila, Italy

[7]To whom correspondence should be addressed at: Department of Biomedical Sciences and Technologies, University of L'Aquila, Via Vetoio, 67100 L'Aquila, Italy. E-mail: ctatone@univaq.it

Limited knowledge exists about changes in follicle quality associated with age. The aim of this work was to investigate whether ageing may cause oxidative stress-mediated alterations in human granulosa cells (GCs) from periovulatory follicles. GCs employed in this study were obtained from follicular aspirates of 20 younger women (range 27–32 years) and 20 older women (range 38–41 years) undergoing an IVF treatment. Results obtained from comparative RT–PCR analysis revealed that the mean relative levels of mRNAs coding for superoxide dismutases, Cu, ZnSOD (SOD1), MnSOD (SOD2) and catalase were significantly decreased in women ≥38 years ($P < 0.05$, Student's t-test). These changes were associated with a reduced expression of SOD1, SOD2 and catalase at the protein level. When examined at an ultrastructural level, most of the GCs from this group showed defective mitochondria and fewer lipid droplets than those observed in the younger group. These results indicate that GCs from older patients suffer from age-dependent oxidative stress injury and are taken as an evidence for reduced defence against reactive oxygen species (ROS) in GCs during reproductive ageing.

Key words: antioxidant enzymes/gene expression/granulosa cells/mitochondria/reproductive ageing

Introduction

Reproductive capacity in women declines dramatically beyond the mid-30s (te Velde and Pearson, 2002). The prevailing concept of female reproductive ageing assumes that the decline of both quantity and quality of the oocyte/follicle pool determines an age-dependent loss of female fertility (Ottolenghi *et al*., 2004). Although the primary cause for this decline is the gradual depletion of oocytes, the decline of oocyte quality is suggested to play an important role. For example, it has been shown that young women undergoing standard IVF with their own oocytes show a success rate comparable with older women (40 years) undergoing IVF with oocytes donated by this younger subset of women (Navot *et al*., 1991). Most of the primordial follicles are formed during fetal development, although a recent study suggested meiotic entry of germline stem cells in the post-natal mammalian ovary (Johnson *et al*., 2004). In human beings, primary oocytes remain arrested at the germinal vescicle (GV) stage until stimulated to grow and resume meiosis at periovulation time. During this period which in women may last ≤50 years, metabolically active GV oocytes and surrounding granulosa cells (GCs) may be exposed to factors deriving from the ageing of the ovaries (Tarin *et al*., 2000). Based on this assumption, the most relevant idea on ovarian ageing involves the accumulation of damage by oxidative stress associated, at least in part, with an increasingly compromised microcirculation around the leading follicle (Friedman *et al*., 1997; Van Blerkom *et al*., 1997).

Reactive oxygen species (ROS) are involved in the modulation of an entire spectrum of physiological reproductive functions such as oocyte maturation, ovarian steroidogenesis, corpus luteal functions and luteolysis. Moreover, they play a role during fertilization, embryo development and pregnancy (Agarwal *et al*., 2005).

In the ovarian tissues, the physiological role of ROS is ensured by a delicate balance between ROS and antioxidant defences. Oocytes and GCs in all follicular stages as well as follicular fluid are endowed with the major antioxidant and detoxifying enzymes (Peterson and Stevenson, 1992; El Mouatassim *et al*., 1999; Suzuki *et al*., 1999; Carbone *et al*., 2003). Cytosolic copper–zinc superoxide dismutase (SOD1, EC 1.15.1.1) and mitochondrial manganese-SOD (SOD2, EC1.15.1.1) reduce superoxide anion to hydrogen peroxide and molecular oxygen (Zelko *et al*., 2002). The removal of hydrogen peroxide is catalysed by catalase (EC 1.11.1.6) that reduces hydrogen peroxide to water (Fridovich, 1986). Therefore, the SODs and catalase serve, in tandem, as front-line antioxidant defences (Scandalios, 2005).

The relationship between ageing and antioxidant enzyme function continues to be a contentious area. We have recently demonstrated that ROS scavenging efficiency in the follicular fluid undergoes a significant decrease during ageing as a result of changes in some antioxidant enzymatic activities (Carbone *et al*., 2003). This condition might be due to age-associated metabolic defects in GCs perhaps as a result of changes in the microenvironment of the aged ovary.

