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Expression of superoxide dismutase, catalase and glutathione peroxidase in the bovine corpus luteum: evidence supporting a role for oxidative stress in luteolysis*

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Apoptosis, a type of physiological or active cell death, has been implicated as a mechanism underlying regression of the corpus luteum (CL) in the rat, bovine, rabbit and ovine ovary. Previous *in vitro* studies of cultured luteal cells have also provided evidence which suggests that reactive oxygen species play an important role in luteolysis in the rodent ovary. To further evaluate the potential role of oxidative stress in luteal cell demise, changes in the expression of several enzymes known to protect cells from oxidative stress were investigated using bovine CL collected from ovaries of non-pregnant (day 21 of the estrous cycle; regressed CL) and pregnant (day 21 of pregnancy; functional CL) animals. Biochemical analysis of genomic DNA extracted from these two pools of CL demonstrated the presence of extensive levels of internucleosomal DNA cleavage characteristic of cell death via apoptosis in regressed, but not in functional, CL. Northern blot analysis of total RNA indicated that functional CL expressed significantly higher levels of mRNA encoding secreted superoxide dismutase (SEC-SOD, 1.9 kb) and manganese-containing or mitochondrial SOD (Mn-SOD, multiple transcripts) as compared to regressed CL. Similarly, levels of mRNA encoding catalase (2.1 kb), an enzyme responsible for detoxification of peroxides to water, were significantly higher in functional versus regressed CL. From these data, we conclude that a decline in expression of specific oxidative response genes occurs during luteolysis, and that maintained expression of these genes in the CL during pregnancy may prevent oxidative damage and delay regression.

Keywords: apoptosis; superoxide dismutase; glutathione peroxidase; catalase; oxidative free radicals; corpus luteum; bovine

Introduction

Injection of domestic animals with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), the physiological signal for luteolysis, causes an inhibition of luteal steroid synthesis followed by a disruption of cellular integrity (Nett *et al.*, 1976; Niswender *et al.*, 1985; Keyes and Wiltbank, 1988; Sawyer *et al.*, 1990). The inhibition of progesterone synthesis is reversible until the structural integrity of the corpus luteum (CL) has been disrupted, after which cell death is inevitable (Pate and Condon, 1984; reviewed in Pate, 1994). Two distinct types of cell death, apoptosis and necrosis, have been identified in the CL during regression. Apoptosis, a form of physiological cell death, occurs during natural or induced luteolysis in the bovine (Juengal *et al.*, 1993) and ovine (Rueda *et al.*, 1995) ovary, as well as during luteal regression in the rat (Orlicky *et al.*, 1992) and rabbit (Dharmarajan *et al.*, 1994).

The onset of apoptosis in the bovine CL, however, was not observed until after the decline in serum concentrations of progesterone (Juengal *et al.*, 1993), suggesting that apoptosis may be involved in cellular demise of the CL only during structural regression. Moreover, in contrast to the reported role of apoptosis in luteolysis during the estrous cycle of the rat, regression of the postpartum CL in this species is apparently not associated with apoptosis (Orlicky *et al.*, 1992). Despite conflicting data concerning the precise role of apoptosis in luteolysis, it is likely that cell death is involved in structural regression of the CL following the loss of progesterone synthesizing capacity. Therefore, a better understanding of the sequence of events that initiate this irreversible process, following the decline in steroidogenesis, would provide a basis for understanding how the CL initiates self-destruction.

One interesting hypothesis concerns the potential role of reactive oxygen species in triggering luteal regression. The generation of oxidative free radicals occurs in all cells as a consequence of normal metabolism and hormone-mediated signalling events involving lipid membrane turnover (reviewed in Yu, 1994). Due to the deleterious effects exerted by reactive oxygen species on many aspects of cellular function and homeostasis, cells possess several defense mechanisms which serve to detoxify or metabolize free radicals and their intermediates. Proteins which play a role in protecting cells from oxidative stress include several forms of superoxide dismutase (SOD). These enzymes convert superoxide anion to hydrogen peroxide, which can then be rapidly metabolized to water via the actions of either glutathione peroxidase (GSHPx) or catalase. If activity of one or more of these enzymes is disrupted, the resultant increase in reactive oxygen species can cause DNA strand breaks, protein destabilization, plasma membrane breakdown and, ultimately, cell death (McConkey *et al.*, 1989; Wong *et*

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al., 1989; Hockenberry *et al.*, 1993; Rosen *et al.*, 1993; Yu, 1994). Interestingly, cells which are oxidatively-stressed display several morphological and biochemical features characteristic of apoptosis (Kerr *et al.*, 1972; Wyllie, 1980), suggesting that reactive oxygen species can trigger physiological cell death.

