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Changes in the expression of Heat Shock Proteins in ovaries from bovines with cystic ovarian disease induced by ACTH

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ABSTRACT

Cystic ovarian disease (COD), which is considered one of the most important causes of reproductive failure in dairy cattle, induces intraovarian changes in the expression of numerous genes. The purpose of this study was to analyze the changes in the expression of Heat Shock Proteins (HSPs) in ovaries from bovines with cystic ovarian disease induced by ACTH. Immunoreactivity for Heat Shock Proteins (HSPs) in ovaries of cows with induced COD showed differential expression patterns in growing follicles from the control group. The immunopositive area for Hsp27 and Hsp60 in granulosa cells showed significant differences between tertiary follicles from normal cycling animals and those from animals with induced COD. The cysts showed increased Hsp27 immunostaining in theca cells in relation to tertiary follicles from normal cycling cows. Hsp70 immunostaining was more intense in cystic follicles than in other follicular categories from animals with induced COD, in both granulosa and theca cells. In granulosa cells, tertiary follicles from the control group showed higher levels of Hsp90 than cysts. These results demonstrate that there are differences in HSP protein expression when COD is induced. In fact, HSP expression would be part of the functional response to the changes in hormones and neurotransmitters induced by stress, indicating that HSPs can control hormonal functions and vice versa.

1. Introduction

Cystic ovarian disease (COD) which is considered one of the most important causes of reproductive failure in cattle, can result in significant economic losses to the dairy industry by delaying conception (Peter, 2004). This disease has been defined as the presence of one or more follicular structures in the ovary/ovaries, with a diameter of at least 20 mm, which persist for more than 10 days in the absence of luteal tissue, interrupting the normal reproductive cycle (Silvia et al., 2002; Peter, 2004; Vanholder et al., 2006). Although its etiopathogenesis is not completely understood, the imbalance between cellular proliferation/apoptosis in the ovary could lead to alterations in the ovarian micro-environment and delay follicle regression after ovulation failure, indicating the presence of an important intra-ovarian component as etiologic factor of COD (Maniwa et al., 2005; Salvetti et al., 2010). Also, many factors such as high yielding, nutritional management, infectious disease and stress have been defined as predisposing causes of COD (Vanholder et al., 2006).

It is well known that adequate welfare conditions promote reproduction, via hormonal stimulators while adverse conditions (for example, extreme temperatures, nutritional deficiencies, and diseases) induce stress and suppress reproduction through stress-related substances. These anomalies induce changes in the expression of numerous genes, including genes encoding Heat Shock Proteins (HSPs) (Sirotkin and Bauer, 2011).

These stress proteins form a diverse group of proteins that are classified according to their molecular weight. Most members of the HSP family perform a chaperone or chaperonin-like function, helping proteins in the intracellular environment to reach the appropriate, final folding state and avoiding folding structures that are not functional and could lead to proteosomal degradation or protein denaturation. Stress-inducing agents often affect the redox state and hydration of the cell, which, in turn, cause increased levels of misfolded proteins that may be deleterious by virtue of their altered biological activities. The cellular response to stress is represented at the molecular level by the induced synthesis of HSPs (Ellis, 1993; Welch, 1993).

A growing body of evidence suggests that HSPs are also closely involved in a number of crucial processes in tumor development

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such as the regulation of cell cycle progression (Helmbrecht et al., 2000), control of apoptotic pathways (Didelot et al., 2006; Garrido et al., 2006; Schmitt et al., 2007) and immune surveillance against cancer (Li, 2001).

HSPs can stabilize environmental effects in response to stress, increase cell proliferation and survival, inhibit apoptosis, permit repair, and prevent cell death. Hsp27 could indirectly affect apoptosis by facilitating the degradation of death regulatory proteins. The clearest evidence of the implication of HSPs in protein degradation comes from neurodegenerative disorders that are characterized by a proteosomal dysfunction and the deposition of improperly folded proteins in fibers, inclusion bodies, and plaques in the nervous system. These events are associated with an increased expression of Hsp27 that are typically found in association with insoluble protein aggregates and ubiquitin (Lanneau et al., 2010). Hsp70, besides Hsp27, have been implicated in the protection against apoptosis induced by a variety of stimuli. Hsp70 and its co-chaperone Hsp40 prevent the translocation of Bax to the mitochondria during apoptosis (Gotoh et al., 2004).

On the other hand, interestingly Hsp60 plays several physiological roles in addition to functioning as a chaperone. One role of the Hsp60-like protein could be, besides the movement of cholesterol, the transport of progesterone from syncytiotrophoblast to maternal blood. Thus, the possible role of the Hsp60-like protein in cholesterol distribution, as in placental steroidogenesis, becomes relevant (Olvera-Sanchez et al., 2011).

Hsp90 is a molecular chaperone protein that regulates signal transduction by nuclear receptors (NR) and protein kinases. This molecular chaperone associates with the unliganded form of estrogen receptors (ESRs), as well as with the androgen receptor, the progesterone receptor, the glucocorticoid receptor, and the mineralocorticoid receptor. Ligand binding to each NR induces a conformational change within the receptor, causing its dissociation from Hsp90, leading to receptor dimerization, interaction with cofactors, DNA binding, and target gene activation. Thus, molecular chaperoning is an essential initial step in the tightly regulated process of ligand-dependent transcriptional control of ESRs and could thus be involved in the regulation of steroidogenesis and follicular development (Johnson and Toft, 1994; Fang et al., 1996; Powell et al., 2010).

