

Alteration in localization of steroid hormone receptors and coregulatory proteins in follicles from cows with induced ovarian follicular cysts

Natalia R Salvetti^{1,2}, Natalia S Alfaro¹, Melisa M L Velázquez^{1,2}, Ayelen N Amweg^{1,2}, Valentina Matiller¹, Pablo U Díaz^{1,2} and Hugo H Ortega^{1,2}

¹Morphological Sciences Department, Faculty of Veterinary Sciences, National University of Litoral (FCV-UNL), R.P. Kreder 2805, 3080 Esperanza, Santa Fe, Argentina and ²Argentine National Research Council (CONICET), Buenos Aires, Argentina

Correspondence should be addressed to H H Ortega at Morphological Sciences Department, Faculty of Veterinary Sciences, National University of Litoral (FCV-UNL); Email: hhortega@fcv.unl.edu.ar

N R Salvetti and N S Alfaro contributed equally to this work

Abstract

Cystic ovarian disease (COD) is an important cause of infertility in cattle. The altered follicular dynamics and cellular differentiation observed in COD may be mediated through a disruption of the expression of steroid receptors and their associated transcriptional cofactors. The aim of this study was to determine the protein expression profiles of ESR1, ESR2, PGR, AR, NCOA3, NCOR2, and PHB2 (REA) in ovarian follicles in an experimental model of COD induced by the administration of ACTH. Ovaries were collected and follicles were dissected from heifers during the follicular phase (control) or from heifers treated with ACTH to induce the formation of ovarian follicular cysts. Ovaries were fixed, sectioned, and stained immunohistochemically for steroid receptors and the associated transcription factors. The relative expression of ESR1 was similar in follicular cysts and in tertiary follicles from both control and cystic cows and was significantly higher than in secondary follicles. The expression of ESR2 in the granulosa was higher in cystic follicles. No differences were seen for PGR. The expression of androgen receptor was significantly increased in tertiary follicles with lower immunostaining in cysts. The expression of NCOA3 was observed in the granulosa and theca with a significantly increased expression in the theca interna of cystic follicles. The highest levels of NCOR2 expression in granulosa, theca interna, and theca externa were observed in cysts. In granulosa cells, NCOR2 levels increase progressively as follicles mature and the treatment had no effect. In summary, ovaries from animals with induced COD exhibited altered steroid receptor expression compared with normal animals, as well as changes in the expression of their regulators. It is reasonable to suggest that in conditions characterized by altered ovulation and follicular persistence, such as COD, changes in the intra-ovarian expression of these proteins could play a role in their pathogenesis.

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Introduction

Cystic ovarian disease (COD) is an important cause of infertility and has been reported in many species, including cows, sheep, and pigs. Many factors, such as stress, nutritional management, and infectious disease, can lead to COD. The primary cause of this disease has not yet been elucidated, although an intra-ovarian component is involved in its pathogenesis (Silvia *et al.* 2002, Peter 2004, Vanholder *et al.* 2006). In this context, ovarian folliculogenesis is a complex process of morphological and biochemical events regulating the growth and differentiation of follicles from the primordial to the preovulatory stage and ovulation. In this process, steroids play a key role in the growth, differentiation, and

function of ovarian follicles. Locally produced androgens, estrogens, and progesterone are involved in the regulation of different follicular functions (Rosenfeld *et al.* 2001, Drummond *et al.* 2002, Schams & Berisha 2002, Brosens 2004, Drummond 2006, Kimura *et al.* 2007, Ortega *et al.* 2009).

The locations of steroid hormone receptors within ovarian follicles have been evaluated using a variety of techniques (Manikkam *et al.* 2001, Cassar *et al.* 2002, Jo *et al.* 2002, Schams & Berisha 2002, Van den Broeck *et al.* 2002a, 2002b, Hampton *et al.* 2004, D'Haeseleer *et al.* 2005). It has been demonstrated that either estrogen or estrogen receptor (ESR) imbalances/disturbances may result in the development of ovarian follicular cysts in cattle (Garverick 1997, Salvetti *et al.* 2007,

Alfaro *et al.* 2012), sheep (Ortega *et al.* 2009), humans (Shushan *et al.* 1996, Jakimiuk *et al.* 2002), and rodents (Salvetti *et al.* 2009a). However, one problem with previous studies is that follicular tissues were collected from abattoirs where the reproductive history of the animals was not known.

On the other hand, recent studies have demonstrated that the transcriptional activity of steroid hormone receptors is determined not only by hormone binding but also by the relative activities of nuclear receptor-associated coactivators and corepressors (Mussi *et al.* 2006). The equilibrium between coregulators and the nature of the ligand combines to determine the state of nuclear receptor activation or inhibition (Park *et al.* 2005). Some of the most important coactivators are the nuclear receptor coactivators (NCOAs), also referred to as steroid receptor coactivators (SRCs). These include NCOA1 (SRC1), NCOA2 (SRC2/GRIP1/TIF2), and NCOA3 (SRC3/pCIP/ACTR/AIB1/RAC-3/TRAM-1) (McKenna *et al.* 1999). On the other hand, one of the most widely studied corepressors, nuclear receptor corepressor 2 (NCOR2) or silencing mediator of retinoid and thyroid receptors (SMRT), was first discovered and identified through its interaction with thyroid and retinoid hormone receptors (Chen & Evans 1995, Hörlein *et al.* 1995). Another important molecule is the repressor of ESR activity (PHB2 (REA)), which interacts

with selected steroid receptors, such as ESR (Montano *et al.* 1999, Delage-Mourroux *et al.* 2000).

Studies examining coregulator expression in the ovary are limited (Hlaing *et al.* 2001, Zhang *et al.* 2003, Hussein-Fikret & Fuller 2005, Chen *et al.* 2008). It is possible that altered coregulator expression may lead to altered transcriptional activation of steroid receptors, with changes in the steroid-mediated actions in follicular development. In this context, NCOAs are overexpressed in some endocrine-dependent organs and tumors and enhance cell proliferation and differentiation (Sarvilinna *et al.* 2006, Mukherjee *et al.* 2007), indicating an important pathophysiological role. Also, coactivator expression is known to be sexually dimorphic (Auger *et al.* 2002, Charlier *et al.* 2002, Duncan & Carruth 2007) and regulated by steroid hormones (Murphy & Segal 1997, Mitev *et al.* 2003, Charlier *et al.* 2006). However, there are limited data regarding the expression of coregulators in the bovine ovary (Hlaing *et al.* 2001), and no studies have been performed to investigate protein localization in different ovarian compartments.