On this basis, the aim of this work was to investigate whether ageing may cause oxidative stress-mediated alterations in human GCs from periovulatory follicles. To this end, we compared the levels of mRNA coding for SOD1, SOD2 and catalase in women from two age groups (younger women, 27–32 years and older women, 38–41 years). The effects of gene expression changes on protein levels of SOD1, SOD2 and catalase were also evaluated. Moreover, we carried out a morphological analysis at the ultrastructural level to assess whether age-related changes in the expression of antioxidant enzymes were associated with oxidative stress cellular injury.

Materials and methods

Patient selection

Forty women undergoing IVF treatment at the Center for Assisted Reproduction of University of L'Aquila were selected for this study after giving written consent. They were categorized on the basis of age: young women (range 27–32 years, $n = 20$) and older women (range 38–41 years, $n = 20$).

Ovarian stimulation was induced in all patients by using the long down-regulation protocol with GnRH analogue (Enantone Depot 3.75 mg, Takeda, Italy) on day 23. Therapy was continued with a daily dose of 225 IU recombinant FSH (Gonal F, Serono, Italy) from day 2 of the subsequent menstruation. Follicular monitoring was performed by serum 17-β estradiol (E_2) measurement and serial transvaginal ultrasound every 2 or 3 days. When at least three or more follicles with a diameter ≥18 mm were seen, patients received 10.000 IU hCG (Gonasi). Oocyte retrieval was performed by transvaginal aspiration 36 h after the hCG administration. The only primary indication for IVF treatment was male factor infertility, and ICSI was performed. Patients enrolled in this study were selected on the basis of their ovarian response. No significant differences existed between young and older women for peak serum β-E_2 levels, day 3 FSH serum concentration, number of follicles (young, 5.8 ± 0.25; old, 5.6 ± 0.22), number of oocytes retrieved and number of GCs collected for each patient ($P > 0.05$). The number of clinical pregnancies decreased from five in the younger group to three in the older group, although this difference was not significantly different.

Isolation of GCs from follicular aspirates and cell culture

For each patient, after removal of the oocytes, follicular aspirates were pooled and centrifuged at 3000 g for 10 min. The supernatant was discarded, and the cell pellet was resuspended in DMEM/Ham's F12 (1:1) (Life Technologies, Gibco BRL, Paisley, UK). GCs were separated from erythrocytes on a 40% Percoll gradient (Sigma, St. Louis, MO, USA). The top layer of the suspension was collected, taking care to avoid the layers containing lymphocytes and red blood cells (RBCs). GCs were concentrated by centrifugation, and the cell pellet was resuspended in fresh medium. Cell suspension consisted of GC colonies and singular cells, which may have included contaminating theca cells and fibroblasts. Therefore, according to Carlberg *et al.* (2000), the cell suspension was passed through a cell strainer (size 40 μm; Becton Dickinson, Labora, Sweden). The retained cells obtained by back washing were counted in a Burker chamber using Trypan blue exclusion and plated in 24-well plates. GCs were cultured in 300 μl of medium DMEM/Ham's F12 (1:1) containing 10% fetal bovine serum and antibiotics (100 IU/ml penicillin and 10 ug/ml streptomycin sulphate, Sigma). After 24 h of culture at 37°C and 5% CO_2, cells were rinsed twice in culture medium to remove any remaining blood cells, leukocytes or paraendothelial cells.

The obtained GCs (number of GCs/patient: young, 121 300 ± 12 231; old, 118 600 ± 7043, $P > 0.5$) were divided into two groups: cells for Real-Time PCR experiments were transferred to tubes, frozen in N_2 and maintained at –80°C until use; cells for electron microscopy were immediately fixed as described below. Cells used in the Real-Time PCR experiments were processed separately for each patient, whereas the western blotting analysis and microscopy experiments employed cells pooled from samples obtained from three patients of the same age group.