Together, these vias would be part of the functional response to the changes in hormones and neurotransmitters induced by stress and there are reports indicating that HSPs can control hormonal functions and vice versa (Salvetti et al., 2009; Bombardier et al., 2009; Sirotkin and Bauer, 2011).

It has been found that HSPs are highly expressed in the female reproductive organs (Ciocca et al., 1996; Driancourt et al., 1999; Velázquez et al., 2010, 2013; Jones et al., 2011). In this sense, the ovarian cycle is associated with changes in HSP expression and these proteins are very important in ovarian physiology, particularly in follicular development through modulation of sex steroid receptors functions (Pratt and Toft, 2003; Salvetti et al., 2009).

Several studies have evaluated the amounts of HSPs in ovaries from laboratory animals (Khanna et al., 1995; Ohsako et al., 1995; Paranko et al., 1996; Maizels et al., 1998; Salvetti et al., 2009), bovine oocytes (Wrenzycki et al., 2002), bovine follicular fluid (Driancourt et al., 1999, 2001; Maniwa et al., 2005) and human ovarian cells (Langdon et al., 1995; Kim et al., 1996), as well as the constitutive expression of these proteins in ovaries of adult cows (Velázquez et al., 2010). Despite the possible participation of HSPs in the molecular mechanism of pathogenesis of several diseases (Yeyati and van Heyningen, 2008; Hirsh and Junger, 2008; Milioti et al., 2008; Saluja and Dudeja, 2008; Romanucci et al., 2008), and although experimental models of cystic ovaries (Salvetti et al., 2009) have been established, no previous integral study on the localization of HSP family members in ovaries from bovines with induced COD has been carried out. In addition, all studies in cattle have been conducted in samples from abattoirs, with the disadvantage of lacking knowledge of the history of the animals. In a previous study, we analyzed the localization and quantification of these proteins and their mRNAs in the ovarian compartment, and their association with healthy and spontaneous cystic follicles (Velázquez et al., 2010, 2011).

On the basis of these observations and considering that HSPs have been described to be involved in stress response mechanisms and reproductive processes, we hypothesized that the altered hormonal response and follicular persistence in animals with induced COD would be an effect of the altered expression of HSPs in these ovaries. The aim of the present study was to determine the protein expression profiles of Hsp27, Hsp60, Hsp70 and Hsp90 in ovarian follicles in an experimental model of COD to investigate their possible involvement in ovarian disorders.

2. Materials and methods

All procedures were performed with the approval of the Institutional Ethics and Security Committee (Facultad de Ciencias Veterinarias – Universidad Nacional del Litoral, Santa Fe, Argentina) and according to the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (Federation of Animal Science Societies, 1999).

2.1. Animals and treatment

Ten nulliparous Argentinean Holstein heifers (18–24 months old; 400–450 kg body weight) maintained under standard husbandry conditions with regular estrous cycles, as corroborated previously by detection of estrus, rectal palpation and ultrasonography, were used. Their estrous cycles were synchronized using the Ovsynch protocol, which has been previously described (Gumen et al., 2003).

Tetracosactrin hexaacetate (Synacthen Depot, Novartis, Basel, Switzerland), a synthetic polypeptide with adrenocorticotropin (ACTH) activity, was used to treat five of the heifers (treated group) as detailed earlier (Salvetti et al., 2010). Briefly, beginning on day 15 of a synchronized estrous cycle, five heifers received subcutaneous injections of 1 mg of ACTH, every 12 h for 7 days (treated group). Other five animals received saline solution (1 ml) (control group) (Fig. 1) (Dobson et al., 2000; Salvetti et al., 2012). Ultrasonography for ovarian examinations was carried out in all animals using a real-time B-mode scanner, equipped with a 7.5 MHz, linear-array, intrarectal transducer (Aloka, SSD 500; Wallingford, CT, USA) (Sirois and Fortune, 1988). Normal folliculogenesis, follicular regression and cyst development were daily monitored by ultrasonography through a complete estrous cycle, until detecting a preovulatory follicle (21–23 days) in the control group and from Day 14 (one day before to treatment) until ovariectomy in the treated group. Cysts identified by ultrasonography were defined following criteria widely described (Silvia et al., 2002; Vanholder et al., 2006). The first day of cyst formation (Day 37 ± 3 days) was the day a follicle attained 20 mm or more in diameter. The ovaries were removed 10 days later, by flank laparotomy. In control animals, ovariectomy was carried out when the dominant follicle reached a diameter >10 mm, in the absence of an active corpus luteum (CL) to obtain normal growing follicles (approximately Day 18).

2.2. Sample collection and tissue preparation

To determine hormonal levels, blood samples were collected from both experimental groups (control and treated), centrifuged at 1000*g* for 30 min, and serum stored at −20 °C until hormone analysis in a parallel work (Ortega et al., 2008).

