Changes in the glucose-6-phosphate dehydrogenase activity in granulosa cells during follicular atresia in ewes

Clara Ortega-Camarillo, Alicia González-González, Marcela Vergara-Onofre¹, Everardo González-Padilla², Alejandro Ávalos-Rodríguez¹, Margarita E Gutiérrez-Rodríguez, Lourdes Arriaga-Pizano³, Miguel Cruz, Luís Arturo Baiza-Gutman⁴ and Margarita Díaz-Flores

Unidad de Investigación Médica en Bioquímica, Hospital de Especialidades, Centro Médico Nacional Siglo XXI, Instituto Mexicano del Seguro Social, Avenida Cuauhtemoc 330, Colonia Doctores, C.P. 06720 México DF, Mexico,

¹Bioquímica de la Reproducción, Departamento de Producción Agrícola y Animal, Universidad Autónoma Metropolitana-Xochimilco, C.P. 04960 México DF, Mexico, ²Facultad de Medicina Veterinaria y Zootecnia, UNAM, C.P. 04510 México DF, Mexico, ³Unidad de Investigación Médica en Inmunología, Hospital de Especialidades, CMN SXXI, Instituto Mexicano del Seguro Social, C.P. 06720 México DF, Mexico and ⁴Laboratorio de Biología del Desarrollo, Unidad de Morfología FES-IZTACALA, UNAM-México, C.P. 54090 México DF, Mexico

Correspondence should be addressed to M Díaz-Flores; Email: mardiaz2001@yahoo.com

Abstract

Apoptosis of granulosa cells during follicular atresia is preceded by oxidative stress, partly due to a drop in the antioxidant glutathione (GSH). Under oxidative stress, GSH regeneration is dependent on the adequate supply of NADPH by glucose-6-phosphate dehydrogenase (G6PD). In this study, we analyzed the changes of G6PD, GSH, and oxidative stress of granulosa cells and follicular fluid and its association with apoptosis during atresia of small (4–6 mm) and large (> 6 mm) sheep antral follicles. G6PD activity was found to be higher in granulosa cells of healthy small rather than large follicles, with similar GSH concentration in both cases. During atresia, increased apoptosis and protein oxidation, as well as a drop in GSH levels, were observed in follicles of both sizes. Furthermore, the activity of G6PD decreased in atretic small follicles, but not in large ones. GSH decreased and protein oxidation increased in follicular fluid. This was dependent on the degree of atresia, whereas the changes in G6PD activity were based on the type of follicle. The higher G6PD activity in the small follicles could be related to granulosa cell proliferation, follicular growth, and a lower sensitivity to oxidative stress when compared with large follicles. The results also indicate that GSH concentration in atretic follicles depends on other factors in addition to G6PD, such as *de novo* synthesis or activity of other NADPH-producing enzymes. Finally, lower G6PD activity in large follicles indicating a higher susceptibility to oxidative stress associated to apoptosis progression in follicle atresia.

Reproduction (2009) 137 979–986

Introduction

The development of morphologically and steroidogenically mature follicles depends on granulosa cell survival (Hsueh *et al.* 1994, 1996). Although hormonal stimuli, intracellular signals, and metabolic processes are determinants of the death of these cells, the inducing factor involved in follicular atresia by way of apoptosis is still unknown (Tilly *et al.* 1992). Apparently, the intracellular redox state is a critical factor affecting the apoptosis cascade by altering the equilibrium between cell survival signals and the progression to death (Jolly *et al.* 1997).

Reactive oxygen species (ROS) play a key role in intracellular signaling, and recent evidence has suggested their participation in apoptosis and follicular atresia (Jolly *et al.* 1997). Electron transport associated with steroidogenesis, in addition to mitochondrial metabolism, constitutes important pathways for the

production of free radicals in steroidogenically active cells, such as granulosa cells of antral follicles (Rapoport *et al.* 1995, Hsueh *et al.* 1996, Tilly 1996), in spite of the fact that these radicals are efficiently neutralized by antioxidant molecules (Aten *et al.* 1992). Under certain stimuli, this equilibrium can be disrupted, leading to a state of oxidative stress. Hence, it has been proposed that inadequate protection against ROS constitutes a trigger for follicular atresia. Therefore, the survival of follicles subjected to gonadotropin stimulation is related to an increase in the expression of antioxidant enzymes (Tilly & Tilly 1995), while treatment with antioxidants blocks granulosa cell apoptosis in cultured follicles (Tilly 1996). Among the most noteworthy deleterious effects of ROS on follicular cells is the inhibition of induction of steroidogenesis by gonadotropic hormone with the loss of follicular function (Margolin *et al.* 1990).