SOD1, SOD2 and catalase mRNA quantification by Real-Time PCR
RNA extraction

Total RNA was isolated from GCs using ABI Prism Nucleic Acid PrepStation (PE Applied Biosystems, Foster City, CA, USA) according to manufacturer

recommendations. Briefly, cells were washed twice in calcium/magnesium-free phosphate-buffered saline (PBS) and then lysed with 2× nucleic acid purification lysis solution at the final concentration of 1× with calcium/magnesium-free PBS. Lysed samples were transferred to a 96-well purification tray and placed on the instrument consisting of a specific membrane that physically captures the RNA passing through with wash solutions under precisely controlled vacuum conditions. A 'Method of isolation of total RNA from Cultured cells' was run. The isolated RNA was eluted in 100 μl Nucleic Acid Purification Elution Solution.

cDNA synthesis

For cDNA synthesis, 50 μl of total RNA was directly processed with the High-Capacity cDNA Archive Kit (PE Applied Biosystems). For each sample, 50 μl of total RNA was added to 10 μl of 10× RT Buffer, 4 μl of 25× dNTP mixture, 10 μl of 10× Random Primers, 5 μl of MultiScribe RT enzyme (50 U/ml) and RNAse-free water to reach a final volume of 100 μl. The reactions were incubated in a GeneAmp PCR System 9700 (PE Applied Biosystems) at 25°C for 10 min, 42°C for 59.59 min, 95°C for 5 min and then at 4°C.

Real-Time PCR

Finally, a so-called Singleplex Real-Time PCR was performed for the Relative Quantitation of SOD1, SOD2 and catalase gene expression versus GAPDH, by using TaqMan™ technology (PE Applied Biosystems) on the ABI Prism 9700HT Sequence Detection System Instrument (PE Applied Biosystems), connected to Sequence Detector Software (SDS version 2.0, PE Applied Biosystems) for collection and analysis of data.

Primer pairs and TaqMan probes for all target genes and those for the GAPDH reference gene were provided from Applied Biosystems as 20× mix ready to use at a final concentration 1×, corresponding to final concentrations of 100 nM TaqMan probe and 80 nM primers.

According to manufacturer recommendations, 25 μl reactions were performed in a MicroAmp Optical 96-well reaction plate (PE Applied Biosystems) using 12.5 μl TaqMan Universal PCR Master Mix 2× (PE Applied Biosystems), 1.25 μl Assay-on-Demand™ Gene Expression Product 20× for human SOD1 target gene or 1.25 μl human SOD2 target gene or 1.25 μl human catalase target gene (TaqMan MGB probe, FAM™ dye-labeled, PE Applied Biosystems) or 1.25 μl Pre-Developed TaqMan Assay Reagent 20× for human GAPD (GAPDH) reference gene (TaqMan MGB probe, VIC™ dye-labelled, PE Applied Biosystems) and, for each sample, 1.5 μl of the cDNA reaction mix, and RNAse-free water to reach the final 25 μl reaction volume.

PCR was performed at 50°C for 2 min and at 95°C for 10 min and then run for 45 cycles at 95°C for 15 seconds and at 60°C for 1 min. All reactions were performed in triplicate and repeated twice.

The Relative Quantification of SOD1, SOD2 and catalase gene expression was evaluated with data from SDS software using the arithmetical formula 2^{-DDCt}, according to the comparative Ct method, representing the amount of target, normalized to the GAPDH endogenous control (reference) and relative to a sample used as the basis for comparative results (Calibrator). A sample from the younger group was chosen randomly and used as a calibrator in all the replicates. Data were presented as fold induction of transcripts for target genes SOD1, SOD2 and catalase in GCs versus GAPDH gene expression in GCs.

For the DDCt calculation to be valid, relative efficiency plots were performed to verify that the efficiency of the target amplification and the efficiency of the reference amplification were approximately equal.

SOD1, SOD2 and catalase protein quantification by immunoblotting
Antibodies

Anti-Cu–Zn SOD (rabbit polyclonal) and bovine anti-rabbit peroxidase-conjugated secondary antibody were from Santa Cruz (Santa Cruz Biotechnology Inc., CA, USA), and anti-Mn SOD (rabbit polyclonal) was from Stressgene (San Diego, CA, USA). Anti-catalase (rabbit polyclonal) was from Rockland (Gilbertsville, USA) and anti-β-actin was from Sigma.