Based on these observations, we hypothesized that cattle with COD may have a disrupted expression of ovarian steroid receptors and their coregulators associated with an altered follicular dynamic and cellular differentiation. As steroid hormones are important intra-ovarian regulators of follicular development and

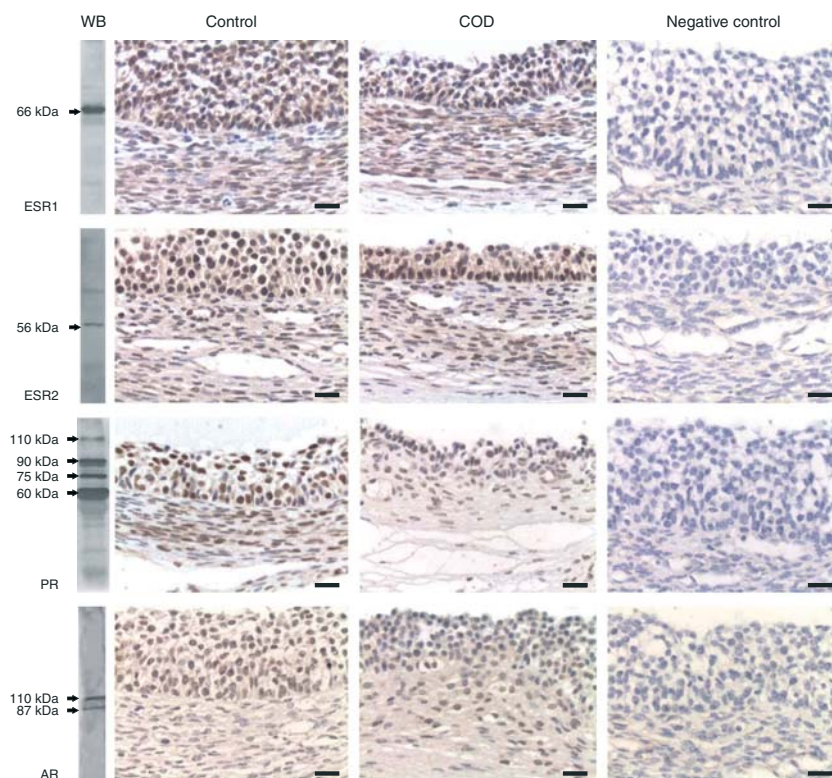


Figure 1 Representative images of ESR1, ESR2, PGR, and AR immunostaining in antral follicles of normal cycling animals and cystic follicles of COD-induced animals. Negative controls demonstrating the specificity of the antibody are also shown. Verification of antibody specificity by western blot analyses of ovarian homogenate is shown on the left column. Bars = 20 μ m.

ovulatory processes, the aim of this study was to determine the protein expression profiles of steroid receptors, NCOA3, NCOR2, and PHB2 in ovarian follicles in an experimental model of COD compared with heifers with regular estrous cycles in order to investigate the mechanisms underlying follicular cyst formation.

Results

Experimental model

The successful induction of COD was confirmed by ultrasonography, ovarian morphology, and intrafollicular and serum hormone levels. All the treated animals showed healthy developing follicles, as well as follicles showing different degrees of atresia and one large cyst (in one ovary) with a complete granulosa cell layer. Corpora lutea were absent in all cases. The ovaries from control animals exhibited follicles in various stages of development, including primary, secondary, and tertiary follicles, as well as atretic follicles and corpora lutea. In addition, COD induction was confirmed by serum and follicular fluid hormone analysis. Steroid concentrations in follicular fluid showed that all follicles were categorized as estrogen-active with no differences in estrogen concentrations between cystic and preovulatory (control). Mean progesterone concentrations were significantly higher in the follicular fluid of preovulatory follicles than in induced cysts. The progesterone:estradiol ratio did not differ between cystic and preovulatory follicles (parallel study, Ortega *et al.* (2008) and Salvetti (2010)).

Antibody specificity

The results from western blot analysis of follicular wall homogenates are summarized in Figs 1 and 2. Western blot analysis showed positive bands of appropriate

molecular weight for each of the proteins studied. The ESR1 and ESR2 antibodies detected a single band at 66 and 55 kDa respectively. Progesterone receptor (PGR) showed three isoforms corresponding to PGRB (116 kDa), PGRA (94 kDa), and PGRC (60 kDa). Androgen receptor (AR) antibody detected a band at 110 kDa (ARA) and another band at 87 kDa (ARB). The NCOA3, NCOR2, and PHB2 antibodies each detected a single band at 160, 150, and 37 kDa respectively corresponding to the expected molecular weights of the coregulatory proteins. In the absence of the primary antibodies, no specific bands were detected.

Immunohistochemistry

To obtain information regarding the localization of steroid receptors and coregulatory proteins in different follicular structures, their expression was evaluated by immunohistochemistry and quantitative image analysis. In this sense, immunohistochemistry is currently the only technique that allows an integrative analysis about the cellular localization of proteins in nearby tissue compartments when it is not possible to separate cell populations to perform other, more sensitive and specific techniques. This technique also allows determining the location within an integrated context.

Steroid receptor expression

Expression of ESR1 was localized to the nucleus of the granulosa cells of primary follicles and to both the theca interna and the granulosa cells of secondary follicles (Fig. 3). The intensity of ESR1 staining in the nuclei of granulosa cells was greater than that in theca cells ($P < 0.05$). The immunostaining was increased as the follicular development progressed in the granulosa and theca cell compartment ($P < 0.05$). The immunostaining

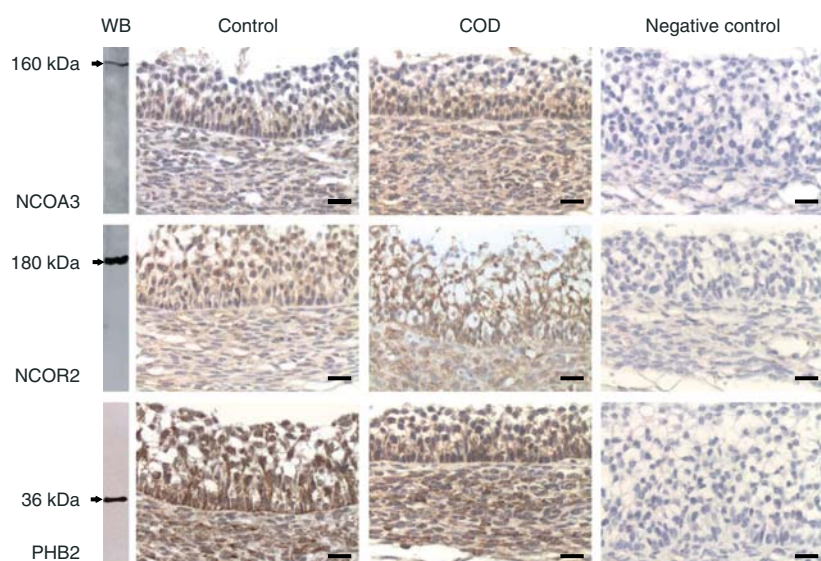


Figure 2 Representative images of NCOA3, NCOR2, and PHB2 immunostaining in antral follicles of normal cycling animals and cystic follicles of COD-induced animals. Negative controls demonstrating the specificity of the antibody are also shown. Verification of antibody specificity by western blot analyses of ovarian homogenate are shown on the left column. Bars = 20 μ m.