Changes in the homeostasis of the reduced form of glutathione (GSH) and in the intracellular redox equilibrium constitute some of the first events in apoptosis (Lu 1999). GSH contributes to antioxidant metabolism through its action, either as a proton donor or as a cofactor for nucleophilic conjugations. It has been demonstrated that it detoxifies a wide range of oxygen and nitrogen reactive species. The antiapoptotic effect of FSH is mediated in part by increased synthesis of GSH, resulting in a decrease in ROS production and survival of granulosa cells in cultured preovulatory follicles (Tsai-Turton & Luderer 2006). Alterations in the reduced/oxidized GSH (GSH/GSSG) ratio precede the induction of the apoptotic signal in the mitochondrion and fragmentation of chromatin, coinciding with an increase in caspase-3 expression. Restoration of the GSH/GSSG equilibrium with the administration of niacinamide and/or GSH inhibits apoptosis (Lu 1999, Pias 2002). GSH participates in diverse processes related to oocyte maturation (Nasr-Esfahani & Johnson 1992), follicular growth and development (Agarwal *et al.* 2003), and its concentration is dependent on the day of the estrous cycle (Kim *et al.* 2007).

The antioxidant activity of GSH depends on its reduced form, which is regenerated from GSSG through the action of glutathione reductase. In this context, glucose-6-phosphate dehydrogenase (G6PD) has been proposed as a cell enzyme aiding in survival against oxidative stress by generating NADPH required for GSH regeneration (Leopold & Loscalzo 2000, Filosa *et al.* 2003, Jiang *et al.* 2003, Díaz-Flores *et al.* 2006). G6PD activity increases in growing cells and prevents cell death during oxidative stress (Tian *et al.* 1998, 1999); hence, its inhibition brings about a drop in NADPH levels, a decrease in cell growth (Endo *et al.* 1993), and an exacerbation of damage caused by free radicals (Filosa *et al.* 2003). Likewise, it has been demonstrated that an increase in its expression provides better protection against cell death caused by H_2O_2 , through the increase in GSH (Salvemini *et al.* 1999, Filosa *et al.* 2003). The aforementioned information led us to suppose that a decrease in G6PD activity must be associated with the start and progression of apoptosis in follicular atresia. Thus, the present study is aimed at determining the temporal relationship between G6PD activity, GSH concentration, protein oxidation levels, and the degree of apoptosis in granulosa cells during the atresia of antral follicles of two different sizes.

Results

Apoptosis frequency and oxidative stress in atretic follicles

The results show that the degree of atresia is directly proportional to the percentage of apoptotic cells and inversely proportional to viable cells. Figure 1A depicts

Figure 1 Apoptosis index and ROS damage in granulosa cells from atretic follicles. (A) Representative cytogram of granulosa cells stained with Annexin-V-FITC/PI, from large follicles with different degrees of atresia. (B) Percentage of apoptotic cells. * $P < 0.001$ when comparing atresia 3 versus atresia 1 and 2; ** $P < 0.01$ when comparing atresia 1 versus atresia 2. (C) Carbonyl groups exposed. Determination of dinitrophenylhydrazones on proteins oxidized. * $P < 0.001$ when comparing atresia 3 versus atresia 1 and 2; ** $P < 0.05$ when comparing atresia 1 versus atresia 2.

the results of the cytometric analysis. Cells that initiated apoptosis are shown in the right lower quadrant (Annexin-V-FITC⁺/PI⁻), while those in the upper right quadrant correspond to late apoptosis (Annexin-V-FITC⁺/PI⁺). In both types of follicle, a progressive increase in the apoptosis index of granulosa cells can be observed with respect to the degree of atresia (Fig. 1B). The highest percentage of apoptotic cells was found in atresia of three follicles. This was significantly higher (2.1-fold; $P < 0.001$) than that found in atresia 2 (71.1 ± 7.3 vs $33.3 \pm 2.5\%$ respectively) for the large follicles, whereas for the small follicles, the percentage was 3.1-times higher than that in atresia 2 (61.5 ± 7.7 vs $23.7 \pm 5\%$ respectively). In both conditions, the apoptotic index was 7.9-times higher in atresia 3 when compared with those in atresia 1 in large follicles and small follicles respectively).

Figure 1C shows that protein oxidation was significantly higher in large atretic follicles 2 and 3 versus large healthy follicles ($P<0.001$). In small follicles, a 3.3-fold increase was observed in atresia 3, and 2.4-times in atresia 2 ($P<0.001$) with respect to atresia 1, although the difference between atresia 1 and 2 follicles was not significant. Atretic large follicles depicted a higher oxidative damage than smaller follicles (5.7 ± 1.8 vs 3.7 ± 0.5 $\mu\text{mol/mg}$ protein respectively).