In the ovary, oxidative stress has recently been implicated as a mechanism underlying apoptosis in granulosa cells during follicular atresia (Tilly, 1993; Tilly and Tilly, 1995; Tilly *et al.*, 1995). A role for superoxide radicals (SOR) in regulating luteal cell function was first proposed based on changes in SOR levels that occur in the rat ovary during the estrous cycle (Laloraya *et al.*, 1988). Levels of SOR are elevated within rat luteal cells *in vitro* in response to $PGF_{2\alpha}$ (Sawada and Carlson, 1991, 1994), coincident with a loss of steroidogenic capacity. These data, along with recent findings that inducers of oxidative stress inhibit luteal cell synthesis of progesterone and protein (Gatzuli *et al.*, 1991; Musicki *et al.*, 1994), argue strongly in favor of a role for reactive oxygen species in triggering luteal cell demise.

Based on these data, we hypothesized that increased oxidative stress during luteolysis could result from decreased expression of enzymes that metabolize the superoxide radical and/or hydrogen peroxide. The accumulation of reactive oxygen species would then trigger a cascade of events leading to luteal regression, initially by an inhibition of luteal cell steroid production followed by an induction of structural collapse. To provide further support for the hypothesis that oxidative stress is involved in luteolysis, we analysed changes in the levels of mRNA encoding SOD, catalase and GSHPx during the late stages of luteolysis in CL isolated from non-pregnant cows. Healthy CL collected from ovaries of pregnant cows were used as points of comparison, and changes in SOD, catalase and GSHPx mRNA levels were then correlated to the functional (progesterone output) and structural (apoptosis) integrity of the luteal cells.

Results

Identification of apoptosis in regressed CL

Biochemical analysis of genomic DNA extracted from functional CL collected on day 21 of pregnancy (PREG) revealed the presence of only intact, high molecular weight (MW) DNA (Figure 1A). In contrast, genomic DNA prepared from regressed CL of day 21 non-pregnant cows (OPEN) displayed internucleosomal fragmentation in multiples of 185-basepair (bp) characteristic of cell death via apoptosis (Figure 1A). Quantitative analysis revealed that the extent of radionucleotide incorporation into low MW (<15 kb) DNA prepared from functional CL of pregnant cows was 22 ± 12% of the levels measured in DNA samples prepared from regressed CL of non-pregnant cows ($P<0.01$; Figure 1B). Analysis of serum progesterone concentrations confirmed that non-pregnant cows with regressed CL possessed relatively low levels of circulating progesterone (0.05 ± 0.04 ng/ml) when compared with levels in pregnant animals possessing fully-functional CL (6.16 ± 1.19 ng/ml; mean ± SEM, $n = 3$, $P<0.001$).

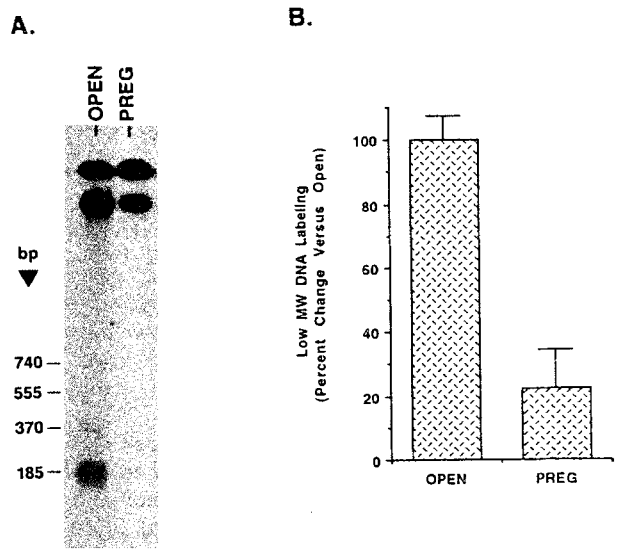

Figure 1 Biochemical analysis of DNA integrity in isolated bovine corpora lutea (CL). Genomic DNA was extracted from CL collected from cows on day 21 of the estrous cycle (OPEN; regressed CL) or on day 21 of pregnancy (PREG; functional CL), radiolabeled on 3'-ends and resolved through a 2% agarose gel. Samples were analysed by autoradiography (**A**, representative of data obtained in three replicate experiments) followed by β-counting of low MW DNA (**B**, mean ± SEM, $n = 3$) for the presence of oligonucleosomal DNA fragments characteristic of cell death via apoptosis