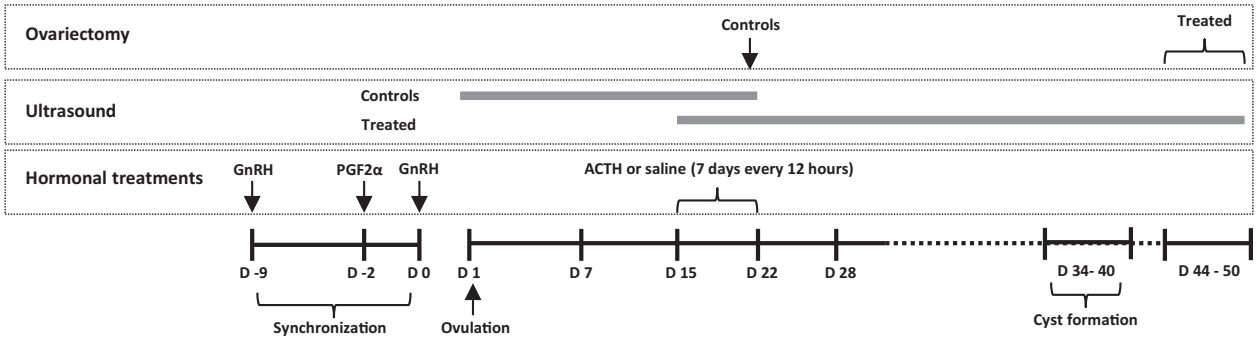

Fig. 1. Experimental design for the induction of ovarian cysts. Holstein heifers were synchronized and treated with ACTH or saline, every 12 h, from days 15 to 21 of the estrous cycle. Follicular dynamics were followed by daily ultrasound scanning, and ovariectomy was performed to obtain preovulatory follicles from control animals and cysts persisting for at least 10 days from treated animals.

Table 1
Antibodies, suppliers and dilutions used for immunohistochemistry.

Antibodies	Clone/source	Source	Dilution
Primary antibodies			
Hsp27	Polyclonal	Abcam, Cambridge, MA, USA	1:600
Hsp60	Polyclonal	Abcam, Cambridge, MA, USA	1:400
Hsp70	BRM22	BioGenex, San Ramon, CA, USA	1:200
Hsp90	AC88	Abcam, Cambridge, MA, USA	1:400
Secondary antibodies			
Biotinylated- anti-rabbitIgG	Goat Polyclonal	Zymed (San Francisco, CA, USA)	1:100
Biotinylated-anti-mouse IgG	Goat Polyclonal	Chemicon (Temecula, CA, USA)	1:100

During the macroscopic examination of the ovaries, the follicular diameter was measured using calipers to corroborate previous ultrasound measurements, and follicular fluid from each follicle was collected and stored at −20 °C for hormone determinations (Ortega et al., 2008). The ovaries were fixed in 4% neutral buffered formalin.

For histological studies, paraffin-embedded sections (4 μm thick) were obtained from both ovaries from each animal using a microtome and mounted on slides previously treated with 3-aminopropyltriethoxysilane (Sigma–Aldrich, St. Louis, MO, USA). Follicle classes were distinguished using criteria previously established and included primary, secondary (preantral), tertiary (antral) (Nomina Histologica, 1994) and cystic follicles (Silvia et al., 2002).

2.3. Immunohistochemistry

Protein expression of Hsp27 and Hsp60 was detected with polyclonal antibodies, whereas that of Hsp70 and Hsp90 was detected with monoclonal antibodies, using dilutions listed in Table 1. To test the specificity of the primary antibodies used in this study, previous assays by western blot were carried out with ovarian tissue extracts (Velázquez et al., 2010).

The indirect immunohistochemistry method was performed as previously detailed (Salvetti et al., 2004; Ortega et al., 2009; Velázquez et al., 2010). The antigen–antibody reaction was visualized by the extravidin-peroxidase method (Sigma–Aldrich) and 3,3-diaminobenzidine (DAB, Invitrogen, Life Technology, CA, USA) was used as chromogen. Finally, the slides were washed in distilled water, counterstained with Mayer's hematoxylin, dehydrated, and mounted. To verify specificity, adjacent negative control sections were subjected to the same immunohistochemical method, replacing the primary antibody with mouse or rabbit non-immune serum. To exclude the possibility of non-suppressed endogenous peroxidase activity, some slides were incubated with DAB alone.

2.4. Image analysis

Images were digitized using a CCD color video camera (Motic 2000, Motic China Group, China) mounted on a conventional light microscope (Olympus CX31, Olympus Co., Japan). To obtain representative data from immunohistochemical labeling, the images were obtained from at least 20 microscopic fields of primary, secondary and tertiary follicles from both groups and of cystic follicles from animals with induced COD. Images were analyzed using the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, MA, USA) as detailed earlier (Velázquez et al., 2010).

The percentage of immunopositive area was calculated separately for each follicular compartment (granulosa and theca cell layers) in each slide as a percentage of total area evaluated through color segmentation analysis, which extracts objects by locating all objects of a specific color (brown stain) (Ortega et al., 2009).

2.5. Statistics

A statistical software package (SPSS 11.0 for Windows, SPSS Inc., Chicago, Illinois, USA) was used to analyze the data. The statistical analysis of HSP expression within each experimental group (treated or control) was assessed by one-way ANOVA, followed by Duncan's multiple range tests as a multiple comparison test. To compare HSP expression between the corresponding categories from control and treated animals, Student *t*-test was conducted. Dunnett test was applied to compare each follicular category with cysts. Differences between groups were considered significant if $p < 0.05$. The results are expressed as mean ± SEM.