G6PD activity and GSH concentration in granulosa cells of atretic follicles

G6PD activity in healthy small follicles (atresia 1) was statistically higher ($P<0.001$), markedly dropping (1.8 times) in atresia 2, and even more in atresia 3 (2.8 times). This decrease paralleled the GSH drop associated with the progression of atresia (23.9 ± 4.2 , 9.5 ± 2.7 , and 6.8 ± 1.3 $\mu\text{mol/mg}$ protein, atresia 1, 2, and 3 respectively). However, no significant differences were observed in G6PD activity in the large follicles at different degrees of atresia, although this enzyme did have a tendency to decrease in granulosa cells dependent on the degree of atresia, being more evident in atresia 3 (1.3 times; Fig. 2A). On the other hand, GSH concentrations in these follicles decreased in a much larger proportion: 1.9- and 2.8-fold in atresia 2 and 3 respectively as compared with atresia 1 ($P<0.001$; Fig. 2B).

Figure 2 G6PD activity and GSH concentration in granulosa cells from differently sized atretic follicles. (A) G6PD activity. (B) GSH concentration. * $P<0.001$ when comparing atresia 1 versus atresia 2 and 3. ** $P<0.05$ when comparing atresia 2 versus atresia 3. The graph presents the mean $\pm$ s.d.

Figure 3 DHEA concentration in the liquid of differently sized atretic follicles. *Means significant differences in atresia 3 follicles versus atresia 1 and 2 ($P<0.01$). Bars represent mean $\pm$ s.d.

Steroid hormones

Figure 3 depicts that a high and statistically significant increase in DHEA concentrations occurs only in the fluid of atresia 3 follicles. A 2.3-fold increase was observed in atresia 3 with respect to atresia 2, and 2.9-times versus atresia 1 in large follicles ($P<0.01$). A similar pattern was observed in small follicles.

G6PD activity, GSH concentration, and carbonyl groups in the liquid of atretic follicles

Specific activity of G6PD and GSH concentrations in the atretic follicular fluid were lower than those found in granulosa cells (already of 10- and 5-times respectively) in all kinds of follicles. Figure 4B depicts G6PD activity in the fluid of large follicles, which is 1.1- and 1.6-times lower in atresia 2 and 3 respectively, than in atresia 1, but without significant differences. The follicular liquid of healthy small follicles (atresia 1) possesses higher G6PD activity than that of large follicles, which dropped significantly ($P<0.001$) only in atretic small follicles. In parallel, GSH concentrations also dropped sharply (1.4-fold in atresia 2 and 2.2-fold in atresia 3; Fig. 4C) in both large and small follicles. Figure 4A shows a significant increase in the degree of protein oxidation (0.6 ± 0.1 , 1.9 ± 0.4 , 3.4 ± 0.8 $\mu\text{mol/mg}$ protein in atresia 1, 2, and 3 respectively) in large follicles, with no differences found between follicular sizes.

Discussion

In this study, we used sheep antral follicles of two different sizes – small (4–6 mm) and large follicles (> 6 mm) – to assess changes in G6PD, GSH, and oxidative stress associated to the increase in apoptosis of granulosa cells during follicular atresia. The percentage of apoptotic granulosa cells and the degree of oxidative stress increased during progression toward atresia, in addition to a decrease in GSH concentration. G6PD-specific

Figure 4 ROS-induced oxidation of proteins, GSH concentration, and G6PD activity in liquid of differently sized atretic follicles. (A) Oxidation of proteins in the follicular liquid was higher in atresia 3 than that in atresia 1 and 2 ($*P < 0.01$). (B) G6PD activity according to the degree of atresia, $**P < 0.001$ when comparing atresia 1 versus atresia 2 and 3 in small follicles. (C) GSH concentration decreased along with atresia progression. $*P < 0.01$ when comparing atresia 1 versus atresia 2 and 3. Bars represent mean $\pm$ s.d.

activity is dependent on follicular size with higher activity occurring in small healthy follicles than that in the non-atretic large ones. In the small follicles, activity dropped concomitantly with progression toward follicular atresia, whereas, in the large follicles, no significant changes were seen during atresia. These results indicate that the antioxidant mechanisms of the small follicles are more efficient since their potential to produce NADPH is greater than that in large follicles. It also suggests that a decrease in G6PD activity and GSH depletion are involved in the induction of increased oxidative stress leading to apoptosis during small follicle atresia.

We observed that oxidative stress prevails in follicular atresia, as manifested by the higher oxidation of proteins due to the free radical effects, in addition to an increase in the apoptosis index (Fig. 1B and C). These results demonstrate that the oxidizing environment in the atretic follicles contributes to the damage of macromolecules and is associated to the apoptotic cascade in granulosa cells. Therefore, activation of the antioxidant system becomes relevant for cell survival and prevention of apoptosis. In this context, GSH plays a pivotal role as a protector against ROS (Agarwal *et al.* 2003), particularly in the ovary where its depletion increases antral follicular atresia in rats (Tsai-Turton & Luderer 2006), and in the apoptosis of granulosa cells induced by toxicants (Tsai-Turton *et al.* 2007a, 2007b). In this study, GSH concentrations in granulosa cells and in the follicular liquid were inversely related to the severity of the atresia (Figs 2B and 4C).