SOD, catalase and GSHPx gene expression in the CL

Northern blot analysis of total RNA extracted from bovine CL demonstrated that the levels of mRNA encoding the structural protein, β-actin, remained constitutive during luteolysis in non-pregnant cows when compared with β-actin mRNA levels in functional CL collected from cows on day 21 of pregnancy ($P>0.05$, quantitative data not shown; Figure 2). Therefore, β-actin mRNA levels were used to normalize all remaining data obtained by northern and slot blot analysis of oxidative stress response gene expression.

Evaluation of SOD mRNA levels indicated that functional CL (day 21 of pregnancy) possessed significantly higher levels of mRNA encoding both the secreted (SEC-SOD; transcript size of 1.9 kb), and manganese-containing or mitochondrial (Mn-SOD; transcript sizes of 3.7, 1.9 and 1.5 kb), forms of SOD when compared with those levels detected in regressed CL isolated from non-pregnant cows (Figures 3A and 4A, respectively). Following normalization against β-actin, levels of SEC-SOD and Mn-SOD mRNA in functional CL were estimated to be 445 ± 11% ($P<0.001$; Figure 3B) and 858 ± 14% ($P<0.001$; Figure 4B), respectively, of those levels measured in regressed CL.

A similar analysis of catalase gene expression revealed that levels of the 2.1 kb catalase mRNA transcript in functional CL collected on day 21 of pregnancy were 154 ± 4% of those levels detected in regressed CL of non-pregnant animals ($P<0.05$; Figure 5A and B). In contrast, levels of the 1.2 kb GSHPx mRNA transcript were higher in regressed, as compared with functional, CL (Figure 6A). Following normalization against β-actin, quantitative analysis of hybridization signal intensities indicated that functional CL contained

44 ± 4% less GSHPx mRNA versus those levels measured in regressed CL ($P<0.05$; Figure 6B).

Discussion

Prostaglandin $F_{2\alpha}$ serves as the physiological luteolysin in several species, although its mechanism of action is not completely understood. The inhibition of luteal steroid synthesis mediated by $PGF_{2\alpha}$, which precedes structural collapse of the CL, is reversible until the integrity of the luteal cell has been compromised (reviewed in Pate, 1994). This disruption of luteal cell integrity has been reported to occur, at least in part, through activation of the apoptotic or cell-suicide process (Kerr *et al.*, 1972) in the rat (Orlicky *et al.*, 1992), bovine (Juengal *et al.*, 1993), rabbit (Dharmarajan *et al.*, 1994) and ovine (Rueda *et al.*, 1995) ovary.

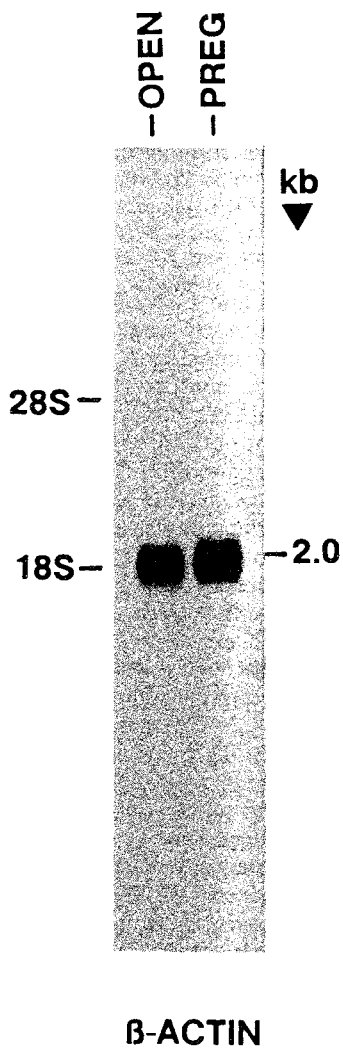

Figure 2 Expression of β-actin mRNA in regressed and functional corpora lutea. Northern blot analysis was conducted using 10 μg of total RNA prepared from regressed (OPEN) or functional (PREG) CL isolated from ovaries of cows described in legend to Figure 1. These data, which are representative of results obtained in three replicate experiments, indicate that β-actin mRNA levels remain constitutive during luteolysis. The estimated transcript size in kilobases, (kb) and migration distances of the 18S and 28S ribosomal RNAs are indicated