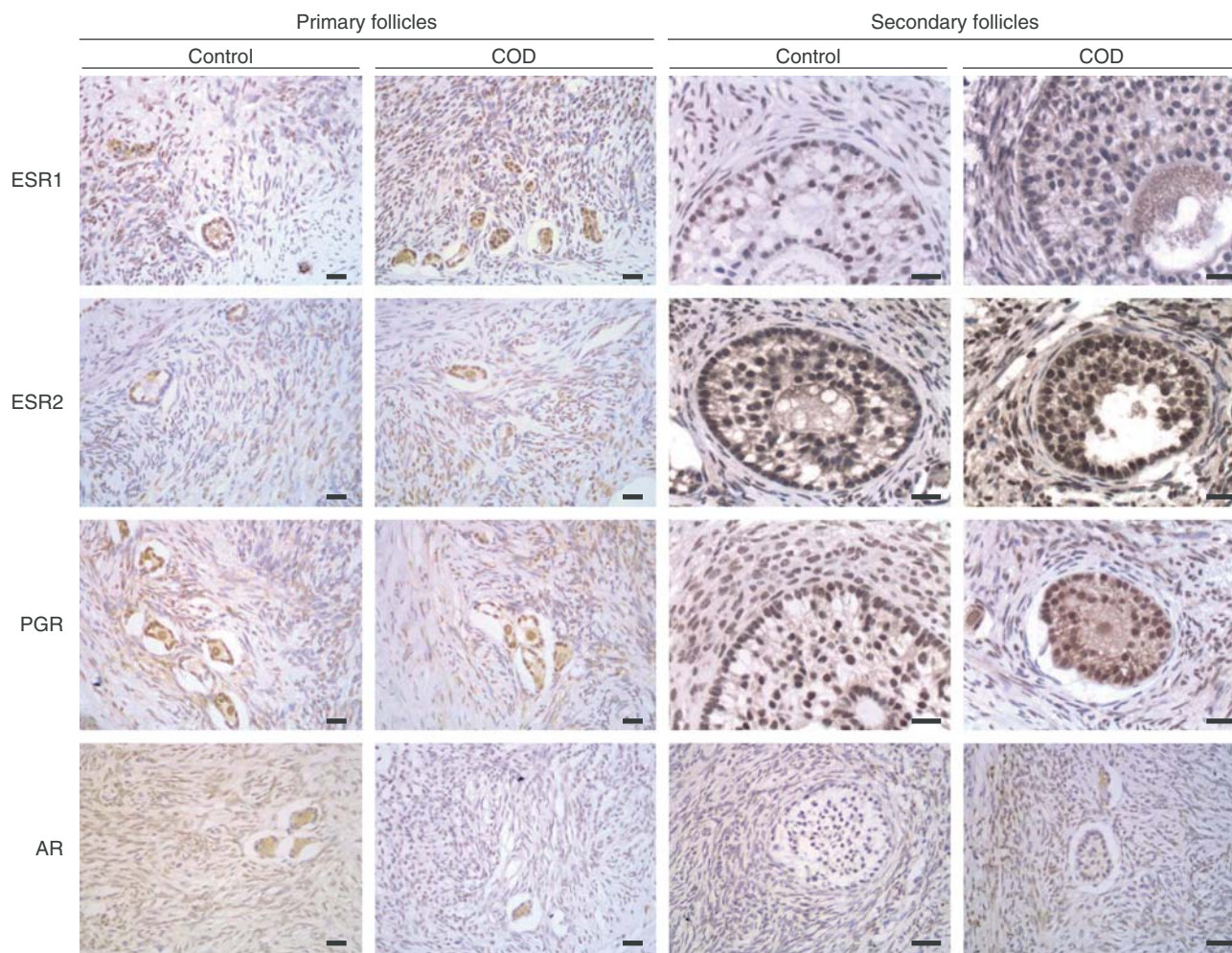


Figure 3 Representative images of ESR1, ESR2, PGR, and AR immunostaining in primary and secondary follicles of normal cycling and COD-induced animals. Bars=20 μ m.

in cystic follicles was similar to that in tertiary follicles, without differences in the same follicular categories between groups (Figs 1 and 4).

ESR2 was detected in the nucleus of granulosa and theca cells of all the follicular categories studied, and the intensity of immunostaining was higher in the granulosa cells of tertiary follicles than in those of the primary and secondary follicles ($P<0.05$; Figs 1, 3 and 4). In animals with induced COD, the immunoexpression of ESR2 was diminished in the tertiary and cystic follicles in relation to that in the tertiary follicles from control animals ($P<0.05$; Fig. 4). Theca cells were scarcely stained, but the tertiary follicles and cysts from COD animals were less stained in theca interna than tertiary follicles from normal animals ($P<0.05$; Figs 1, 3 and 4).

PGR was observed in both the nucleus and the cytoplasm of granulosa cells. Maximum staining intensity was observed in tertiary follicles ($P<0.05$). The expression of PGR was lower in the granulosa cell layer of cystic follicles than in that of the tertiary follicles ($P<0.05$). The level of immunostaining in theca cells

was lower than that in granulosa cells ($P<0.05$), and no difference was evident across the different follicle stages (Figs 1, 3 and 4).

AR was localized in the nucleus, with predominant expression in granulosa cells and weak staining in theca cells. The intensity of immunostaining for AR was lower in both cysts and tertiary follicles from cows with induced COD than in tertiary follicles from control cows ($P<0.05$; Figs 1, 3 and 4).