The difference in G6PD-specific activity in the small and large follicles, observed in this study, constitutes a relevant finding. Small follicle granulosa cells revealed a higher activity of this enzyme (almost twice as high) than large follicles, and only in the small follicles did G6PD activity decrease concomitantly with GSH depletion, dependent on the degree of atresia. These results point to the relevance of this enzyme in cellular proliferation, steroidogenesis, and the antioxidant protection of small follicles (Fig. 2A), and suggests that in these follicles, the increase in oxidative stress and the decrease in GSH concentrations associated with atresia are in part a consequence of reduced NADPH availability due to the drop in G6PD activity.

G6PD as a supplier of NADPH is indispensable for replenishing GSH in oxidative stress conditions of diverse cells (Leopold & Loscalzo 2000, Filosa *et al.* 2003, Jiang *et al.* 2003). The over-expression of G6PD protects against H_2O_2 -induced cellular death through GSH production (Salvemini *et al.* 1999) and, by contrast, the deficiency of G6PD increases susceptibility to oxidative stress and apoptosis (Pandolfi *et al.* 1995, Filosa *et al.* 2003, Jiang *et al.* 2003). This indicates that cells with high G6PD activity respond efficiently to NADPH requirements for maintaining cellular redox states, antioxidant systems, and cell survival.

The lower activity of G6PD in large follicles could be due to the lower rate of cell proliferation of these follicles in relation to small follicles (Jablonka-Shariff *et al.* 1994, 1996). Also, this could indicate a lower capacity of large follicles to respond to oxidative stress and explains the higher degree of protein oxidation observed during atresia of this type of follicles (Jablonka-Shariff *et al.* 1994). Our results are consistent with the regulation of GSH concentration by *de novo* synthesis, as well as by NADPH-dependent recycling under oxidative stress conditions, since G6PD activity in large atretic follicles did not show concomitant changes associated to the decrease in GSH concentration.

NADPH can be supplied by malate dehydrogenase and isocitrate dehydrogenase, which in rat ovary depict an activity equal or higher to that shown by other tissues (Flint & Denton 1970). More studies are required for establishing the relevance of these enzymes in the response to oxidative stress in granulosa cells.

On the other hand, it is known that the antiapoptotic effect of FSH in cultured follicles (Tilly & Tilly 1995) is partially due to its stimulatory effect on the *de novo* synthesis of GSH (Tsai-Turton & Luderer 2006). Glutamate cysteine ligase (GCL), which catalyzes γ -glutamylcysteine synthesis, is needed for the *de novo* synthesis of GSH and expressed in the granulosa cells of healthy follicles. Its expression is stimulated by gonadotropin treatment (Tsai-Turton & Luderer 2005), whereas atretic follicles are devoid of GCL (Luderer *et al.* 2003). Hence, the increase in GCL levels can increase GSH levels in healthy preovulatory follicles.

The decrease in G6PD activity during atresia in small follicles can be explained either by a decrease in the enzyme's concentration, oxidative modifications (Oliver *et al.* 1987), or by the increase in one of its inhibitors, DHEA (Tian *et al.* 1998). In this study, we observed an increase in the concentration of oxidized proteins of granulosa cells and follicular liquid. It is thought that G6PD could be part of these oxidized proteins (Figs 1C and 4A). Treatment of G6PD with an *in vitro* oxidizing system or with secondary products of lipid oxidation, such as hydroxynonenal, induces oxidative changes in this enzyme that increases its heat instability and leads to the loss of its biological activity (Oliver *et al.* 1987, Ninfali *et al.* 2001).

DHEA is a known inhibitor of G6PD and its effects on follicular development have been scarcely studied. After its administration, there is an increase in apoptosis and in the number atretic follicles of the mouse (Kim *et al.* 2003). However, in small follicles, the drop in DHEA occurs later than the decrease in G6PD.

Similar changes were observed in the follicular fluid during follicular atresia, i.e. decrease in GSH concentration and in G6PD activity along with the increase in protein oxidation. Very low G6PD activity was found in the follicular liquid, which could be coming from dead cells or erythrocytes, since at this time the follicles are more vascularized. On its behalf, GSH could be playing an important antioxidant role in the follicular liquid, since it is responsible for safeguarding the integrity and viability not only of the oocyte but also of the follicular cells (Jozwik *et al.* 1999). The decrease in GSH level in the follicular liquid could explain the increase in proteins modified by free radicals in the atretic follicles (Fig. 4A) as a consequence of the inefficient protection against ROS.