Western blot analysis

GC pellets were lysed in a buffer containing 50 mM Hepes (pH 7.2), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 30 mM

$Na_4P_2O_7$, 1 mM Na_3VO_4, 1 mM phenylmethylsulphonyl fluoride, 10 µg/ml leupeptin and 5 µg/ml aprotinin. The protein concentration was determined by a Bio-rad protein assay using bovine serum albumin (BSA) as the standard (Bio-rad laboratories S.r.l., Italy). Samples containing the same amount of protein (15 µg) were run on 12% polyacrylamide gels according to Laemli (1970). Protein bands were transferred onto polyvinylidene difluoride sheets by wet electrophoretic transfer according to Towbin et al. (1979). Non-specific binding sites were blocked overnight at 4°C with 5% no-fat dry milk in Tris-buffered saline (TBS) containing 0.05% Tween 20/TBS. Membranes were incubated with the primary antibody at the appropriate dilution, according to the manufacturer's instructions for 2 h at room temperature, followed by incubation with peroxidase-conjugated secondary antibody (1:2000 in blocking solution) for 1 h at room temperature. After rinsing, the specific immune complexes were detected by an ECL kit (Amersham Pharmacia Biotech). The same blot was stripped and reprobed with anti-β-actin polyclonal antibody. The semi-quantification of immunoreactive bands was performed using Adobe Photoshop version 4.0 (Adobe Systems, USA). The mean pixel intensity of a preselected area, set to include the largest band, was obtained and adjusted for background intensity of our gel. The mean intensity of the bands in younger women was used as the baseline value to which it was arbitrarily assigned the value of 1. The intensity of the bands in older women was calculated relative to this value. The experiment was performed in duplicate.

Transmission electron microscopy

After isolation, GC pellets obtained from two groups of three younger patients (mean number of cells/pellet ± SD, 385 000 ± 21 200) and three older patients (mean number of cells/pellet ± SD, 340 000 ± 28 300) were washed in PBS and fixed in suspension with 2.5% glutaraldehyde (Fluka, from Sigma) in PBS (pH 7.2) for 1 h at 4°C. They were then centrifuged at 13500 g for 25 min (4°C), and the resulting pellets were post-fixed with 1% osmium tetroxide (E.M.S., Fort Washington, PA, USA) in Tyrode solution (pH 7.2) for 1 h at 4°C, dehydrated in an ethanol series and embedded in Durcupan ACM resin (Fluka, from Sigma). Semithin sections (1 µm) were cut with a Sorvall Porter-Blum MT2-B ultramicrotome at intervals of 50 µm throughout each specimen, stained with toluidine blue and screened for the presence of GCs by light microscopy. Ultrathin sections (50–70 nm) corresponding to selected sections containing GCs were mounted on single slot formvar–carbon-coated copper grids. After staining with 5% uranyl acetate in 70% ethanol and lead citrate, random GCs with a visible nucleus were photographed with a Jeol JEM 100C transmission electron microscope operated at 60 kV (microscope magnification for a general view ×3000 and for detail photographs ×10 000, ×25 000, ×40 000).

Morphometry

Negatives were scanned with an image scanner (SnapScan 1236, Agfa, Leverkusen, Germany) connected to a personal computer system. Areas of the GCs and their nucleus as well as the cytoplasmic fraction taken up by mitochondria and lipid droplets were measured and calculated with NIH Image 1.36b software. In addition to these morphometric variables, a comparison between the two groups of age was made by calculating the proportion of cells with defective mitochondria.

Statistical analysis

Quantitative data are expressed as the mean ± SEM of at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by the unpaired Student's t-test. Proportions were compared using the z-test. All analyses were performed using the Sigma-Stat software (Jandel Scientific Corporation, San Rafael, CA, USA). A P value <0.05 was considered statistically significant.