Coregulatory expression

Representative members of the steroid receptor coregulator families, NCOA3, NCOR2, and PHB2, were chosen and their expression was evaluated by immunohistochemistry and quantitative image analysis. Immunostaining of NCOA3 was primarily found in the nucleus and cytoplasm of granulosa and differentiated theca cells, whereas its expression was significantly weaker in theca cells of secondary follicles and theca externa of tertiary follicles than other structures ($P<0.05$; Figs 2 and 5). The most

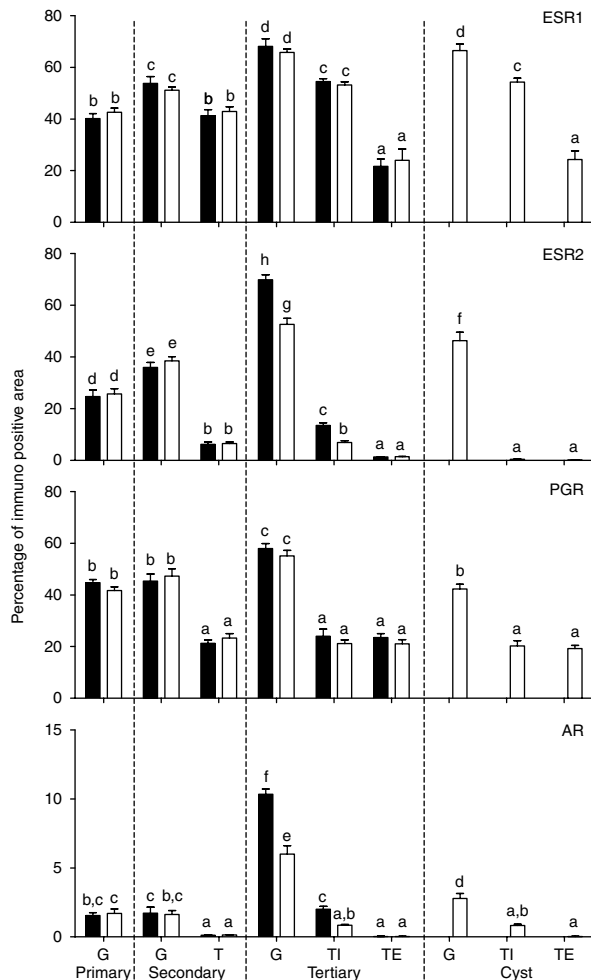


Figure 4 Relative expression (measured as percentage of immuno-positive area) of steroid receptors in primary, secondary, tertiary, and cystic follicles of control (black bars) and COD-induced animals (open bars). Significant differences are shown by differing letters. Bars with different superscript letters show significant differences. Values represent the mean \pm S.E.M. G, granulosa cells; T, theca cells; TE, theca externa; TI, theca interna.

intense immunostaining was observed in the theca interna of cystic follicles ($P < 0.05$; Fig. 6).

A moderate nuclear and cytoplasmic (perinuclear) expression of NCOR2 was observed in granulosa cells, with a significant increase from primary to tertiary follicles ($P < 0.05$; Figs 2, 5 and 6). The theca cells showed a weak immunostaining. In cystic follicles, a high level of cytoplasmic and nuclear immunostaining was evident in both granulosa and theca cell layers compared with other follicle categories ($P < 0.05$; Figs 2, 5 and 6).

Immunostaining for PHB2 was intense in both the perinuclear cytoplasm and the nucleus in granulosa cells from follicles at all stages of development. Staining was much less intense in thecal tissue. There were no differences in staining intensity among groups (Figs 2, 5 and 6).

Discussion

This study is the first to describe the immunolocalization of steroid receptor coregulators in ovarian follicles during normal and cystic follicular growth in cows. The significance of the changes in the cell type-specific expression pattern of steroid receptors and their coregulators is discussed below.

With the increase in the size of the follicles, the immunostaining of both ESR subtypes in the granulosa cell compartment increased significantly, in agreement with previous observations in cattle and other species (Berisha *et al.* 2000, Cárdenas *et al.* 2001, Salvetti *et al.* 2007). However, no changes were found in the immunoexpression of ESR1 in granulosa or theca cells in any follicular stage of induced cystic ovaries in relation to the control group, and a decrease in the expression of ESR2 was evident in the granulosa and theca interna of tertiary and cystic follicles of the induced COD group. These results agree with the protein and mRNA *ESR2* expression patterns previously obtained in cows with COD (Salvetti *et al.* 2007, Alfaro *et al.* 2012). However, these reports also describe a concomitant increase in ESR1 expression that was not found in this experimental model. It can be assumed that these differences are due to the evolution of the cysts, taking into account the fact that this is the first article about steroid receptor expression, using an experimental model instead of spontaneous cases.

The differences observed between cystic and tertiary follicles from control heifers may be attributed to follicle age and unrelated to cyst formation. However, numerous studies have shown that cysts are not simply inactive persistent structures but are in fact the contrary, still evolved, with important metabolic activity and structural changes (Calder *et al.* 2001, Monniaux *et al.* 2008, Ortega *et al.* 2008, Grado-Ahuir *et al.* 2011, Rodríguez *et al.* 2011). Also, other authors have found similar changes in the protein and mRNA expression of ESR2 isoforms in follicular cysts in women with polycystic ovary syndrome compared with normal size-matched follicles in healthy women (Jakimiuk *et al.* 2002), and in prenatal testosterone-treated ovine females characterized by follicular persistence (Ortega *et al.* 2009).

Although ESR1 and ESR2 bind to endogenous ligands such as 17- β -estradiol with similar affinity and form homo- and heterodimers, they display differential transcriptional activities in a cell- and promoter context-dependent manner (McInerney *et al.* 1998, Pettersson *et al.* 2000). Thus, the differential production of ESR2 will likely provide a cellular microenvironment that regulates the estrogen responsiveness of target genes in a cell-dependent manner (O'Brien *et al.* 1999). As a result, small changes in the ER subtypes ratio may perturb normal follicular development (Mosselman *et al.* 1996, Pettersson *et al.* 1997), including alterations in the balance of proliferation/apoptosis, the expression of