Reactive oxygen species have been implicated as a trigger for functional luteolysis (Gatzuli *et al.*, 1991; Riley and Behrman, 1991; Sawada and Carlson, 1991, 1994; Musicki *et al.*, 1994), as well as for apoptosis in

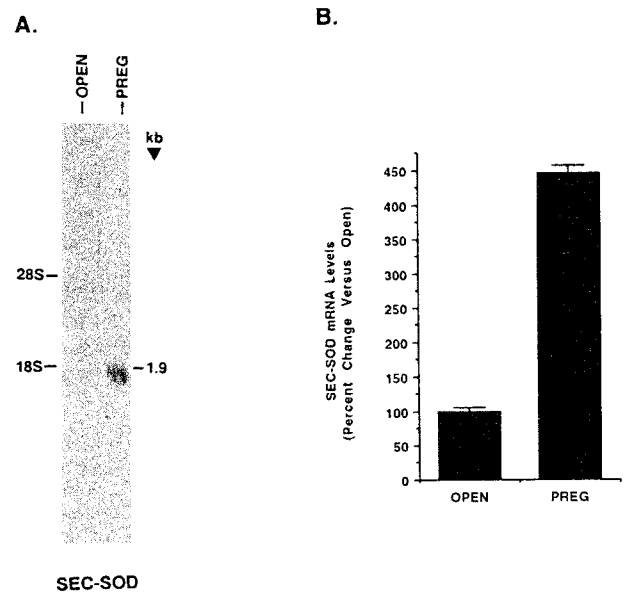

Figure 3 Expression of secreted superoxide dismutase (SEC-SOD) mRNA in regressed and functional corpora lutea. Total RNA was isolated from regressed (OPEN) or functional (PREG) CL, and then used for northern blot (**A**; 10 μg/lane, representative of data obtained in three replicate experiments) or slot blot (**B**; mean ± SEM, $n = 3$) analysis of SEC-SOD mRNA levels. In **A**, the estimated transcript size (in kb) and migration distances of the 18S and 28S ribosomal RNAs are indicated. The data presented in **B** were normalized against β-actin mRNA levels to account for equality of sample loading

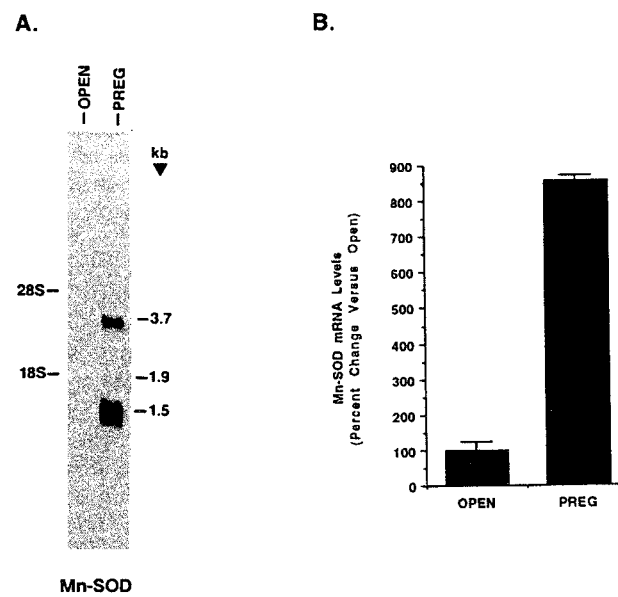

Figure 4 Manganese-containing superoxide dismutase (Mn-SOD) gene expression in the regressed (OPEN) and functional (PREG) corpus luteum. Total RNA samples were prepared from bovine CL and analysed as described in legend to Figure 3 for changes in Mn-SOD mRNA levels during luteolysis. **A** depicts a representative northern blot autoradiogram (estimated sizes of transcripts in kb, and the migration distances of the 18S and 28S ribosomal RNAs, are indicated), whereas the data presented in **B** were obtained by slot blot mRNA hybridization analysis following normalization against β-actin mRNA levels in each sample (mean ± SEM, $n = 3$)