Results

SOD1, SOD2 and catalase gene expression in GCs of younger and older patients

To evaluate mRNA levels of SOD1, SOD2 and catalase in young and old patients, we carried out RT–PCR analysis on GCs from pooled follicular aspirates of each patient. Comparisons of the mean relative mRNA levels between the two groups are shown in Figure 1. These
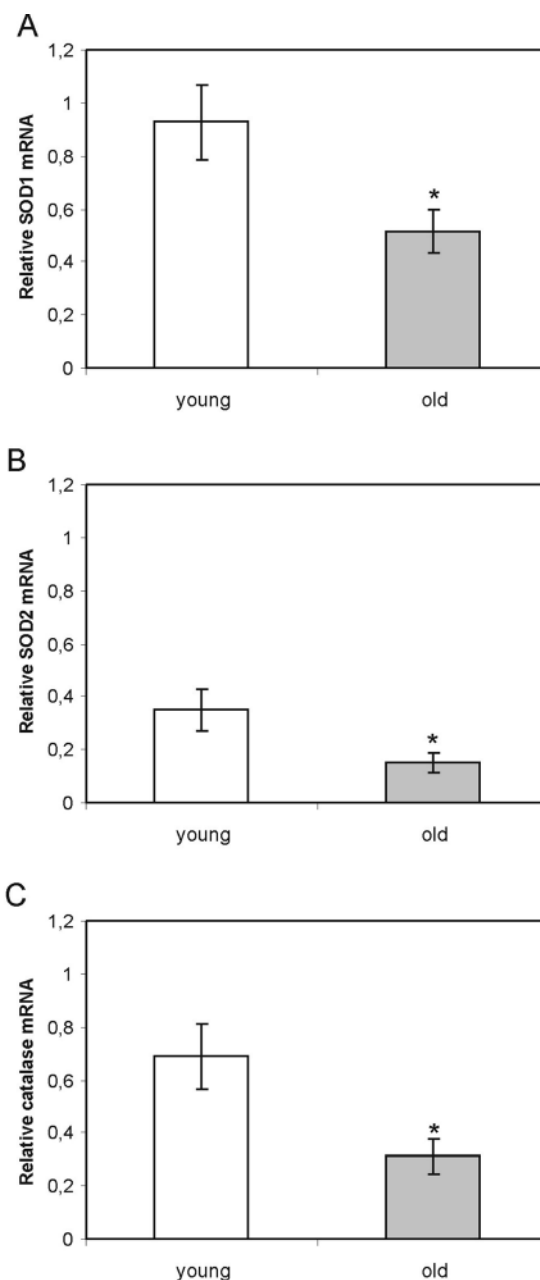

Figure 1. SOD1 (**A**), SOD2 (**B**) and catalase (**C**) mRNA expression levels in granulosa cells from young and old patients. Values are means ± SEM from comparative real-time results obtained from younger ($n = 10$) and older ($n = 10$) patients enrolled in the study. Student's t-test statistical analysis for younger versus older patients: *$P < 0.05$.

data demonstrated that the levels of SOD1, SOD2 and catalase mRNA were down-regulated in women ≥38 years. For these patients, the relative level of SOD1 mRNA was 0.52 ± 0.08, whereas for women ≤32 years it was 0.93 ± 0.14 (Figure 1A). The difference was statistically significant ($P = 0.022$). Similarly, SOD2 expression was found to undergo a significant decrease in GCs retrieved from patients ≥38 years. In this group, the mean relative level of SOD2 mRNA was 0.15 ± 0.04, whereas in the younger group it was 0.35 ± 0.08 ($P = 0.038$) (Figure 1B). Comparison between younger and older patients revealed that decreased levels of transcripts for superoxide dismutases were associated with a decreased level of catalase expression in older women. As shown in Figure 1C, the relative level of catalase mRNA

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in this group of patients was 0.31±0.07, whereas for younger women it was 0.78 ± 0.13 ($P = 0.006$).

SOD1, SOD2 and catalase protein expression in GCs of younger and older patients

To compare intracellular levels of SOD1, SOD2 and catalase proteins in GCs of younger and older women, we performed immunoblotting analysis. As shown in Figure 2, results revealed that in the older group the bands corresponding to each these enzymes exhibited a density lower than that in younger women. In particular, semiquantitative analysis showed that in the older group SOD1 and SOD2 protein levels decreased to about 50 and 70% of that of the younger group, respectively, and catalase protein was reduced to about 40%.