In addition, atretic follicles also presented lower estrogen values as compared with healthy ones (not shown), which also reduces their antioxidant mechanisms (Urata *et al.* 2006). Estrogen concentration

is also related to the incidence of apoptosis in granulosa cells (Valdez *et al.* 2005) by regulating the expression of the BCL2 family antiapoptotic proteins (Garcia-Segura *et al.* 1998).

In conclusion, the granulosa cells of the small and large follicles depict different grades of G6PD activity but at the same GSH concentration, which can be related to different degrees of proliferation and sensitivity to oxidative stress. Furthermore, this indicates that GSH concentration in non-atretic follicles depends on additional factors in addition to G6PD activity, such as *de novo* synthesis of GSH or activity of other NADPH-generating enzymes. The lower G6PD activity in the large follicles was associated to a greater susceptibility to oxidative stress, which produced in them a higher degree of protein oxidation during follicular atresia. The induction of apoptosis of granulosa cells during atresia in small follicles is associated to GSH depletion probably due to a low NADPH supply caused by a decrease in G6PD activity.

Materials and Methods

Reagents

Annexin-V-Fluos was obtained from Roche Applied Science and dichlorofluorescein-diacetate from Molecular Probes (Leiden, The Netherlands), Coat-A-count kits for estradiol (E_2) and progesterone (P_4) quantification were purchased from Diagnostic Products Corporation (Los Angeles, CA, USA), and DHEA RIA DSL 9000 active was acquired from Diagnostic Systems Laboratories (Webster, TX, USA). GSH, glucose-6-phosphate, NADP, and other analytical grade reagents were purchased from Sigma Chemical Co.

Procurement and classification of follicles

Ovaries from non-gestating sheep were obtained from a local slaughterhouse and transported to the laboratory (transport time never exceeded 60 min) in 0.01 M PBS (pH 7.4) at 4 °C. Once the antral follicles were obtained, they were separated by size into two groups (small (4–6 mm) and large (> 6 mm) follicles), were washed twice in cold PBS, and classified under a stereomicroscope (magnification 120 $\times$), dependent on the degree of atresia into one of three groups: atresia 1) healthy follicles abundantly vascularized, of brilliant appearance, and without the apparent detachment of the granulosa layer; atresia 2) follicles with a slight decrease in vascularization and with small detachments of the granulosa layer and atresia 3) follicles with a marked decrease in vascularization and abundant detachments of the granulosa layer and of grayish appearance (Rosales-Torres *et al.* 2000). To corroborate the morphological criteria for the classification of follicular atresia, E_2 and P_4 concentrations were determined in the cell-free follicular liquid.

Sample processing

After classification, the follicular liquid was extracted using an insulin syringe (25×16 mm needle), and immediately centrifuged at 600 *g* for 10 min at 4 °C to separate the granulosa cells. Follicles were opened to recover the granulosa cells adhered to the walls (Ballesteros *et al.* 1992). After washing the granulosa cells with PBS, viability was determined with Trypan blue (follicles that showed percentage viability of granulosa cells higher than 90% were included), and the apoptosis index was measured using flow cytometry. Remnant cells were homogenized (glass-Teflon) in 250 µl of PBS (pH 7.4) and centrifuged at 20 000 *g* for 10 min at 4 °C in order to separate the cell debris from the supernatant. Protein and GSH concentration, G6PD activity, and carbonyl groups levels in proteins were determined on the same day. The cell-free follicular liquid (obtained by centrifugation at 12 000 *g* for 20 min) was subjected to a similar process and a fraction was stored at -70 °C for E₂, P₄ and DHEA quantification.

Apoptosis index

Cells were washed twice in cold PBS by centrifugation at 250 *g* for 5 min, and were resuspended with 0.1 ml of the staining solution: 10 µl of Annexin-V-Fluos (10 µg/ml; conjugated with FITC) and 10 µl propidium iodide (PI, 50 µg/ml) in 1 ml HEPES buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM CaCl₂) and incubated for 15 min in the absence of light. Afterward, 0.4 ml of the HEPES buffer was added, proceeding then with the flow cytometry analysis (BD FACSAria; Becton Dickinson System & Co., San José, CA, USA). We analyzed 30 000 cells per follicle. Fluorescence emission was measured at 515 nm for FITC and at 610 nm for PI, with excitation at 488 nm. Data were analyzed using the FACSDIVA program for flow cytometry. Simultaneous staining with Annexin-V-FITC and PI highlighted discriminate cells with more severe plasmatic membrane alterations having undergone late apoptosis or necrosis. The sum of Annexin-V-FITC⁺/PI⁻ and Annexin-V-FITC⁺/PI⁺ yields the total apoptosis index.