3. Results

3.1. HSP profiles of normal ovaries

Immunodetection of Hsp27 in samples from the control group showed differential expression following follicular development,

in both granulosa and theca cells and moderate reactivity was observed in the cytoplasm of stromal cells. In granulosa cells, Hsp27 expression decreased as folliculogenesis advanced, whereas in theca cells, protein expression was significantly lower in tertiary than in secondary follicles ($p < 0.05$; Fig. 2A and B).

Regarding Hsp60 immunodetection, no significant differences were observed among the follicular categories of the control group. This expression pattern was observed both in granulosa and theca cells (Fig. 2A and B) with a lower specific immunostaining in stromal cells.

Immunoexpression of Hsp70 in the control group showed significant differences among follicular categories both in granulosa and theca cells ($p < 0.05$, Fig. 2A and B). In particular, the greatest percentage of immunopositive area for theca cells was found in tertiary follicles (Fig. 2B) but there was no differences between tertiary and other follicles in granulosa cells (Fig. 2A). Stromal cells of the ovary showed a weak reactivity for Hsp70.

On the other hand, Hsp90 immunostaining was increased in follicular cells of tertiary follicles and the significant differences were observed in relation to secondary follicles, both in theca and granulosa ($p < 0.05$, Fig. 2A and B). The nucleus as well as the cytoplasm of other cells that comprise the ovary showed moderate Hsp90 staining.

3.2. HSP profiles of ovaries from animals with COD

In the granulosa cells from animals with COD, neither Hsp27 nor Hsp60 immunostaining showed differences among the follicles studied (Fig. 3A). In contrast, in theca cells from animals with COD, induced cysts showed an Hsp27 labeling pattern similar to that of tertiary follicles and an Hsp60 labeling pattern that was more intense in tertiary follicles than in the remaining follicles (Fig. 3B).

Hsp70 immunostaining in samples from animals with COD was higher in cystic follicles than in other follicular categories, in both granulosa and theca cells and increased as folliculogenesis advanced (Fig. 3A and B).

Hsp90 immunostaining in granulosa and theca cells of cysts from animals with COD was similar to that found in tertiary follicles but different from that of secondary follicles ($p < 0.05$; Fig. 3A and B).

3.3. Differential expression in animals with COD

HSP immunostaining in ovaries with induced COD demonstrated patterns different from those found in growing follicles from the control group (Fig. 4). In both granulosa and theca cells, the percentage of immunopositive area for Hsp27 showed significant differences between tertiary follicles from the control group and those from the treated group (Fig. 6). In addition, significant differences were observed between the normal secondary follicles and those from treated animals, in theca cells ($p < 0.05$, Figs. 4–6). Moreover, in theca cells, cysts showed a significant increase in the number of immunostained cells for Hsp27 in relation to tertiary follicles from the control group, moreover the differences found in relation to secondary follicles of cystic ovaries. By contrast, in

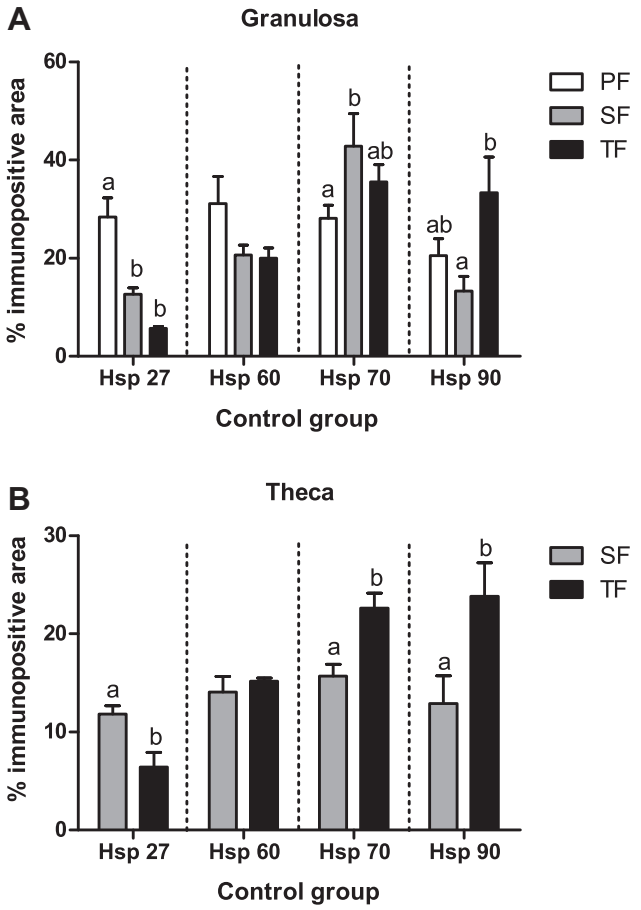

Fig. 2. Relative expression (measured as percentage of immunopositive area) of HSPs in primary (PF), secondary (SF) and tertiary (TF) follicles from control animals, in granulosa (A) and theca (B) cells. Different letters indicate significant differences. Values represent the mean ± SEM.