Morphological characterization of GCs in younger and older patients

The outcomes of the main morphological variables analysed are summarized in Table I. The morphological comparison of GCs obtained from the two groups of age revealed that the mean areas of GC and GC nuclei were not significantly different. Likewise, in both groups, the majority of cells appeared polyhedral in shape and showed nuclei usually large, round and eccentric with a single prominent nucleolus and

some patches of condensed chromatin along the nuclear membrane. The mitochondria appeared round-shaped in both groups of age, and the cytoplasmic fraction taken up by these organelles was not significantly different in GCs of the two groups of age (Figure 3, Table I). However, a marked difference was observed between younger and older GCs when mitochondrial morphology was compared. In the younger group, cristae were clearly visible and appeared of a tubular type (Figure 3A and D), whereas in most of the cells of the older group cristae morphology could not be easily identified, being matrix high electron-dense and cristae were barely visible (Figure 3C). This pattern was associated with the presence of mitochondria with damaged membranes (Figure 3C and F). Moreover, some cells in the older group showed mitochondria with vacuolization and the degeneration of both cristae and matrix (Figure 3B and E). As reported in Table I, the proportion of cells showing the defects mentioned above greatly increased in the older group. No differences for the appearance of smooth endoplasmic reticulum and Golgi complexes were observed for the two groups (data not shown). Lipid droplets showed a low electron density except for occasional highly electron-dense droplets in the younger group (Figure 3G). Morphometric analysis revealed that in the cytoplasm lipid droplets made up a large fraction in both the younger and the older groups. However, the fraction taken up by lipid droplets was significantly reduced in the older group (Table I).
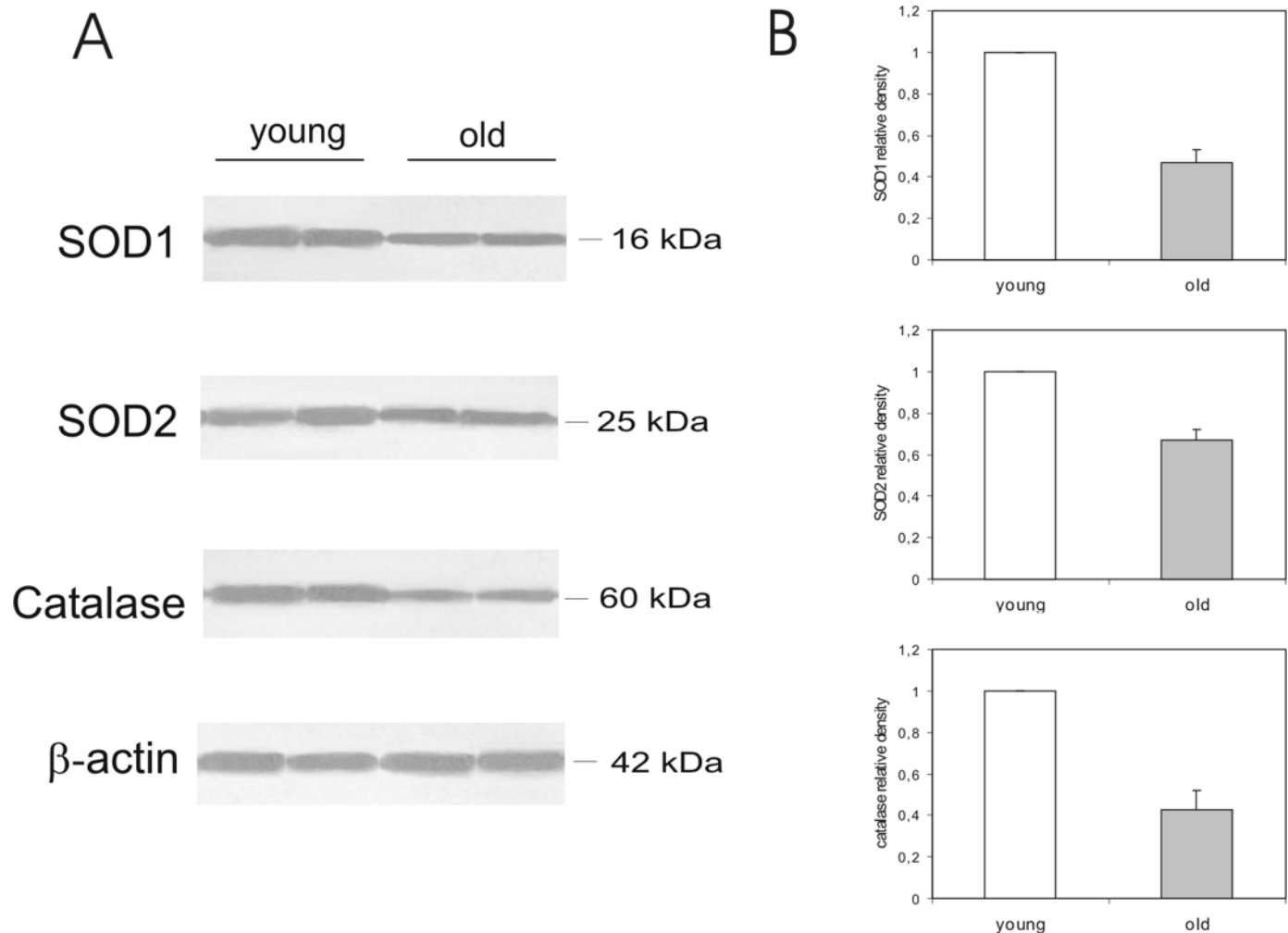
Figure 2. Western blot analysis (**A**) and quantification (**B**) of SOD1, SOD2 and catalase proteins in granulosa cells from younger and older women. The mean intensity of the bands in younger women was used as the baseline value and was arbitrarily assigned the value of 1. In each experiment, the intensity of the bands in older women was calculated relative to this value. β-Actin was used as a housekeeping protein. Data are expressed as means ± SEM.