GSH concentration

Fractions of follicular liquid and granulosa cells were supplemented with an equal volume of 5% (v/v) metaphosphoric acid, and centrifuged at 3000 *g* for 10 min at 4 °C. GSH concentration was measured in the supernatant using a GSH assay Kit (Calbiochem, San Diego, CA, USA).

Enzyme activity assay

G6PD activity was measured by quantifying NADPH production in the follicular liquid and granulosa cell homogenate. After centrifugation for 30 min at 15 000 *g*, the supernatants were collected and G6PD activity was analyzed spectrophotometrically (Rudack *et al.* 1971). Proteins were quantified

using the Lowry method (Lowry *et al.* 1951) with BSA as standard. Enzyme activity was expressed as mU per mg protein per minute under assay conditions. 1 mU equals 1 nmol of NADPH produced per minute.

Protein carbonyl groups

Proteins were precipitated using 20% (w/v) trichloroacetic acid (TCA), centrifuged at 500 *g* for 10 min at 4 °C, washed thrice with 5% (w/v) TCA, and thrice with chloroform. The precipitate was resuspended in 1 ml of 2% (w/v) 2,4-dinitrophenylhydrazine in 200 mM HCl and incubated at 37 °C for 60 min with stirring at an interval of 5 min. Afterward, proteins were precipitated with 20% (w/v) TCA, centrifuged, and washed with 5% (w/v) TCA and finally washed with ethanol/ethyl acetate (1/1 v/v). The pellets were dissolved in 6 M guanidine-HCl, incubated for 10 min at 37 °C. Absorbance was measured at 370 nm to detect dinitrophenylhydrazone formation. Molar extinction coefficient for DPNH ($E=22\,000\text{ M}^{-1}\text{ per cm}$) was used for calculating carbonyl concentration (Halliwell & Gutteridge 1992).

Steroid hormone determination

Concentrations of E₂, P₄, and DHEA were measured by radioimmunoanalysis, using the Coat-A-count E₂, Coat-A-count P₄, and DHEA RIA DSL 9000 active kits, following the manufacturer's instructions. The intra-assay CVs were 5.07, 2.89, and 3.05%, while the inter-assay CVs were 4.9, 5.9, and 10.8% for E₂, P₄ and DHEA respectively.

The E₂/P₄ ratio in healthy (atresia 1) follicles was >1 and <1 in atretic follicles (data not shown). These results agree with previously reported data (Burke *et al.* 2005) and support the morphological classification of follicles.

Statistical analysis

Two or three follicles of each size were assessed on every fourth day. All the determinations were done in duplicate. Data were analyzed by two-way ANOVA using a general linear model with the SAS software (SAS User's Guide, 197; SAS Institute Inc., Cary, NC, USA). Statistically significant differences among groups were determined applying Tukey's test. Results are expressed as mean ± s.d. $P<0.05$ was considered statistically significant.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Funding

This study was supported in part by FP-2006/9/I/394 Fondo para el Fomento de la Investigación, IMSS and the Consejo Nacional de Ciencia y Tecnología, Mexico (grant 2006-C01-54878).

Acknowledgements

We thank Dr Constantino López for his expert assistance and allowing for the use of the flow cytometer.