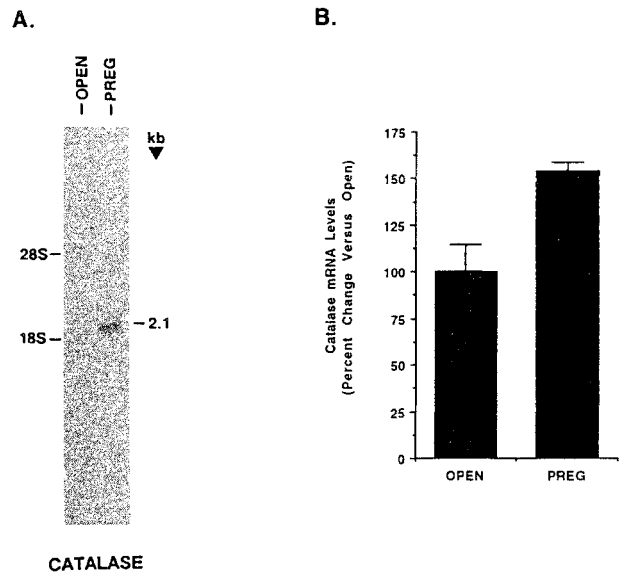

Figure 5 Expression of the catalase gene in the bovine corpus luteum. Analysis of catalase mRNA levels in regressed (OPEN) and functional (PREG) CL were conducted as described in legend to Figure 3. **A** depicts a representative northern blot autoradiogram (estimated transcript size in kb, and migration distances of 18S and 28S ribosomal RNAs, are indicated), whereas **B** represents quantitative determinations following slot blot analysis and normalization of data against β-actin mRNA levels in each sample (mean ± SEM, $n = 3$)

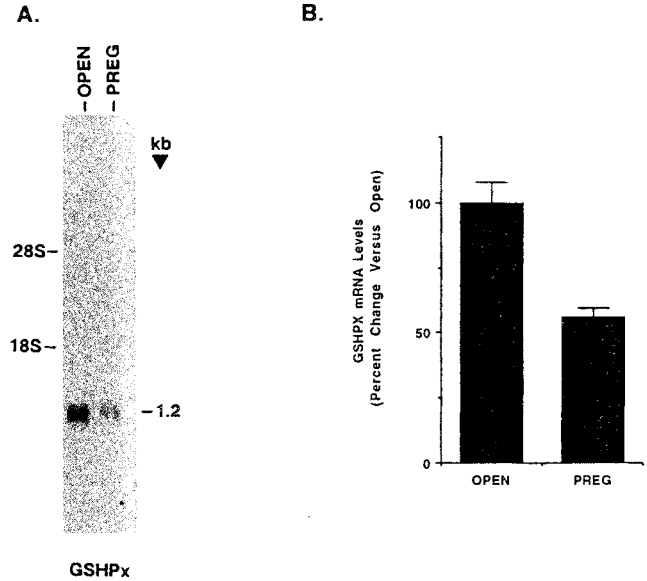

Figure 6 Glutathione peroxidase (GSHPx) gene expression in the bovine corpus luteum. Northern blot (**A**; representative autoradiogram with estimated transcript size in kb, and migration distances of 18S and 28S ribosomal RNAs, indicated) and slot blot (**B**; mean ± SEM, $n = 3$) analysis of GSHPx mRNA levels in regressed (OPEN) and functional (PREG) CL collected from bovine ovaries. The analyses were conducted as described in legend to Figure 3, and the quantitative data presented in **B** were normalized against β-actin mRNA levels in each sample

ovarian follicles (Tilly and Tilly, 1995) and cells of extragonadal origin (McConkey *et al.*, 1989; Wong *et al.*, 1989; Hockenberry *et al.*, 1993; Rosen *et al.*, 1993). We therefore hypothesized that oxidative stress in luteal cells at the time of regression may result from reduced expression of enzymes which function to protect cells from reactive oxygen species and their intermediates. To add support to the hypothesis that oxidative stress is involved in luteolysis, we collected CL from cows on day 21 of the estrous cycle (regressed CL) or pregnancy (functional CL) for analysis of functional integrity (progesterone production), structural integrity (apoptosis) and expression of oxidative stress response genes.