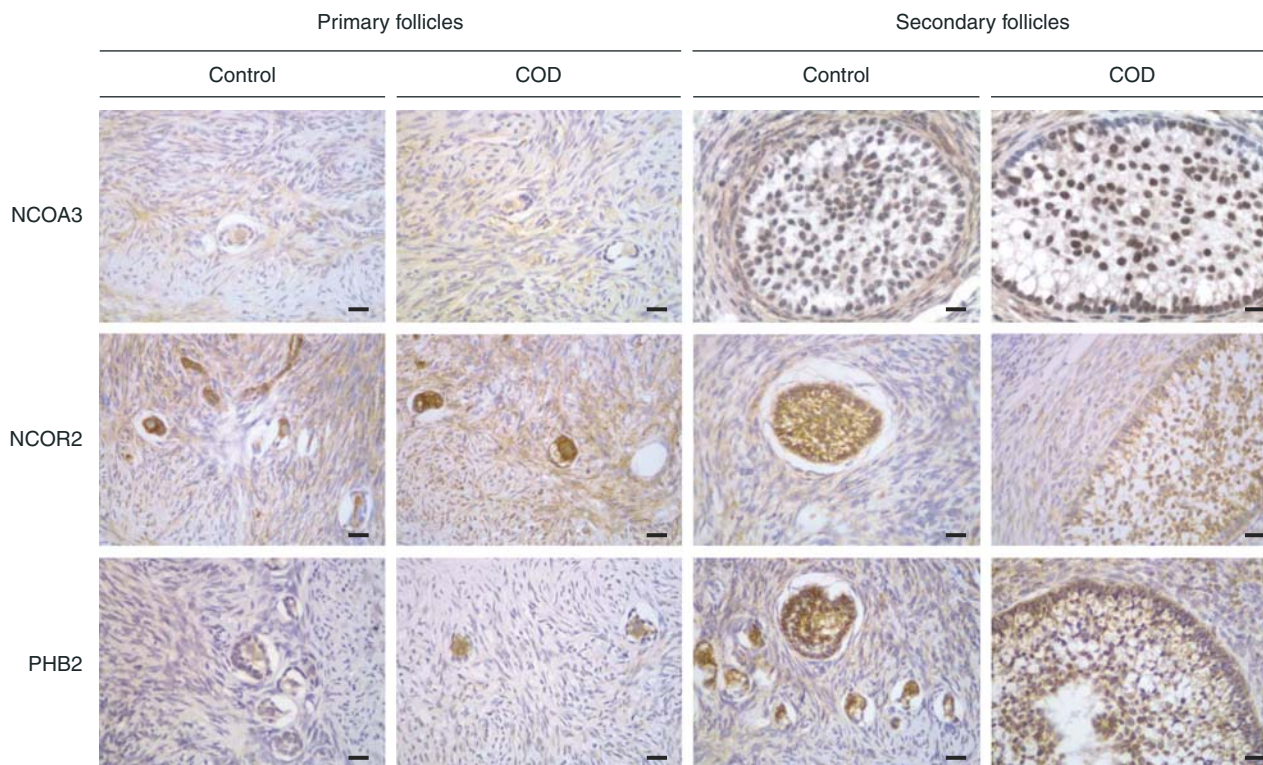


Figure 5 Representative images of NCOA3, NCOR2, and PHB2 immunostaining in primary and secondary follicles of normal cycling and COD-induced animals. Bars = 20 μ m.

gonadotropin receptors, and the disturbance of enzyme action and metabolism, all of which are signs of COD (Calder *et al.* 2001, Isobe & Yoshimura 2007, Salvetti *et al.* 2010).

It has been shown that AR mRNA is expressed in bovine follicles, and it has been suggested that it increases during early follicle development (Hampton *et al.* 2004). A significant increase in AR mRNA in the granulosa cells of cystic follicles (Alfaro *et al.* 2012) has been associated with androgens playing a role in follicular differentiation and growth (Hillier & Tetsuka 1997, Vendola *et al.* 1998, Walters *et al.* 2008). Although the results of this study do not agree with these findings, the discrepancy between the mRNA and protein expression levels could be due to posttranslational regulation of protein expression (Sette *et al.* 2010).

We observed a clear decrease in AR expression in tertiary and follicular cysts in animals with COD. In early stages of follicular development, it has been described that androgen synergistically enhances FSH-induced P450aromatase expression, through its receptors predominantly located in granulosa cells of preantral and early antral follicles. As follicular differentiation progresses, the expression of P450aromatase in pre-ovulatory follicles increases in response to increasing stimulation by gonadotropins, whereas the expression of AR decreases, taking to androgens mainly as a substrate for estrogen synthesis. For healthy follicular

development, a smooth transition of androgen utilization from action (via AR) to metabolism (via P450aromatase) is necessary (Tetsuka & Hillier 1997). The decrease in AR lead to a greater proportion of androgens (testosterone) for the aromatization in granulosa cells with the resulting increase in follicular and circulating estrogens, as observed in ACTH-induced PCO experimental model (Dobson *et al.* 2000, Salvetti 2010).

Previous studies have shown that growing and cystic follicles from COD animals show a decrease not only in proliferation but also in apoptosis *in situ*, and the expression of pro-apoptotic genes in relation to the anti-apoptotic ones (Salvetti *et al.* 2009a, 2010). It is known that alterations in the proliferation of granulosa cells and the fate of the follicles, atresia or formation of cysts, are related to multiple growth factors and hormones (gonadotropins and steroid hormones), and specifically, to steroid hormones. The altered equilibrium of ESR1 to ESR2 and an altered AR expression in cystic follicles might be factors contributing to the development of follicular persistence (Ortega *et al.* 2008, Salvetti *et al.* 2010).

In this sense, estrogens act through two major receptor subtypes, which associate to form homo- or heterodimers. Although ESR1 homodimers induce increased transcriptional activity in relation to ESR1/ESR2 heterodimers or ESR2 homodimers, and given that we have observed a decrease in the latter subtype in tertiary and

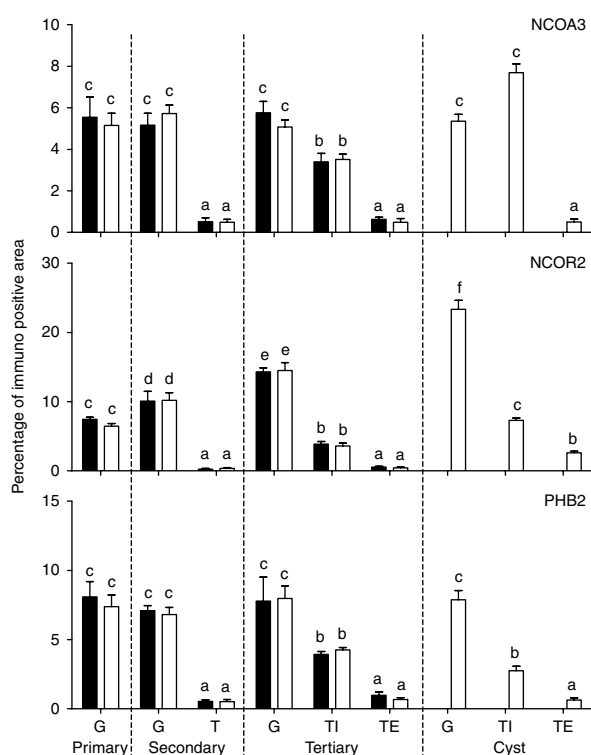


Figure 6 Relative expression (measured as percentage of immuno-positive area) of coregulatory proteins in primary, secondary, tertiary, and cystic follicles of control (black bars) and COD-induced animals (open bars). Significant differences are shown by differing letters. Bars with different superscript letters show significant differences. Values represent the mean \pm S.E.M. G, granulosa cells; T, theca cells; TE, theca externa; TI, theca interna.