Table I. Morphological comparison of granulosa cells (GCs) obtained from younger and older patients

Variable	Younger	Older	P value
Mean area GCs (μm^2)	120.6 ± 7.8	121.8 ± 6.6	>0.05
Mean area GC nuclei (μm^2)	26.4 ± 2.6	33.2 ± 3.3	>0.05
Mean nucleus/cell ratio	0.22	0.27	>0.05
Cytoplasmic fraction of			
Mitochondria (%)	14 ± 8.65	11.4 ± 8.6	>0.05
Lipid droplets (%)	28 ± 4.6	16.7 ± 4.3	<0.001
Cells with defective mitochondria	8.2%	63.3%	<0.001

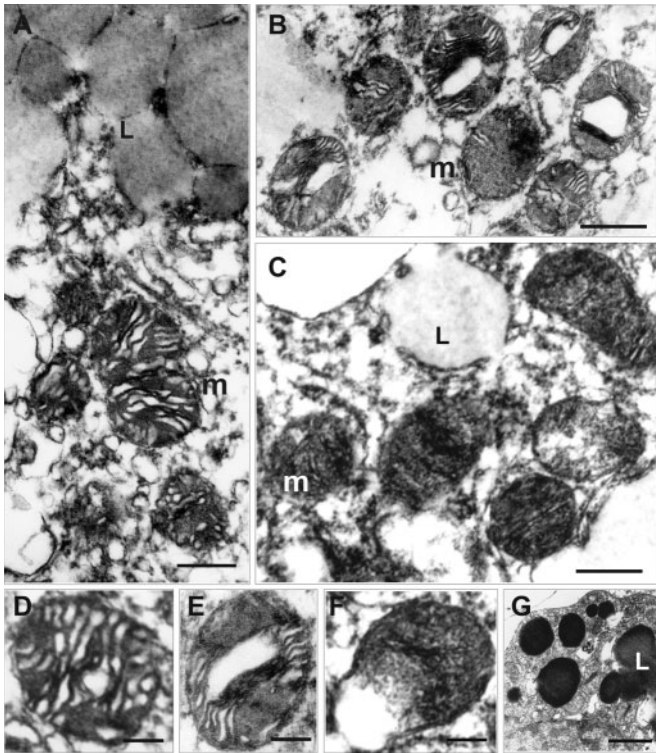Values are the means (±SD).

Figure 3. Representative electron micrographs showing ultrastructural details of granulosa cells in younger and older women: mitochondria in the younger group with intact tubular cristae (**A, D**); defective mitochondria in the older group showing vacuolization and degeneration of both cristae and matrix (**B, E**), cristae barely visible and a high electron-dense matrix (**C**) or ruptured membranes (**C, F**); and highly electron-dense lipid droplets in younger cells (**G**). L, lipid droplets; m, mitochondria. Bar, A–C (0.5 μm), D–F (0.25 μm), G (1 μm).