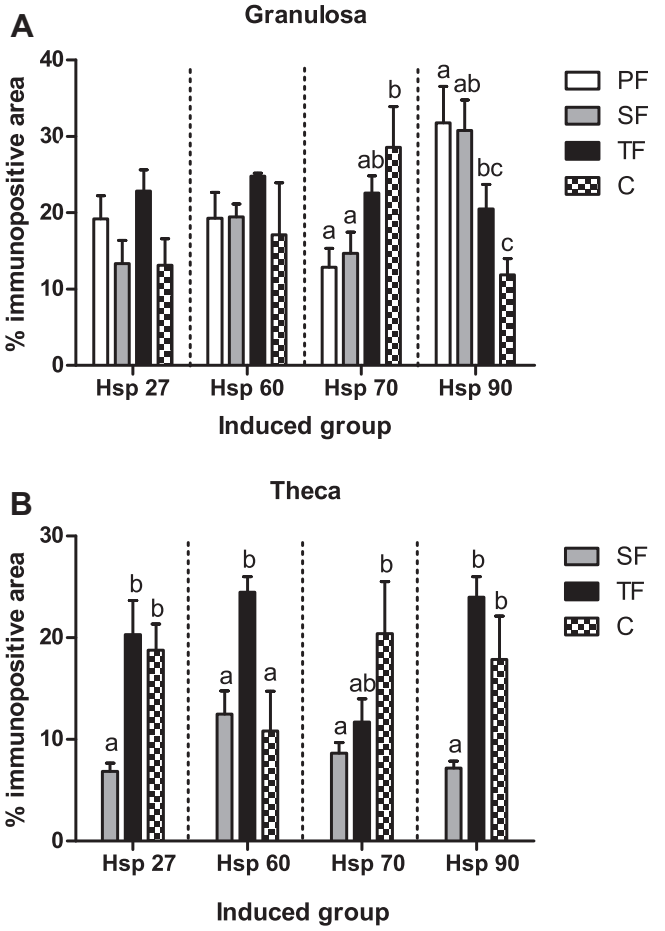

Fig. 3. Relative expression (measured as percentage of immunopositive area) of all HSPs in primary (PF), secondary (SF), tertiary (TF) and cystic follicles (C) of animals with induced COD, in granulosa (A) and theca (B) cells. Different letters indicate significant differences. Values represent the mean ± SEM.

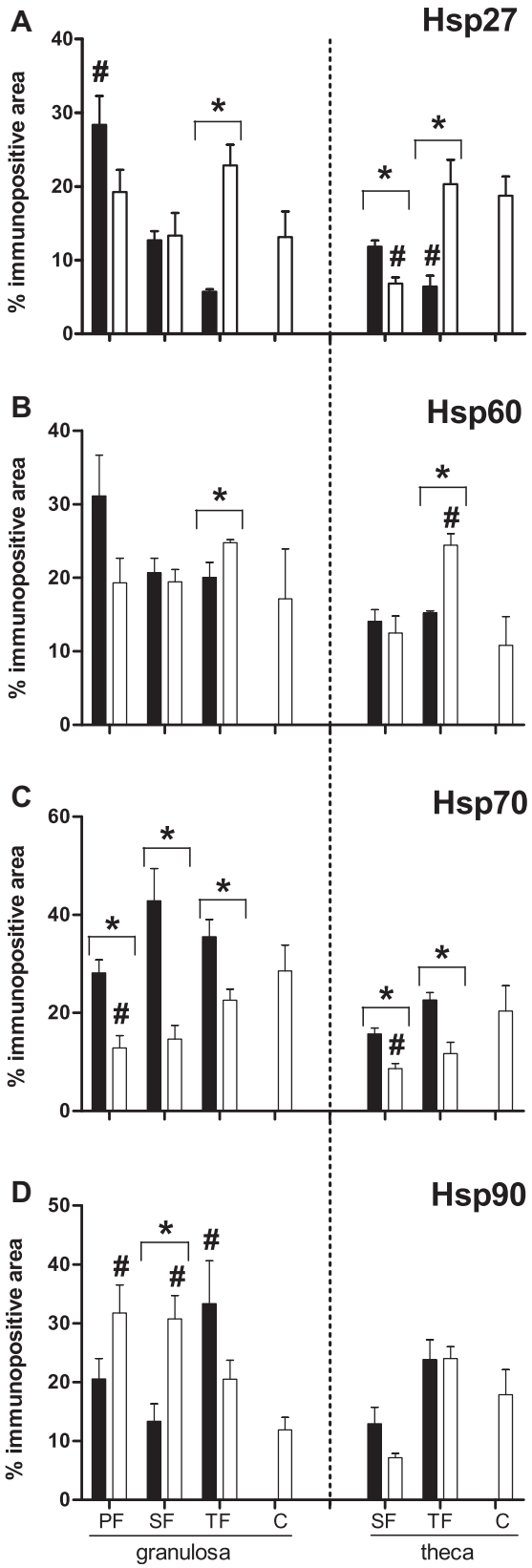

Fig. 4. Relative expression (measured as percentage of immunopositive area) of Hsp27, Hsp60, Hsp70 and Hsp90 in primary (PF), secondary (SF) and tertiary (TF) follicles of control (black bars) and animals with induced COD (open bars), both in granulosa and theca cells. Values are expressed as the mean ± SEM. *Values were statistically different at $p < 0.05$ as compared to the corresponding follicular category. #Values were statistically different at $p < 0.05$ as compared to cysts.

granulosa cells, the cysts showed lower immunostaining than primary follicles ($p < 0.05$; Figs. 4 and 6).