cystic follicles in animals with COD, it could be inferred that there is greater activation of estrogen-dependent genes in ovarian follicles from these animals. This would lead to a decrease in the rates of apoptosis and increased cell proliferation. Now, while apoptosis levels are decreased in the ovaries of animals with COD, proliferation is also lower (Isobe & Yashimura 2007, Salvetti *et al.* 2010). This could be explained by the concomitant changes found in this work in coregulatory proteins, as well as in many other factors regulating folliculogenesis such as insulin-like growth factor 1 (IGF1), IGF2, and gonadotropins (Ortega *et al.* 2008, Rey *et al.* 2010, Rodríguez *et al.* 2011).

Different studies have demonstrated a cell-specific expression of PGR in the bovine ovary (Jo *et al.* 2002, Van den Broeck *et al.* 2002b). However, there is disagreement as to its expression in ovarian cysts. Salvetti *et al.* (2007) showed that no changes were observed in PGR protein expression in cystic follicles, whereas Alfaro *et al.* (2012) described a higher expression of PGR mRNA, particularly the B isoform, in the theca cells of cystic follicles compared with healthy follicles. It was found that protein expression

diminished in the granulosa cells, while it remained constant in theca cells. These differences with other studies may be due to the specificity of the antibody used in previous immunohistochemistry studies, which did not detect all the isoforms. Also, the sensitivity of PCR, the posttranslational regulation of protein expression, and the length of persistence of the cysts studied may be other factors contributing to these discrepancies (Anderson *et al.* 1992, Garverick 1997, Sette *et al.* 2010). The evaluation by western blot analysis of PGR isoforms in complete follicular wall showed a decrease in the expression of the isoform A2 in cystic follicles induced by ACTH in relation to normal large antral follicles without changes in the isoform B between these categories (Salvetti 2010). However, studies in an experimental model of PCOD in rats showed an increase in the ovarian expression of isoform A, without changes in isoform B (Salvetti *et al.* 2009b). There is probably an alteration in the balance PGRA/PGRB leading to alterations in the action of progesterone on follicles in these animals.

Animals with COD usually present high levels of circulating estrogen and LH (Dobson *et al.* 2000, Vanholder *et al.* 2006), which could cause alterations in PGR expression (Alfaro *et al.* 2012). The fact that the expression pattern seen in the normal ovary becomes disrupted in ovarian pathologies and the predominance of one PGR isoform seen in ovarian disease (Graham & Clarke 2002, Alfaro *et al.* 2012) suggest that changes in the ratio of PGR isoform expression could regulate the biological activity of progesterone, thereby resulting in functional hormone withdrawal in the absence of changes in serum concentrations or total progesterone-binding activities of the reproductive tissues (Schams *et al.* 2003, Amrozi *et al.* 2004, Goldman *et al.* 2005).

In this study, the first evidence for the presence of steroid receptor coregulatory proteins in the ovary of cows is presented, and their localization is described in the follicular compartments. Granulosa cells of different follicle stages showed an intense immunostaining of NCOA3, while there was a significant increase in its expression in theca interna of cystic follicles. A similar expression pattern was observed for PHB2, except that it did not show an increase in the theca interna of cystic follicles. On the other hand, NCOR2 showed a gradual increase in its expression associated with follicular development, with a higher expression in cystic follicles. In domestic animals, Hlaing *et al.* (2001) analyzed the presence and distribution of mRNAs encoding several transcriptional cofactors in the ovary of sheep, cows, and pigs. Using northern blotting, the expression of NCOA1, NCOA2, NCOA3, EP300 (P300), NRIP1 (RIP140), SPA, and NCOR2 mRNA was determined in the ovaries of all the three species. In addition, by *in situ* hybridization, NCOA1, NRIP1, and SPA were localized in ovarian granulosa, theca, and stromal cells of the sheep ovary (Hlaing *et al.* 2001).

Despite its importance, the functional regulation of coregulatory proteins remains poorly understood within an ovarian physiological context. Altered coregulator expression levels, such as the overexpression of coactivators, may lead to the increased transcriptional activation of steroid receptors, with an enhanced response in hormone-dependent growth. The levels of coregulator gene expression in ovarian structures have not been fully characterized. An amplification of NCOA3 has been observed in ovarian tumors and was associated with cell differentiation and growth (Bautista *et al.* 1998, Tanner *et al.* 2000, Hussein-Fikret & Fuller 2005). Also, in breast cancer, the amplification of NCOA3 correlates with high expression levels, increased tumor sizes, and ER and PGR positivity, supporting the role of this coactivator in the development and progression of ER-dependent tumors (Anzick *et al.* 1997, Bautista *et al.* 1998). Mice bearing a homozygous deletion of NCOA1 exhibit partial resistance to multiple hormones including estrogen, progesterone, androgen, and thyroid hormones (Edwards 2000), suggesting that different members of the p160 family have overlapping redundant functions that may affect multiple hormonal responses. On the other hand, PHB2 differs from other coregulators in that it is ER selective among the nuclear hormone receptors, acting as a negative feedback regulatory mechanism to control ER-dependent gene expression in normal cells (Mussi *et al.* 2006). However, in this study, no significant differences in its expression were observed in cystic ovaries.

Although the genes encoding NCOR2 are ubiquitously expressed, their levels are variable across various tissues and cell lines, including ovarian tumors (Havrilesky *et al.* 2001). We found that NCOR2 expression was higher in granulosa cells than in theca cells with a significant increase from primary to tertiary follicles. However, a significant upregulation in the expression of NCOR2 was found in the theca and granulosa cells of cystic ovaries compared with that observed in the normal ovary. The findings suggest that NCOR2 could have a role as a corepressor of estrogen, androgens, and progesterone in the cystic ovary, modulating the response to steroid hormones in the microenvironment affected by the imbalance in the steroid receptor expression. We found an increase in the expression of NCOR2 in granulosa and theca cells of follicular cysts, concomitantly with an increase in the expression of NCOA3 in the theca interna of cysts. Both coregulatory proteins act on the transcriptional activity of various ligand-dependent transcriptional activators, including androgen and ESRs. Considering the overall regulatory balance, we can assume that there is a higher transcriptional activity of estrogen and androgen-dependent genes via ESR1 and AR receptors respectively in the theca interna cells of follicular cysts, which would be diminished in the granulosa due to increased expression of corepressor NCOR2. This would explain that

observed previously in relation to the increase in cell differentiation of theca cells, which in many cases are luteinized in follicular cysts, and the decreased levels of apoptosis, factors that contribute to persistent follicles in animals with COD (Ortega *et al.* 2007a, 2007b, 2008, Rey *et al.* 2010, Salvetti *et al.* 2010, Velázquez *et al.* 2010, 2011).