References

- Agarwal A, Salen RA & Bedaiwy MA 2003 Role of reactive species in the pathophysiology of human reproduction. *Fertility and Sterility* **79** 829–843.
- Aten RF, Duarte KM & Behrman HR 1992 Regulation of ovarian antioxidant vitamins, reduced glutathione, and lipid peroxidation by luteinizing hormone and prostaglandin F_{2α}. *Biology of Reproduction* **46** 401–407.
- Ballesteros LM, Rosales-Torres AM, Rosado A & Hernández-Pérez O 1992 Activity, compartmentation and subcellular distribution of lysosomal enzymes during follicular maturation in the goat. *Animal Reproduction Science* **27** 129–139.
- Burke CR, Cárdenas H, Mussard ML & Day ML 2005 Histological and steroidogenic changes in dominant ovarian follicles during oestradiol-induced atresia in heifers. *Reproduction* **129** 611–620.
- Díaz-Flores M, Ibañez-Hernández MA, Galvan RE, Durán-Reyes G, Medina-Navarro R, Ortega-Camarillo C, Vilar-Rojas C, Cruz M & Baiza-Gutman LA 2006 Glucose-6-phosphate dehydrogenase activity and NADPH/NADP⁺ ratio in liver and pancreas are dependent on the severity of hyperglycemia in rat. *Life Sciences* **78** 2601–2607.
- Endo T, Aten RF, Leykin L & Behrman HR 1993 Hydrogen peroxide evokes antisteroidogenic and antigonadotropic actions in human granulosa luteal cells. *Journal of Clinical Endocrinology and Metabolism* **76** 337–342.
- Filosa S, Fico A, Pagliarunga F, Balestrieri M, Crooke A, Verde P, Abriescia P, Bautista JM & Martini G 2003 Failure to increase glucose consumption through the pentose-phosphate pathway results in the death of glucose-6-phosphate dehydrogenase gene-deleted mouse embryonic stem cells subjected to oxidative stress. *Biochemical Journal* **370** 935–943.
- Flint APF & Denton RM 1970 The role of nicotinamide-adenine dinucleotide phosphate-dependent malate dehydrogenase and isocitrate dehydrogenase in the supply of reduced nicotinamide-adenine dinucleotide phosphate for steroidogenesis in the superovulated rat ovary. *Biochemical Journal* **117** 73–83.
- García-Segura LM, Cardona-Gómez P, Naftolin F & Chowen JA 1998 Estradiol upregulates Bcl-2 expression in adult brain neurons. *Neuroreport* **9** 593–597.
- Halliwell B & Gutteridge JMC 1992 Biologically relevant metal ion-dependent hydroxyl radical generation. *FEBS Letters* **307** 108–112.
- Hsueh AJW, Billig H & Tsafiriri A 1994 Ovarian follicle atresia: a hormonal controlled apoptotic process. *Endocrine Reviews* **15** 707–724.
- Hsueh AJW, Eisenhauer KM, Chun S-Y, Hsu SY & Billig H 1996 Gonadal cell apoptosis. *Recent Progress in Hormone Research* **51** 433–455.
- Jablonka-Shariff A, Fricke PM, Grazul-Bilska AT, Reynolds LP & Redmer DA 1994 Size, number, cellular proliferation, and atresia of gonadotropin-induced follicles in ewes. *Biology of Reproduction* **51** 531–540.
- Jablonka-Shariff A, Reynolds LP & Redmer DA 1996 Effects of gonadotropin treatment and withdrawal on follicular growth, cell proliferation, and atresia in ewes. *Biology of Reproduction* **55** 693–702.
- Jiang J-Y, Macchiarelli G, Tsang BK & Sato E 2003 Capillary angiogenesis and degeneration in bovine ovarian antral follicles. *Reproduction* **125** 211–223.
- Jolly PD, Tisdall DJ, De'ath G, Heath DA, Lun S, Hudson NL & McNatty KP 1997 Granulosa cell apoptosis, aromatase activity, cyclic adenosine 3',5'-monophosphate response to gonadotropins, and follicular fluid steroid levels during spontaneous and induced follicular atresia in ewes. *Biology of Reproduction* **56** 830–836.
- Jozwik M, Wolczynski S, Jozwik M & Szamatowicz M 1999 Oxidative stress markers in preovulatory follicular fluid in humans. *Molecular Human Reproduction* **5** 409–413.
- Kim HH, Shaipanich M, Hasselblatt K & Yeh J 2003 Induction of apoptosis and ovarian cyst formation in the mouse ovary by dehydroepiandrosterone (DHEA). *Journal of Medicine* **34** 101–112.
- Kim MK, Hossein MS, Oh HJ, Fibrianto HY, Jang G, Kim HJ, Hong SG, Park JE, Kang SK & Lee BC 2007 Glutathione content of *in vivo* and *in vitro* matured canine oocytes collected from different reproductive stages. *Journal of Veterinary Medical Science* **69** 627–632.
- Leopold JA & Loscalzo J 2000 Cyclic strain modulates resistance to oxidant stress by increasing G6PDH expression in smooth muscle cells. *American Journal of Physiology. Heart and Circulatory Physiology* **279** H2477–H2485.
- Lowry OH, Rosebrough NJ, Farr AL & Randall RJ 1951 Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* **193** 265–275.
- Lu SC 1999 Regulation of hepatic glutathione synthesis: current concepts and controversies. *FASEB Journal* **13** 1169–1183.
- Luderer U, Díaz D, Faustman EM & Kavanagh TJ 2003 Localization of glutamate cysteine ligase subunit mRNA within the rat ovary and relationship to follicular apoptosis. *Molecular Reproduction and Development* **65** 254–261.
- Margolin Y, Aten RF & Behrman HR 1990 Antigonaladotropic and antisteroidogenic actions of peroxide in rat granulosa cells. *Endocrinology* **127** 245–250.
- Nasr-Esfahani MM & Johnson MH 1992 Quantitative analysis of cellular glutathione in early preimplantation mouse developing *in vivo* and *in vitro*. *Human Reproduction* **7** 1281–1290.
- Ninfali P, Ditroilo M, Capellacci S & Biagiotti E 2001 Rabbit brain glucose-6-phosphate dehydrogenase: biochemical properties and inactivation by free radicals and 4-hydroxy-2-nonenal. *Neuroreport* **12** 4149–4153.
- Oliver CN, Ahn B-W, Moermant EJ, Golstein S & Stadman ER 1987 Age-related changes in oxidized proteins. *Journal of Biological Chemistry* **262** 5488–5491.
- Pandolfi PP, Sonati F, Rivil R, Mason P, Grosveld F & Luzzatto L 1995 Targeted disruption of the housekeeping gene encoding glucose 6-phosphate dehydrogenase (G6PD): G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress. *EMBO Journal* **14** 5209–5215.
- Pias EK 2002 Apoptosis in mitotic competent undifferentiated cells is induced by cellular redox imbalance independent of reactive oxygen species production. *FASEB Journal* **16** 781–790.
- Rapoport R, Sklan D & Hanukoglu I 1995 Electron leakage from the adrenal cortex mitochondrial P450_{scc} and P450_{c11} systems: NADPH and steroid dependence. *Archives of Biochemistry and Biophysics* **317** 412–416.
- Rosales-Torres AM, Ávalos-Rodríguez A, Vergara-Onofre M, Hernández-Pérez O, Ballesteros LM, García-Macedo R, Ortiz-Navarrete V & Rosado A 2000 Multiparametric study of atresia in ewe antral follicles: histology, flow cytometry, internucleosomal DNA fragmentation, and lysosomal enzyme activities in granulosa cells and follicular fluid. *Molecular Reproduction and Development* **55** 270–281.
- Rudack D, Chisholm EM & Holten D 1971 Rat liver glucose 6-phosphate dehydrogenase. Regulation by carbohydrate diet and insulin. *Journal of Biological Chemistry* **246** 1249–1254.
- Salvemini F, Franzé A, Lervolino A, Filosa S, Salzano S & Ursini MV 1999 Enhanced glutathione levels and oxidoreistance mediated by increased glucose-6-phosphate dehydrogenase expression. *Journal of Biological Chemistry* **274** 2750–2757.
- Tian W-N, Braunstein LD, Pang J, Stuhlmeier KM, Xi Q-C, Tian X & Stanton RC 1998 Importance of glucose-6-phosphate dehydrogenase activity for cell growth. *Journal of Biological Chemistry* **273** 10609–10617.
- Tian W-N, Braunstein LD, Apse K, Pang J, Rose M, Tian X & Stanton RC 1999 Importance of glucose-6-phosphate dehydrogenase activity in cell death. *American Journal of Physiology. Cell Physiology* **276** C1121–C1131.
- Tilly JL 1996 Apoptosis and ovarian function. *Reviews of Reproduction* **1** 162–172.
- Tilly JL & Tilly KI 1995 Inhibitors of oxidative stress mimic the ability of follicle-stimulating hormone to suppress apoptosis in cultured rat ovarian follicles. *Endocrinology* **136** 242–252.
- Tilly JL, Kowalski KI, Schomberg DW & Hsueh AJW 1992 Apoptosis in atretic ovarian follicles is associated with selective decreases in messenger ribonucleic acid transcripts for gonadotropin receptors and cytochrome p450 aromatase. *Endocrinology* **131** 1670–1676.