Day 21 pregnant cows possessed fully-functional, healthy CL as evidenced by high serum progesterone concentrations and the absence of luteal apoptosis (no internucleosomal DNA cleavage). In contrast, serum progesterone concentrations in non-pregnant animals on day 21 of the estrous cycle were extremely low, indicative of non-functional CL. Moreover, the isolated CL from these cows displayed extensive levels of internucleosomal DNA cleavage characteristic of cell death via apoptosis, confirming a loss of cellular structural integrity as well. Northern blot analysis of oxidative stress response gene expression in these tissues demonstrated the presence of relatively abundant levels of secreted and manganese-containing (mitochondrial) SOD mRNA in functional CL of pregnant animals. However, the levels of these messages were almost non-detectable in regressed CL, suggesting that cells within the regressed CL have a markedly impaired capacity to metabolize the superoxide radical. Taken with previous data which indicate that elevated SOR levels induced by physiological ($PGF_{2\alpha}$; Riley and Behrman, 1991; Sawada and Carlson, 1991, 1994) or pharmacological (xanthine oxidase; Gatzuli *et al.*, 1991) approaches disrupt luteal cell function, the present findings support the concept that oxidative stress is likely a fundamental event associated with luteolysis. It is important to point out, however, that this conclusion is based on the assumption that changes in SOD mRNA levels in the bovine CL reflect comparable changes in enzymatic activity of SOD protein. This limitation in the interpretation of the data warrants clarification in future studies.

To further analyse the relationship between oxidative stress response gene expression and luteal regression, an evaluation of mRNA levels for two key enzymes responsible for promoting conversion of peroxides to water (catalase and GSHPx) was conducted. Consistent with the loss in SOD expression during luteolysis, catalase mRNA levels were significantly lower in regressed, as compared to functional, CL. These findings would indicate that cells in the regressed CL may also have a reduced capacity to detoxify peroxides in addition to a loss of superoxide dismutase function. Surprisingly, the last series of experiments revealed that GSHPx mRNA levels were more abundant in regressing CL of non-pregnant animals as compared with functional CL obtained from their pregnant counterparts. Although the reason for this discrepancy is unclear at present, it is likely that the markedly reduced expression of SOD (which converts SOR to peroxides) in regressing CL would lead to a significant accumulation of SOR without further conversion to hydrogen peroxide. Thus, under these conditions, catalase and GSHPx activities (which convert peroxides to water) would likely not serve as critical determinants of luteal cell fate.

In summary, we conclude that a decline in secreted superoxide dismutase, manganese-containing superoxide dismutase and catalase gene expression occurs during luteal regression in the bovine ovary. These findings, along with previous data which indicate that increased levels of reactive oxygen species can be detrimental to luteal cell function, support the concept that oxidative stress plays a central role in luteolysis. Although the cause-effect relationship between luteal regression and oxidative stress remains to be fully elucidated, these data provide a basis for future studies on the emerging importance of reactive oxygen species in regulating ovarian function.

Materials and methods

Animals and tissue collection

Western range cattle (Angus crossbred, 5–7 years of age) were given a single intramuscular injection of 25 mg of prostaglandin $F_{2\alpha}$ (Lutalyse; Upjohn Company, Kalamazoo, MI) to synchronize estrous cycles (Rueda *et al.*, 1993). Animals were monitored twice daily for estrus. Cows randomly assigned to the pregnant group were artificially inseminated 12 h following standing estrus. Reproductive tracts were collected from cows on day 21 of the estrous cycle (open group, $n = 3$) or day 21 of pregnancy (pregnant group, $n = 3$). The CL were removed, snap-frozen in liquid nitrogen and stored at −70°C until analysis. To monitor luteal function, blood samples were collected via jugular venipuncture prior to tissue collection for determination of circulating concentrations of progesterone. Pregnancy was verified by retrieval of a conceptus following uterine flushing (Rueda *et al.*, 1993).