Although this study was not designed to directly determine the role of steroid receptor cofactors in the COD, the results indicate that cofactors may be involved in altered folliculogenesis, ovulation, differentiation, and follicular persistence as a component of the pathogenesis in ovarian alterations in cattle. In summary, the results of this study are the first evidence for the presence of steroid receptor coregulatory proteins in cows with COD. Furthermore, variation in the relative levels and pattern expression of ESR and AR was observed in ovarian compartments associated with an experimental model of COD. While the findings of the current study provide evidence that an altered steroid signaling system may be present in bovine cystic follicles, additional studies are necessary to understand the potential associations with other follicular regulators.

Materials and Methods

Animals and treatment

All the procedures were approved by the Institutional Ethics and Security Committee (Faculty of Veterinary Sciences, Universidad Nacional del Litoral, Argentina) and are consistent with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science Societies, 1999). Ten nulliparous Argentinean Holstein heifers (18–24 months old; 400–450 kg body weight; maintained under standard husbandry conditions) with regular estrous cycles, previously corroborated by detection of estrus, rectal palpation, and ultrasonography, were used. The estrous cycles were synchronized using the OVSYNCH protocol: the animals were injected with GNRH (Receptal, Intervet, Buenos Aires, Argentina, 4 µg/ml, 5 ml/animal) on day 0, prostaglandin F_{2α} (Iliren, Intervet, 0.2 mg/ml, 5 ml/animal) on day 7, and GNRH on day 9. Observations for estrus began 24 h after the second GNRH treatment and were made every 12 h. The time of the first detection of estrous behavior was designated day 0 of the estrous cycle (Gumen *et al.* 2003).

Beginning on day 15 of a synchronized estrous cycle, five heifers received s.c. injections of 1 mg/ml (1 ml/animal) tetracosactrin hexaacetate (Synacthen Depot, Novartis), a synthetic polypeptide with ACTH activity, every 12 h for 7 days. Five control animals received saline (1 ml; Dobson *et al.* 2000, Ortega *et al.* 2008, Salvetti *et al.* 2010). Ovarian ultrasonographic examinations were performed in all animals as described previously, using a real-time, B-mode scanner equipped with a 7.5 MHz, linear-array, intrarectal transducer (Aloka, SSD 500; Wallingford, CT, USA; Sirois & Fortune 1988). The growth and regression of follicles >5 mm, corpora lutea, and cysts were monitored and daily ovarian ultrasonography

was performed through a complete estrous cycle (21–23 days in the control group) and from day 14 (day 0, day of estrus) until day 48 (treated group). Cysts were detected in all animals by ultrasonography and defined as any follicular structure with a diameter equal to or >20 mm present for 10 days or more, without ovulation or corpus luteum (CL) formation (Dobson *et al.* 2000). The first day of cyst formation was the day a follicle attained a diameter of 20 mm or more, and the ovaries were removed 10 days later by flank laparotomy. In the heifers used as controls, ovariectomy was conducted when the dominant follicle reached a diameter >10 mm, in the absence of an active CL, to obtain normal growing follicles (approximately day 18). Blood samples were centrifuged at 1000 *g* for 30 min and serum was stored at –20 °C until hormone analysis (data not shown; parallel study, Ortega *et al.* (2008)).

Tissue sampling and classification of follicles

During the macroscopic examination of the ovaries, the external follicular diameter was measured with calipers to corroborate ultrasound measurements, and follicular fluid from each follicle was aspirated and stored separately at –20 °C. Small samples from ovarian tissues of each group were immediately frozen at –80 °C until used in western blotting for the determination of the specificity of the antibodies used in immunohistochemistry. The health status of the follicles was confirmed by hormonal concentrations (relation estrogen:progesterone) in follicular fluid (Ortega *et al.* 2008, Salvetti 2010).

After taking follicular fluid and small samples for molecular biology techniques, ovaries were fixed in 4% buffered formaldehyde for 8 h at 4 °C and then washed in PBS. For light microscopy, fixed tissues were dehydrated in an ascending series of ethanol, cleared in xylene, and embedded in paraffin. Sections (5 µm thick) were mounted on slides previously treated with 3-aminopropyltriethoxysilane (Sigma–Aldrich) and primarily stained with hematoxylin–eosin for a preliminary observation of all ovarian structures (Ortega *et al.* 2008, Salvetti *et al.* 2010). For the immunohistochemical studies, follicles were classified into the following groups: primary, secondary, tertiary, and cystic follicles (Silvia *et al.* 2002, Ortega *et al.* 2008, Salvetti *et al.* 2010). Primary follicles were composed of one oocyte surrounded by one cuboid granulosa cell layers, without an antrum. Secondary follicles were composed of one oocyte surrounded by two or more granulosa cell layers, without an antrum. Tertiary follicles were composed of one oocyte surrounded by a stratified epithelium of granulosa cells (cumulus oophorus) with the follicular antrum present and with a size range of 3–9 mm without signs of apoptosis in their granulosa (Jaiswal *et al.* 2004, D’Haeseleer *et al.* 2005, Adams *et al.* 2008). Only cystic follicles with a complete granulosa cell layer within the sections examined and without signs of luteinization were used.

Antibodies

Antibodies for steroid receptors were purchased from commercial sources, as listed in Table 1. To evaluate steroid receptor coregulator expression, affinity-purified rabbit polyclonal antibodies provided by Dr J G Ramos (Laboratorio de

Table 1 Antibodies, suppliers, and dilutions used for immunohistochemistry (IH) and western blotting (WB).