On the other hand, regarding Hsp60 immunoreactivity, there were significant differences in tertiary follicles from control animals and those from animals with COD. These differences were evidenced both in granulosa and theca cells ($p < 0.05$; Fig. 6). The cysts presented significant differences for Hsp60 immunostaining only in relation to tertiary follicles from animals with COD, in theca cells (Figs. 4 and 6).

The expression pattern observed for Hsp70 showed less staining in all follicular categories from animals with induced COD than that from the control group, both in granulosa and theca cells ($p < 0.05$; Figs. 4–6). Primary and secondary follicles from cows with COD showed significantly less Hsp70 immunostaining than that observed in cysts, in granulosa and theca cells, respectively ($p < 0.05$; Fig. 4).

Hsp90 immunostaining was greater in granulosa cells of primary and secondary follicles than that observed in cysts from animals with induced COD. Also, there were significant differences between secondary follicles from the control and treated group (Figs. 4 and 5). Also, tertiary follicles from the control group showed higher levels of Hsp90 than cysts, in that follicular compartment (Fig. 6), while theca cells showed no differences in Hsp90 immunostaining between experimental groups (Fig. 4).

4. Discussion

Considering previous reports in relation to the participation of HSPs in the functional response to hormones and neurotransmitters (Driancourt et al., 1999; Jayachandran and Miller, 2002; Bombardier et al., 2009; Sirotkin, 2010), we evaluated the association between these proteins and induced COD in cows, since the anomalies that occur due to this reproductive disorder may cause changes in the expression of several genes, including genes encoding HSPs (Sirotkin and Bauer, 2011).

Numerous authors have previously evaluated the expression of HSPs in the ovary and their participation in the reproductive functions of individuals under stress conditions (Guzeloglu et al., 2001; Maniwa et al., 2005; Salvetti et al., 2009; Velázquez et al., 2010, 2011; Sirotkin and Bauer, 2011; Park et al., 2012). However, this is the first study about the expression of HSPs in cows with induced COD.

We found lower Hsp27 immunostaining as folliculogenesis advanced, in both granulosa and theca cell layers in the control group. In contrast, the growing follicles from cystic ovaries showed an Hsp27 expression similar to that observed in induced cysts. Perhaps, the stress ovarian conditions during development of COD could be influencing the normal expression of HSPs observed in this study for control group and it could explain the differences found between the HSP levels of both groups, which were most clearly observed between tertiary control follicles and cysts.

Numerous reports sustain a role for Hsp27 as an important regulator of signal transduction pathways, differentiation and apoptosis (Beere, 2004; Kostenko and Moens, 2009; Rayner et al., 2010). It has been suggested that a disorder in cell proliferation and apoptosis is associated with the development of follicular cysts in cows (Isobe and Yoshimura, 2007; Salvetti et al., 2010) and in this case, the marked difference between Hsp27 immunostaining of tertiary follicles and cysts shows that the disorder mentioned above could be happening in theca cells. Moreover, some of these results are similar to those from studies carried out in bovine ovaries from abattoirs, where the levels of Hsp27 varied with follicular development (Velázquez et al., 2010).

A peculiar association has been found between Hsp60 and the transport of mitochondrial proteins by Paranko et al. (1996). These