Discussion

Reproductive ageing is accompanied by a change in the antioxidant enzymatic pattern in follicular fluids which impairs ROS scavenging efficiency (Carbone et al., 2003). In this study, we extended our investigation to GCs and found that IVF patients of advanced reproductive age exhibit a reduced expression of genes mainly involved in the neutralization of ROS. These results, being associated with the presence of cellular modifications at an ultrastructural level, are taken as an evidence of reduced defences against ROS and are discussed in terms of oxidative stress injuries in relation to the reproductive ageing process in women.

In the last decade, the study of the global expression profiling of ageing organs has demonstrated that somatic ageing is associated with an alteration of gene expression (Johnson et al., 1999; Finkel and Holbrook, 2000). However, so far, changes in gene expression associated with female reproductive ageing have been reported only in mouse oocytes where, beyond a reduced expression of genes related to 'energy pathways' and mitochondrial function, a decreased expression of transcription factors has been observed (Hamatani et al., 2004). Thus, our results describing an age-related decrease in SOD1, SOD2 and catalase mRNA content represent the first evidence that reproductive ageing can also affect gene expression in GCs. As this alteration is associated with a reduced content of the relative proteins, age-dependent down-regulation of the expression of SODs and catalase may result in the impairment of antioxidant defences which could seriously impair GC functions and structures. This hypothesis is strengthened by the fact that in GCs the antioxidant enzymes analysed in this study are known to play a crucial role in the protective mechanisms against superoxide anions and hydrogen peroxide generated during the synthesis of steroid hormones (Behrman and Aten, 1991; Campisi et al., 1994). The activity of catalase in GCs is stimulated by FSH in concomitance with an increase in E_2 levels (Behl and Pandey, 2002), and the expression of SOD at protein level correlates with that of the transcription factor involved in the expression of the steroidogenic P450 enzyme (Sasaki et al., 1994; Suzuki et al., 1999).

The presence of an increased number of cells with defective mitochondria has been previously described in a cohort of primordial follicles in women of advanced age (de Bruin et al., 2004), although at a lower extent than that observed in luteinizing GCs. This suggests that the alterations observed in this study may have occurred after the initiation of follicle growth but may be the consequence of mitochondria with pre-existing subtle damage in the resting follicle pool. Furthermore, they may reflect an adaptive response to a condition of oxidative stress arisen as a consequence of a reduced oxygen supply to maturing follicles (Friedman et al., 1997; Van Blerkom et al., 1997). This hypothesis is supported by the observation that GC mitochondrial alterations closely resemble those previously described in muscle cells from rats exposed to a hypoxic stress (Amicarelli et al., 1999).

As far as we know, little information exists about the ultrastructure of luteinizing human GCs. According to Rotmensch et al. (1986), mitochondrial cristae of the tubular type appeared in a population of GCs whose morphology was associated with high steroidogenic activity. Nevertheless, in the same study, cristae of the lamelliform type could be observed in GCs obtained from small follicles containing unfertilizable oocytes. Based on these observations, in this work, cristae resembling those showed by Rotmensch et al. (1986) were considered 'tubular', a common form in steroid-producing cells (Prince, 2002). According to Rotmensch et al. (1986), highly electron-dense lipid droplets could occasionally be found in younger cells but were not present in older GCs where we also observed a lipid fraction less abundant than that in the younger group. Although a previous study reported that follicular E_2 and free progesterone and testosterone levels do not significantly differ with ageing (Sadraie et al., 2000), the present data suggest that the possibility that ageing may affect steroidogenic activity in GCs would require further investigation.

In conclusion, we propose that, in GCs, reproductive ageing is associated with the down-regulation of genes involved in the front-line defence against ROS, a condition leading to the accumulation of oxidative damage which seems to involve mainly mitochondria. Although conflicting results exist about age-dependent increase of mtDNA deletions in human GCs (Seifer et al., 2002; Au et al., 2005), present findings supports the view that, as in the somatic ageing process, these organelles play a major role in the regulation of female reproductive ageing. Given the key role of GCs in the functional maturation of the follicle and the oocyte (Albertini and Barrett, 2003), it may be speculated that age-related changes jeopardizing physiological

processes in these cells may represent one of the main causes for the reduced developmental competence of oocytes produced during advanced reproductive ageing.

Acknowledgements

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