Antibodies	Clone/source	Dilution
Primary antibodies		
ESR1	Clone 1D5 (BioGenex, San Ramon, CA, USA)	IH: 1:40 WB: 1:50
ESR2	Polyclonal. (BioGenex)	IH: 1:250 WB: 1:500
PGR	Clone PR88 (BioGenex)	IH: 1:40 WB: 1:100
AR	Polyclonal (Affinity BioReagents, Golden, CO, USA)	IH: 1:40
NCOA3	Polyclonal (provided by Dr J G Ramos)	WB: 1:50 IH: 1:50
NCOR2	Polyclonal (provided by Dr J G Ramos)	WB: 1:50 IH: 1:40
PHB2	Polyclonal (provided by Dr J G Ramos)	WB: 1:50 IH: 1:100
Secondary antibodies		
Biotinylated anti-rabbit IgG	Goat polyclonal (Zymed, San Francisco, CA, USA)	IH: 1:100
Biotinylated anti-mouse IgG	Goat polyclonal (Chemicon, Temecula, CA, USA)	IH: 1:100
Anti-mouse IgG peroxidase	Goat polyclonal (GE-Amersham)	WB: 1:500
Anti-rabbit IgG peroxidase	Goat polyclonal (GE-Amersham)	WB: 1:1000

Endocrinología y Tumores Hormonodependientes, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Argentina) were used (Varayoud *et al.* 2008, Monje *et al.* 2009). Briefly, the antigens were expressed in *Escherichia coli* JM109 (Stratagene Corp., La Jolla, CA, USA) as glutathione-S-transferase fusion proteins, using a pGEX4T-3 vector (Stratagene Corp.). The NCOA3 antigen included the region corresponding to amino acids 581–650 of the rat sequence (accession no. XP_215947), whereas the NCOR2 antigen included amino acids 1946–1995 (accession no. XP_341073) and the PHB2 antigen included amino acids 156–299 (accession no. NM_001013035). The interspecies homology between the target peptide of each antibody and the corresponding bovine protein was tested using Basic Local Alignment Search Tool (BLAST Software; <http://www.ncbi.nlm.nih.gov/BLAST>) to confirm antigen specificity. Also, the specificity of the antiserum was tested via western blot, as described later.

Immunohistochemistry

The details, suppliers, and concentrations of the antibodies used are reported in Table 1. Each antibody was assayed in five sections (minimum) of each ovary from each heifer. A streptavidin–biotin immunoperoxidase method was performed as described previously (Salveti *et al.* 2004, Ortega *et al.* 2009). Briefly, after deparaffinization, microwave pre-treatment (antigen retrieval) was performed by incubating the

sections in 0.01 M citrate buffer (pH 6.0). The endogenous peroxidase activity was inhibited with 3% (v/v) H₂O₂ in methanol, and nonspecific binding was blocked with 10% (v/v) normal goat serum. All sections were incubated with the primary antibodies for 18 h at 4 °C and then for 30 min at room temperature with biotinylated secondary antibodies selected specifically for each of the two types of primary antibodies used (monoclonal or polyclonal). The antigens were visualized by the streptavidin–peroxidase method (BioGenex, San Ramon, CA, USA), and 3,3-diaminobenzidine (Liquid DAB-Plus Substrate Kit; Zymed, San Francisco, CA, USA) was used as the chromogen. Finally, the slides were washed in distilled water and counterstained with Mayer's hematoxylin, before being dehydrated and mounted.

To verify the immunoreaction specificity, adjacent control sections were subjected to the same immunohistochemical methods, replacing primary antibodies with rabbit and mouse nonimmune sera. The specificity of the secondary antibodies was tested by incubation with the primary antibodies raised against human antigens with a proven negative reaction to the tissues of cattle: anti-CD45 (Clone: PD7/26; Dako, Carpinteria, CA, USA) and anti-Ki-67 (polyclonal, rabbit anti-human Ki-67; Dako). To exclude the possibility of nonsuppressed endogenous peroxidase activity, some sections were incubated with DAB alone.

Western blotting

To test the specificity of the primary antibodies, ovarian tissue extracts were separated by SDS–PAGE (15% resolving gel), as described previously (Ortega *et al.* 2009, Salvetti *et al.* 2010). Proteins were transferred onto nitrocellulose membranes (Amersham), blocked for 1 h in 2% nonfat milk in Tris-buffered saline containing 0.05% Tween 20 (Sigma–Aldrich Corp.), and then incubated overnight at 4 °C with specific primary antibodies (Table 1). Following washing, membranes were treated for 1 h with the corresponding secondary peroxidase-conjugated antibody (Table 1). The immunopositive bands were detected by chemiluminescence, using the ECL-plus system (GE-Amersham) on hyperfilm-ECL film (GE-Amersham).

Image analysis

The images were analyzed using the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, MA, USA). For the immunohistochemistry technique, images were digitized using a CCD color video camera (Motic 2000, Motic China Group, Xiamen, China) mounted on a conventional light microscope (Olympus BH-2, Olympus Co.), using an objective magnification of $\times 40$. The microscope was prepared for Koehler illumination. This was achieved by recording a reference image of an empty field for the correction of unequal illumination (shading correction) and calibrating the measurement system with a reference slide to determine background threshold values. The reference slides contained a series of tissue sections stained in the absence of a primary antibody. The positive controls were used as interassay controls to maximize the levels of accuracy and robustness of the method (Ranefall *et al.* 1998, Ortega *et al.* 2009, 2010).

The methodological details of image analysis as a valid method for quantifying have been described previously (Baravalle *et al.* 2007, Ortega *et al.* 2007a, 2007b, 2008, 2009, 2010, Salvetti *et al.* 2007). The main strength of the imaging approach used in this study is the visualization of the *in situ* localization of proteins within the cells of interest. The image analysis score was calculated separately in each follicular wall layer (granulosa and theca cells) from at least 50 images of the primary, secondary, and tertiary follicles from ovaries of both groups and cystic follicles from animals with induced COD.

The percentage of the immunopositive-stained area was used to determine protein expression by immunohistochemistry and calculated as a percentage of the total area evaluated through the color segmentation analysis, which extracts objects by locating all objects of the specific color (brown stain). The brown stain was selected with a sensitivity of 4 (maximum 5) and a mask was then applied to separate the colors permanently. The images were then transformed to a bi-level scale TIFF format.

Statistical analysis

The number of individuals per group was obtained from a sample size calculation that evaluated the number of individuals necessary to produce an estimate of the immunoreactivity that would fall within 0.4 units of the real value. A statistical software package (SPSS 11.0 for Windows, SPSS, Inc., Chicago, IL, USA) was used to perform the statistical tests. The differences between the groups of data were assessed by one-way ANOVA, followed by Duncan's multiple range tests. $P < 0.05$ values were considered significant. Results are expressed as mean \pm S.E.M.

In these analyses, the dependent variable was percentage of immunopositive-stained area for each of the seven proteins studied, and the independent variable (fixed factor) was each tissular compartment (treated or not; 15 in total). This comparison among all compartments allowed us to detect changes in the expression of the proteins analyzed not only due to treatment but also due to follicular development and compartments.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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