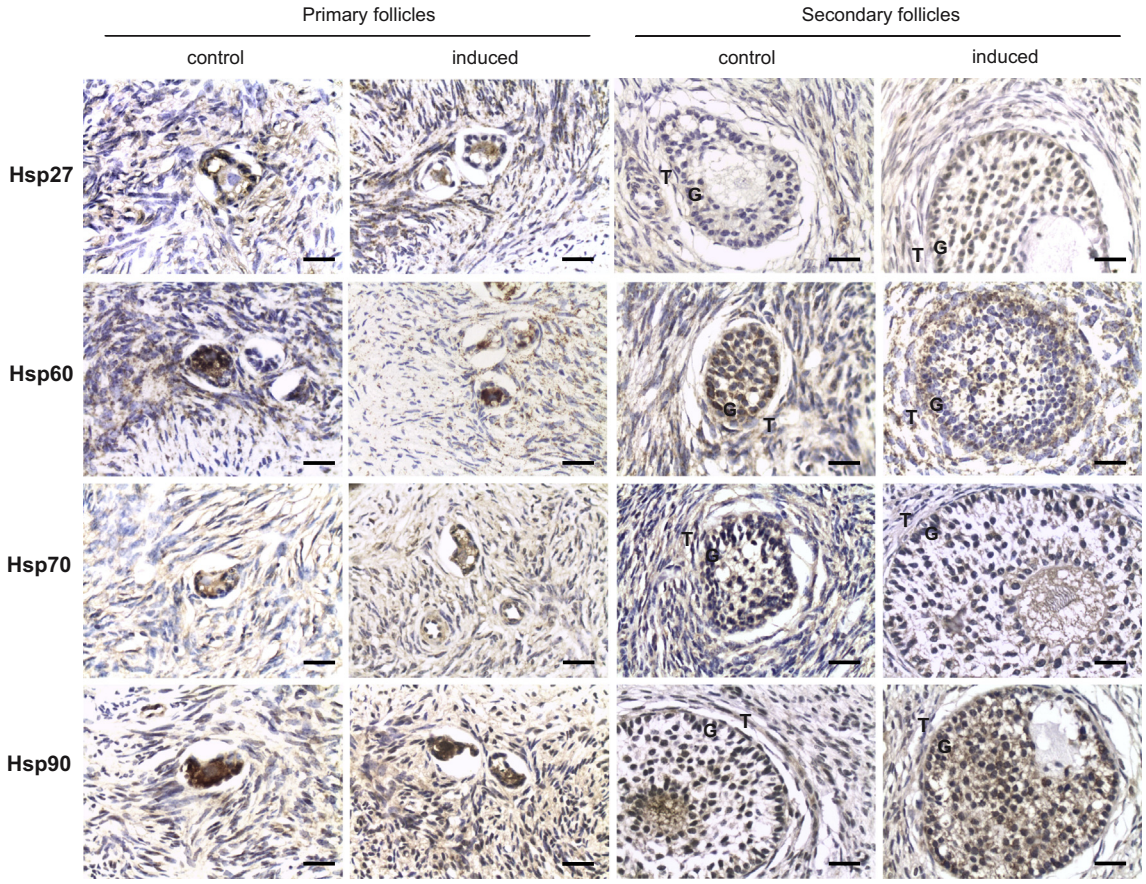

Fig. 5. Representative images of Hsp27, Hsp60, Hsp70 and Hsp90 immunostaining in primary and secondary follicles of control animals and animals with induced COD. T: theca cells, G: granulosa cells. Bars = 25 μm.

authors described their implication in folliculogenesis in prepubertal rats, where the intense staining was observed in oocytes of the primordial and growing preantral follicles. In the present work, Hsp60 immunoreactivity was similar along normal folliculogenesis in the control group, in both granulosa and theca cells. However, in the cows with induced COD, cystic follicles showed staining similar to that of the remaining categories in granulosa cells, while tertiary follicles exhibited the highest percentage of immunostaining in theca cells.

Recent reports have indicated a significant role for Hsp60 during inflammatory processes given its ability to establish a connection among the immune system components (Quintana and Cohen, 2011). This highlights the implication of this protein during ovulation, since it has been described as an inflammatory process. In this study the significant differences were observed between tertiary follicles from the control group and those from the animals with induced COD, which could be reflecting the deregulation in this follicular stage and thus be one of the reason by which ovulation does not occur and the follicle remains up to develop the cyst.

In contrast, Hsp60 protein levels varied markedly in cystic follicles in relation to the remaining categories when samples were obtained from slaughterhouses (Velázquez et al., 2010, 2011). Perhaps, the lack of knowledge of cyst evolution, the unknown nutritional conditions of the animals from abattoirs and other stress factors could be influencing the expression of HSPs and may explain the differences observed between results obtained from spontaneous cysts and induced cysts by ACTH in this work (Calder et al., 2001; Vanholder et al., 2006).

The widely recognized ability of Hsp70 to inhibit apoptosis is a feature may contribute to the protective effect against cell death. In fact, the underlying ability of HSPs to maintain cell survival correlates with the inhibition of the mechanism of caspase activation (Garrido et al., 1999; Beere et al., 2000; Mosser et al., 2000; Beere, 2004, 2005). Hsp70 immunostaining in tertiary follicles from the control group was similar to that of the other follicular categories analyzed, but in the theca the greatest relative amount was observed in tertiary follicles. Ovaries from animals with induced COD showed an increased immunoexpression as folliculogenesis advanced, in both granulosa and theca cell layers. The comparison between follicles from the control and COD animals showed clear differences, which could be associated with the disturbance of intracellular mechanisms that regulate cell growth and expression of some factors such as Hsp70 in disorders like COD (Peter, 2004). In this sense, Hsp70 immunostaining was greater in cysts than in primary follicles from ovaries with induced cyst. According to this increase, Maniwa et al. (2005) have found in bovines a greatest relative amount of Hsp70 in follicular fluid of cysts in relation to large follicles of about 10 mm in diameter from ovaries without cystic follicles. In a previous work, we have found the greatest percentage of immunopositive cells in granulosa cells of cysts, which confirms the results obtained in this study (Velázquez et al., 2010). Also, in another previous work, Hsp70 immunoreactivity was evident in all follicular cells of the categories studied, but more intense in granulosa and theca cells of cystic follicles of rats with induced polycystic ovarian syndrome (PCOS),as in the present study (Salvetti et al., 2009). Taking account together these results we could hypothesize that the increased level of Hsp70 detected in this study could explain the decreasing apoptosis in follicular cysts and delayed regression of these structures reported by Salvetti et al. (2010) in cows with COD. Moreover, the ovary could be responding to stress