Genomic DNA isolation and analysis

Genomic DNA was extracted from CL as described previously (Gross-Bellard *et al.*, 1973; Tilly and Hsueh, 1993) and quantitated by spectrophotometry at an absorbance of 260 nm. An equivalent amount of genomic DNA from each treatment group was radiolabeled on 3′-ends with [α^{32}P]-dideoxy-ATP (ddATP, 3000 Ci/mmol; Amersham, Arlington Heights, IL) using 25 U terminal transferase enzyme (Boehringer-Mannheim, Indianapolis, IN) as described (Tilly and Hsueh, 1993). Samples were then separated by electrophoresis through 2% agarose gels (500 ng of labeled DNA per well) for approximately 3.5 h at 65 V. The gels were dried for 2 h without heat in a slab gel dryer, sealed in plastic wrap and exposed to Kodak X-Omat films at −70°C for autoradiographic analysis. Following autoradiography, low MW DNA fractions (<15 kb) were excised from the gel, mixed with 3 ml scintillation fluid (Scintiverse BD, Fisher Scientific, Pittsburgh, PA), and counted in a β-counter to provide a quantitative estimate of the degree of internucleosomal DNA cleavage among samples as described (Tilly and Hsueh, 1993).

Preparation of cRNA and cDNA probes

Complementary DNAs encoding ovine SEC-SOD (Rueda *et al.*, 1994), ovine Mn-SOD (Rueda *et al.*, 1994), ovine GSHPx (Rueda *et al.*, 1994), and rat catalase (Tilly and Tilly, 1995) were utilized for these studies. Due to the extremely high degree of nucleotide homology which exists between these genes across species (in most cases greater than 85–90%), combined with the use of antisense RNA probes under highly stringent hybridization conditions, there were likely no problems with the specificity of recognition of the bovine mRNA species by these heterologous probes. Moreover, the estimated sizes of the transcripts detected in the present studies were in complete agreement with previous data based on the use of cRNA probes against oxidative stress response gene coding sequences in a homologous system (Tilly and Tilly, 1995).

Antisense RNA probes complementary to mRNA coding sequences were synthesized by *in vitro* transcription from linearized plasmid templates using RNA polymerase, [α^{32}P]-CTP (3000 Ci/mmol; Amersham) and the Gemini II Riboprobe Core System (Promega, Madison, WI) as described (Melton *et al.*, 1984). A human β-actin cDNA probe (American Type Culture Collection, Bethesda, MD; ATCC no. 65129) was radiolabeled with [α^{32}P]-dCTP (3000 Ci/mmol; Amersham) using the random priming method (Feinberg and Vogelstein, 1983), and purified from unincorporated radionucleotides by column chromatography (NucTrap Push Columns; Stratagene, La Jolla, CA).

Extraction and analysis of RNA

Total RNA was extracted by the guanidinium isothiocyanate-phenol-chloroform extraction procedure (Chomczynski and Sacchi, 1987). The quantity and purity of the RNA preparations were estimated by measuring the optical density of each sample at 260 versus 280 nm. Following isolation, RNA samples were analysed by northern or slot blot hybridization analysis using cRNA probes (3×10^6 c.p.m. probe/ml hybridization buffer) under highly stringent conditions at 65°C (Tilly *et al.*, 1992a; Tilly and Tilly, 1995). Following cRNA probe hybridization analysis, the radioactivity on the blots was allowed to decay and the blots were re-hybridized with the radiolabeled β-actin cDNA probe at 42°C under conditions previously described (Tilly *et al.*, 1992b). Changes in SOD, catalase and GSHPx mRNA levels were normalized against β-actin mRNA levels in each sample following scanning densitometry of hybridization signal intensities.

Progesterone radioimmunoassay

Progesterone concentrations in serum samples were determined by radioimmunoassay (RIA) as previously described (Rueda *et al.*, 1993).

Data analysis

All experiments were repeated at least three times. A representative autoradiogram is presented where appropriate for qualitative analysis, whereas quantitative results obtained from RIA of serum progesterone concentrations, β-counting of low MW DNA labeling (DNA analysis) or densitometric analysis of hybridization signal intensities (RNA analysis) represent the mean ± SEM of combined data from the replicate experiments. All data concerning changes in SOD, catalase and GSHPx mRNA levels were normalized relative to levels of β-actin message in each sample. Statistical differences between mean percent values were first analysed by a one-way analysis of variance. When significant differences were noted, the data were further analysed by Scheffe's test. Significance was assigned at the 0.05 level.

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