- Tsai-Turton M & Luderer U** 2006 Opposing effects of glutathione depletion and follicle-stimulating hormone on reactive oxygen species and apoptosis in cultured preovulatory rat follicles. *Endocrinology* **147** 1224–1236.
- Tsai-Turton M & Luderer U** 2005 Gonadotropin regulation of glutamate cysteine ligase catalytic and modifier subunit expression in rat is subunit and follicle stage specific. *American Journal of Physiology. Endocrinology and Metabolism* **289** E391–E402.
- Tsai-Turton M, Luong BT, Tan Y & Luderer U** 2007a Cyclophosphamide-Induced apoptosis in COV434 human granulosa cells involves oxidative stress and glutathione depletion. *Toxicological Sciences* **98** 216–230.
- Tsai-Turton M, Nakamura BN & Luderer U** 2007b Induction of apoptosis by 9,10-dimethyl-1,2-benzanthracene in cultured preovulatory rat follicles is preceded by a rise in reactive oxygen species and is prevented by glutathione. *Biology of Reproduction* **77** 442–451.
- Urata Y, Ihara Y, Murata H, Goto S, Koji T, Yodoi J & Inoue S** 2006 17 β -Estradiol protects against oxidative stress-induced cell death through the glutathione/glutaredoxin-dependent redox regulation of Akt in myocardiac H9c2 cells. *Journal of Biological Chemistry* **281** 13092–13102.
- Valdez KE, Cuneo SP & Turzillo AM** 2005 Regulation of apoptosis in the atresia of dominant bovine follicles of first follicular wave following ovulation. *Reproduction* **130** 71–81.

Received 6 November 2008
 First decision 16 January 2009
 Accepted 24 March 2009