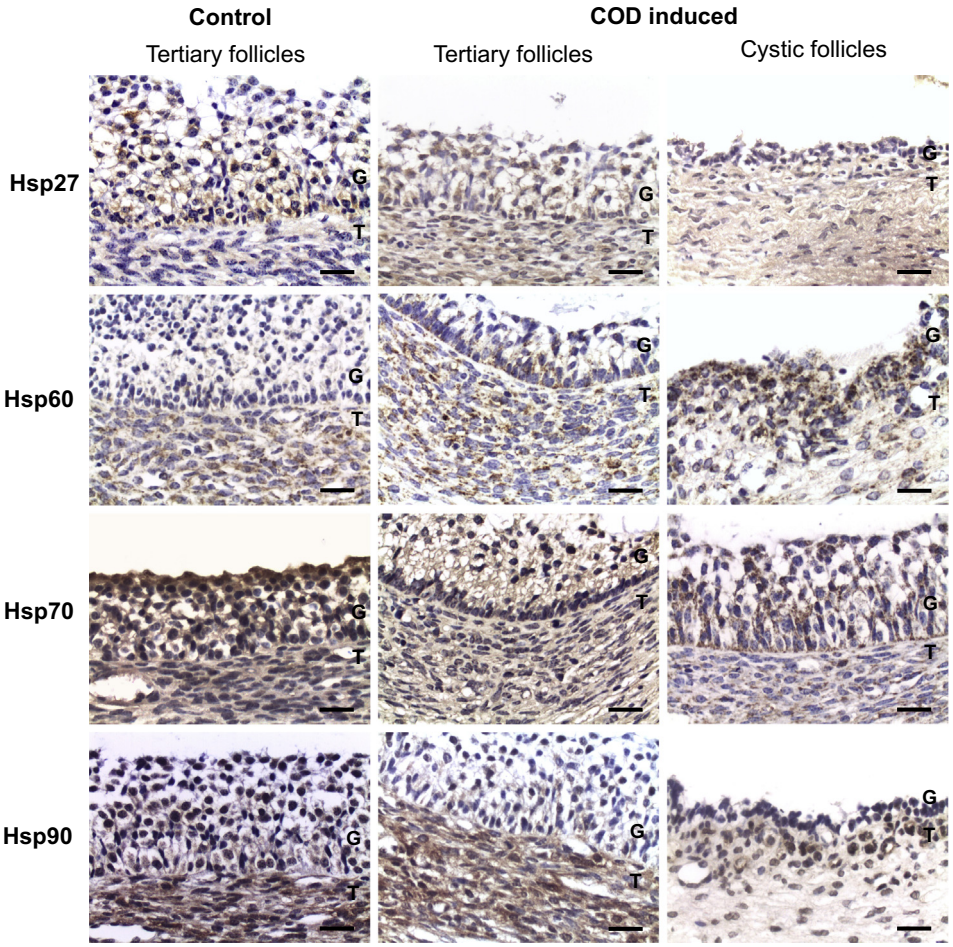

Fig. 6. Representative images of Hsp27, Hsp60, Hsp70 and Hsp90 immunostaining in tertiary follicles from control animals, and tertiary and cystic follicles of animals with induced COD. T: theca cells, G: granulosa cells. Bars = 25 μm.

conditions by this mechanism. Sirotkin and Bauer (2011) have recently demonstrated that synthesis of Hsp70 by porcine ovarian cultured cells is stimulated by stressors like high temperature and malnutrition/serum deprivation, demonstrating the involvement to Hsp70 in ovarian stress response.

The role of Hsp90 as a potential regulatory protein of gene expression has been recognized in relation to its ability to bind steroid receptors in a heterocomplex. In unstressed cells, Hsp90 performs housekeeping functions that control the activity, turnover and trafficking of a variety of proteins (Pratt and Toft, 2003). In this case, ovaries from the control group showed Hsp90 immunoreactivity both in granulosa and theca cells of all the follicular categories analyzed. The immunostaining in granulosa and theca cells of tertiary follicles from the control group was greater than that observed in secondary follicles. Likewise, other authors have found increased Hsp90 immunostaining in follicular fluid during follicular development in bovine ovaries (Driancourt et al., 1999, 2001). However, in the group with induced COD, a tendency to show decreased Hsp90 immunoexpression was observed in granulosa cells of growing follicles while cysts showed weak staining in relation to the remaining follicles. These results could be associated with the alterations observed in hormone steroid receptors, previously reported in ovaries from cows with COD where the authors found diminished levels of mRNA for ESRβ in follicular cells of cysts (Alfaro et al., 2012; Salvetti et al., 2012). This correlation between Hsp90 and ESRβ expression allow us to infer that there is a regulation exerted by this chaperone protein on that functional receptor, especially in stress conditions.

The comparison between experimental groups showed differences only in the granulosa cells of secondary follicles, since Hsp90 immunoexpression was increased in those follicles from the COD group. However, in theca cells we found similar Hsp90 immunoreactivity in tertiary and cystic follicles. In agreement with these results, Park et al. (2012) found increased levels of Hsp90 in ovaries from rats with induced PCOS by ACTH. Thus, they demonstrated the implications of Hsp90 in stress pathway that lead to the development of cystic disease.

The present study broadens previous findings about cystic ovarian disease. In addition, it is the first report about expression of HSPs in animals with experimental induced COD, and thus allows evaluating the changes that occur as follicular development advances in both controls and cystic animals. Also, these results demonstrate that there are differences in HSP protein expression when COD is induced. In fact, their expression would be part of the functional response to the hormonal changes induced by stress, indicating that HSPs can control hormonal functions and vice versa.

Conflict of interest

The authors declare that they have no conflict of interest.

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